The Potential of Secondary Metabolites from Plants as Drugs or Leads Against Protozoan Neglected Diseases – Part I

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Abstract: Infections with protozoan parasites are a major cause of disease and mortality in many tropical countries of the world. Diseases caused by species of the genera *Trypanosoma* (Human African Trypanosomiasis and Chagas Disease) and *Leishmania* (various forms of Leishmaniasis) are among the seventeen "Neglected Tropical Diseases" (NTDs) defined as such by WHO due to the neglect of financial investment into research and development of new drugs by a large part of pharmaceutical industry and neglect of public awareness in high income countries. Another major tropical protozoan disease is malaria (caused by various *Plasmodium* species), which -although not mentioned currently by the WHO as a neglected disease- still represents a major problem, especially to people living under poor circumstances in tropical countries. Malaria causes by far the highest number of deaths of all protozoan infections and is often (as in this review) included in the NTDs.

The mentioned diseases threaten many millions of lives world-wide and they are mostly associated with poor socioeconomic and hygienic environment. Existing therapies suffer from various shortcomings, namely, a high degree of toxicity and unwanted effects, lack of availability and/or problematic application under the life conditions of affected populations. Development of new, safe and affordable drugs is therefore an urgent need.

Nature has provided an innumerable number of drugs for the treatment of many serious diseases. Among the natural sources for new bioactive chemicals, plants are still predominant. Their secondary metabolism yields an immeasurable wealth of chemical structures which has been and will continue to be a source of new drugs, directly in their native form and after optimization by synthetic medicinal chemistry. The current review, published in two parts, attempts to give an overview on the potential of such plant-derived natural products as antiprotozoal leads and/or drugs in the fight against NTDs.

Keywords: Neglected tropical diseases; *Trypanosoma*; *Leishmania*; *Plasmodium*; natural product; monoterpene; sesquiterpene; diterpene; triterpene.

1. INTRODUCTION

The present part I of this review comprises a general description of the diseases, the current state of therapy and need for new therapeuticals, assay methods and strategies applied in the search for new plant derived natural products against these diseases as well as an overview on natural products of terpenoid origin showing activity that might be exploited in this respect. Part II [1] will describe the potential of further classes of natural products -such derived from the shikimate pathway (lignans, coumarins, caffeic acid derivatives); quinones of various structural classes; compounds formed via the polyketide pathways (flavonoids and related compounds, chromenes and related benzopyrans and benzofurans, xanthones, acetogenins from Annonaceae and polyacetylenes); diverse classes of alkaloids- as antiprotozoal leads and drugs.

The names of protozoan organisms mentioned throughout this review are abbreviated as follows: *Trypanosoma brucei; Tb; T. brucei gambiense; Tbg; T. brucei rhodesiense; Tbr; T. brucei brucei; Tbb; T. congolense; Tcon; T. cruzi; Tcr; Leishmania amazonensis; Lam; L. braziliensis; Lbra; L. enriettii; Lenr; L. donovani; Ldon; L. infantum; Linf (syn. L. chagasi; Lcha); L. major; Lmaj; L. mexicana; Lmex; L. panamensis; Lpan; L. peruviana; Lper; L. tropica; Ltro; Plasmodium falciparum; Pfc; P. berghei; Pber; P. vinckei petteri; Pvin; P. vivax; Pviv; P. yoelii; Pyoe.*

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1.1. Neglected Diseases Caused by Protozoan Parasites

Neglected tropical diseases (NTDs) are a group of mostly life-threatening infections affecting more than a billion people worldwide. Most affected are poor populations of remote rural areas, urban slums, or conflict zones, often in underdeveloped and developing countries. People suffering from NTDs hence constitute an unattractive market to private-sector research and development (R&D) investment. These diseases not only directly affect health, but represent a vicious cycle of socioeconomic events which reinforce and feedback on each other leading towards inescapable poverty of a sizable number of the population (http://www.thelancet.com/series/neglected-tropical-diseases).

The global threat posed by NTDs compelled the World Health Organization (WHO) to release its first report on the current situation in 2010 [2]. The number of NTDs enumerated in this report reached 17, including three major protozoan diseases, namely, human african trypanosomiasis (HAT or "sleeping sickness"), Chagas disease and Leishmaniasis (sections 1.1.1.–1.1.3). These diseases affect many millions of people world-wide. Approximately 10 million people were estimated to be infected with Chagas disease in 2009 with an annual estimated number of 10,000 death cases. Leishmaniases cause approximately 50,000 death cases annually. For HAT, the total number of infected people in 2006 was 50,000-70,000 [2], although the number of new infections appears to be falling [3].

Besides these, another major protozoan disease, annually causing even one million or more deaths and other devastating consequences for many people in tropical coutries, is malaria. Although not included in WHO's current list of NTDs, malaria is still considered by many authors, including those of the present review as a neglected disease, as pointed out below (section 1.1.4). Currently existing therapies for the mentioned diseases are either largely based on drugs that are extremely toxic and prone to show severe side effects, or they are not sufficiently available or applicable to the most seriously affected people. Especially in the case of malaria, increasing resistance of the parasites to the existing drugs is another serious problem with existing pharmacotherapies. The search for novel active principles and lead structures for the development of new efficacious, safe and affordable new drugs against all these diseases is hence an extremely urgent task. This section will give an outline on each of the diseases and their specific problematic.

1.1.1. African Trypanosomiasis (Sleeping Sickness)

Human African Trypanosomiasis (HAT, or "sleeping sickness") is a fatal vector-borne disease caused by the kinetoplastid protozoan pathogen *Trypanosoma brucei* (*Tb*) [4-6]. It is endemic in sub-Saharan Africa where it is transmitted by the bite of the Tsetse fly (*Glossina* spp., family Glossinidae). The WHO estimates that as many as 60 million people are at risk to contract HAT, which is caused by two distinct subspecies of *T. brucei (Tb)*, *T. b. gambiense* (West Africa; *Tbg*) and *T. b. rhodesiense* (East Africa; *Tbr*). Following continued control efforts, the number of new cases reported in 2009 by the WHO has dropped below 10 000 for the first time in 50 years and has further decreased in the last year [3]. The number of new infections reported annually, however, is considered to be only a fraction of the true number of infected individuals [2]. *Tbg* accounts for more than 90% of the disease [2].

Both forms begin with a haemolymphatic stage and become fatal when the trypanosomes invade the central nervous system (CNS). HAT is one of the very few infectious diseases with a mortality rate of 100% if left untreated. HAT is found primarily in rural communities and the primitive medical care in these regions likely results in significant underreporting of the disease burden. *Tbr*, besides humans, also infects both wild and domesticated animals. Cattle contribute significantly to human infection as a reservoir, and disease in these animals contributes to malnutrition. Several other subspecies of trypanosomes are also endemic in Africa, but are limited to infection of animals (*T. b. brucei (Tbb)*, *T. congolense* (*Tcon*) and *T. evansi*).

The clinical manifestations of HAT depend both on the subspecies of the parasite and on the stage of infection. *Tb* is an entirely extracellular parasite that in the early stages replicates in the blood, and in latter stages migrates into the central nervous system (CNS) and is found in the cerebral spinal fluid (CSF). Early stage disease is manifest by fever, headache, malaise, weight loss, and arthralgia, with symptoms of fever sometimes accompanied by rigor and vomiting and cycling over several day intervals. Skin lesions or chancre may also be present. The underlying etiology of the cyclic symptoms is antigenic variation of the parasite surface coat (variant surface glycoprotein (VSG)) [7] which occurs at a sufficient frequency to render useless the host immune response. Late stage disease is marked by progressively worsening neurological symptoms including headache, sleep disorders, personality changes (e.g. anxiety, irritability, violence, delirium), motor weakness and visual impairments. *Tbr* causes a virulent, rapidly fatal disease with high parasitemia that kills patients within weeks to months. It progresses quickly to CNS involvement, and leads to multiple organ involvement, including significant cardiac symptoms [8], endocrine and gastrointestinal problems. *Tbg* is characterized by low parasitemia and a slower time course before CNS involvement [9]. Symptoms manifest over many years leading to the classic neurological symptoms and progression to coma and death for which sleeping sickness has been named. Disease diagnosis and stage assessment is rather simple and relies on the microscopic identification of parasites in the blood, lymph or CSF, often requiring better techniques to increase sensitivity, particularly for patients infected with *Tbg* [10].

Drugs are the only therapeutic option for the treatment of HAT as there is no vaccine and no prospects that one will be developed in the near future. Four drugs are registered for the treatment of sleeping sickness and are provided free of charge to endemic countries through a WHO private partnership with Sanofi-Aventis (pentamidine, melarsoprol and eflornithine) and Bayer AG (suramin). During the first stage, pentamidine is used for *Tbg* infections; it is also effective against the haemolymphatic stage of *Tbr*, but suramin shows higher efficacy [11]. HAT is usually managed with a combination of suramin (*Tbr*) and pentamidine (*Tbg*) for early stage disease prior to CNS involvement, and melarsoprol and eflornithine for late stage disease. It is likely that nifurtimox/eflornithine combination therapy (NECT) will supplant eflornithine alone as a treatment option. A just completed clinical trial led the WHO to place NECT on its Essential Medicines List and to the recommendation that it should be considered as the front line treatment for late stage *Tbg* by control programs. Eflornithine is not effective against *Tbr*, thus melarsoprol remains the only option for late stage disease [9].

Suramin (available since 1920) is effective against early stage infection by both *Tbr* and *Tbg* [4, 9]. Its exact mechanism of action is not known. Its use is typically restricted to *Tbr* since pentamidine is available for treatment of *Tbg*. Suramin does not cross the blood brain barrier and is thus ineffective against late stage disease. A range of side effects have been reported and include nausea, vomiting, fatigue and shock followed by renal toxicity and neurological complications such as headache and peripheral neuropathy after several doses [4].

Pentamidine, a diamidine, shows significant antitrypanosomal activity, the mechanism of action being unknown. However, pentamidine is significantly enriched by the parasite (reaching millimolar levels) which may be a key factor in its selective toxicity. It is used for the treatment of early stage *Tbg* but not for *Tbr* where it has reduced activity. Pentamidine is not effective

against late stage HAT, and it has been presumed not to cross the blood brain barrier. Pentamidine causes significant toxicity in at least half of the patients, with life threatening hypoglycemia being the most serious [4].

Melarsoprol, discovered in 1949, is an organic arsenical active against both stages of *Tbg* and *Tbr* [4]. Because of its extreme toxicity it is reserved for late stage treatment of *Tbr* and is recommended for *Tbg* only where eflornithine (see below) is unavailable. It is a prodrug, rapidly converted to melarsin oxide which reacts with many biomolecules by interaction with sulfhydryl groups in both, the parasite and the host, the latter explaining its toxicity [4]. The molecular mechanism behind the trypanocidal activity, however, is not fully understood. Increasing numbers of treatment failures are being reported [12] and field isolates ten-fold less sensitive to the drug have been identified. However, treatment failures may also result from other factors [12, 13].

Eflornithine (D,L-α-difluoromethylornithine) is the only clinically utilized antitrypanosomal agent with a known mechanism of action. It was discovered to have antitrypanosomal activity in 1980 and approved for clinical use in 1990. It inhibits the enzyme ornithine decarboxylase (ODC) [4] which is crucially involved in the biosynthesis of polyamines required for cell growth. Eflornithine is less effective against *Tbr* but it is recommended for treatment of late stage *Tbg* and has been clinically demonstrated to have a better safety profile than melarsoprol [14]. Eflornithine, however, does not efficiently enter the CNS and therefore large doses are required to be administered intravenously for a prolonged period [15]. This regimen is practically impossible to comply with in rural settings. Increasing use of eflornithine as a single agent leaves it exposed to development of resistance. The main side effects of eflornithine include seizures, fever, infections, neutropenia, hypertension and diarrhea. These effects are generally reversible once drug intake is discontinued provided they are properly managed. Septic shock was identified as the cause of the eflornithine related deaths under treatment. Studies on the combination nifurtimox-eflornithine (NECT) using a shortened course of eflornithine have recently been completed with a higher cure rate (96.5%) than with eflornithine alone (91.6%), fewer severe adverse events as well as treatment-related deaths. Undoubtedly, the NECT therapy also is of short and long-term benefit due to reduction of the chance for the development of resistance to eflornithine [9]. NECT has now been considered as the front line treatment for late-stage *T.b. gambiense* infection [9].

Fexinidazole is a 2-substituted 5-nitroimidazole that was rediscovered by the Drugs for Neglected Diseases initiative (DNDi) after screening more than 700 new and existing nitroheterocycles [16]. Fexinidazole is the first new clinical drug candidate in 30 years with the potential to treat advanced-stage sleeping sickness. After promising pre-clinical pharmacological and safety studies, fexinidazole as oral treatment has entered first in-human phase I studies in September 2009 [11, 16].

Oxaboroles also showed activity in animal models of *Tb* infection [17]. Some of these compounds cured murine CNS infection, however they were actively transported from the brain and had to be administered at high doses. New compounds that are not effluxed from the brain were identified, for example a compound designated SCYX-7158 became a pre-clinical candidate at the end of 2009 [11, 17].

There are great future prospects for the discovery of potential antitrypanosomal drugs to combat HAT after the genome sequencing of the kinetoplastid protozoal pathogens (*Tb*, *Tcr* and *Leishmania*) has been completed. Efforts are under way to exploit RNAi (functional only in *Tb*) [18] as well as whole organism screening approaches. A number of inhibitors with antitrypanosomal activity have been identified by both approaches, but none of the programs are yet at the stage of identifying a pre-clinical candidate. Considering the severe disadvantages of existing drugs, there is a clear and pressing need for the development of safer and more effective drugs for the treatment of HAT.

1.1.2. American Trypanosomiasis (Chagas Disease)

Chagas disease (CD), caused by the kinetoplastid parasite *Trypanosoma cruzi* (*Tcr*), is transmitted in about 90% of all cases by faeces of blood sucking Triatomine insects (Triatominae are a subfamily of the family Reduviidae). Further sources of infection are blood transfusion, organ transplantation, laboratory accidents, oral or congenital contaminations [19-21]. CD affects approximately ten million people worldwide, mostly in Latin America where it is endemic but also in other non-endemic areas where infections are due mainly to non-vectorial transmission routes related to population mobility of infected individuals, lack of universal blood bank screenings, vertical transmission (from infected mother to child) and/or organ donation [22]. Although both vectorial and transfusional transmission have sharply declined in the past decades as consequence of governmental policies, a variety of challenges related to public health services and health-care supports, epidemiological control strategies, educational approaches, and development of new therapeutics, still need to be overcome [23-25].

The life cycle of *Tcr* involves hematophagous triatomine insects (vectors) and vertebrate hosts. Bloodstream trypomastigotes ingested by the triatomine differentiate into epimastigotes that proliferate in the insect midgut, differentiating in the posterior intestine into metacyclic forms. Metacyclic trypomastigotes released with faeces of the blood-sucking triatomines are able to infect cells of the mammalian hosts. Intracellularly, they undergo differentiation into amastigotes, which proliferate and transform back into trypomastigotes, which are the main parasite forms released during the host cell lysis [26].

CD is characterized by two consecutive clinical steps. A short and frequently oligosymptomatic acute phase, which involves flu-like symptoms and presents patent parasitemia. The acute stage is associated with relatively low mortality rates (<8%). Immunocompetent individuals control the parasite burden and get into the second, chronic, phase. After several months and even decades, about 30-40% of patients develop clinical symptoms characteristic of this phase, mainly cardiac and/or digestive disturbances [27, 28]. Although the exact mechanisms that trigger chronic manifestations are still unclear, the low but persistent parasitism due to unbalanced immune responses and/or parasite/host genetic heterogeneity have been associated with disease progression [29].

The therapy of CD is based on two old drugs empirically developed about forty years ago - the nitro derivatives benznidazole (Bz) and nifurtimox (Nf). They are recommended for all acute, reactivated and early chronic cases [30] and recent data also suggest the benefits of Bz therapy for later chronic patients [31-33]. However, both nitro compounds are far from being ideal since frequently they are not well tolerated, causing multiple side effects and presenting limited efficacy especially in the later chronic disease [34-39]. Unfortunately, since the clinical introduction of Bz and Nf, only allopurinol and a few azoles like itraconazole, fluconazole and ketoconazole have been used to treat CD, but none really proved substantial efficacy [30, 40-42]. Recently, therapeutic success to treat a chronic CD patient with systemic lupus erythematosus was demonstrated with the use of posaconazole [35, 43, 44]. However, the clinical use of this anti-fungal agent for CD may be limited due to its high costs [35].

There is hence an urgent need for identifying alternative drugs with higher tolerability, broader efficacy, lower costs, suitability for the oral route and shorter time of administration. Presently, drug development efforts for CD lie almost exclusively in pre-clinical research, and only posaconazole and a prodrug of ravuconazole are

planned for future phase II studies [45]. The existing gap between pre-clinical studies and clinical trials must be filled by trans-disciplinary efforts focusing on pooled funds and expertises allowing the identification of new and feasible therapeutic options [46].

In this context, the design of novel candidates may be approached by the search of specific targets in *Tcr* through cellular and molecular approaches aiming to combine high efficacy and low toxicity [32, 35]. The development of inhibitors for a large variety of metabolic pathways is continually being performed, including trypanothione and pyrophosphate metabolism, cysteine protease, purine and ergosterol biosynthesis and phospholipids [44-49]. Alternatively, the screening of already known compounds (from natural and synthetic sources) especially such used in the therapy of other diseases as well as their new derivatives may represent another interesting approach for the search of new therapies for CD [34].

1.1.3. Leishmaniasis

Leishmaniases represent a set of severe human diseases, producing different clinical manifestations depending on the *Leishmania* species involved and the host's immune response [50]. The etiological agents of these diseases consist of about 20 known species of *Leishmania* that have been associated with various disease forms in man [2]. Leishmaniases are endemic in Africa, America, the Indian sub-continent and in sub-tropical southwest Asia as well as the Mediterranean. In 2009, Leishmaniases affected approximately 10 million people world-wide [2, 3] and cause about 50,000 deaths each year.

Leishmania species are obligatory intracellular protozoan parasites residing in macrophages of their mammalian hosts. The extracellular stage, the promastigote, is introduced into subcutaneous tissue in the human host during the bite of an infected sandfly vector, *Phlebotomus* (Old World) and *Lutzomyia* (New World), both subfamily Phlebotominae, family Psychodidae. The promastigote is phagocytosed by a mononuclear phagocyte, after which it converts into the obligatory intracellular form, the amastigote [51].

Two general forms of the disease can occur: visceral leishmaniasis (VL), caused by 2 species of *Leishmania* (*L. donovani* (*Ldon*) and *L. infantum* (*Linf*) = *L. chagasi* (*Lcha*)), and tegumentary leishmaniasis (TL) caused by several dermatropic species of *Leishmania*. This disease is the third largest among the infectious diseases transmitted by vectors, after malaria and filariasis [51]. Leishmaniasis affects around 10 million people worldwide, with an annual incidence of approximately two million new cases, and 350 million are living at risk to be infected. VL causes 500,000 new cases per year globally, 90% of the new cases occuring in just five countries (India, Bangladesh, Brazil, Nepal and Sudan). Tegumentary forms of the disease affect 1,500,000 people. Although not a killing disease as is VL, disfigurement, disability, social and psychological stigma are all severe consequences of TL. In South American countries, *Leishmania amazonensis* (*Lam*) causes a broad spectrum of clinical manifestations, ranging from single cutaneous lesions to multiple, often disfiguring nodules associated with the mucosal form and even occasional visceral complications [52, 53]. This species is described as the unique etiologic agent of anergic diffuse cutaneous leishmaniasis in Brazil, a condition that is associated with specific impairment of the cell-mediated immune response at an early stage of infection. Illness caused by this parasite varies from self-cure to chronic infection.

The development of the leishmanial infection depends on the parasite properties, such as infectivity, pathogenicity and virulence, besides the heterogeneity of the host regulatory response resulting in different clinical manifestations [54]. The host immune response participation could be affecting the answer to the treatment and the disease progression.

The recommended drugs for the treatment of leishmaniasis are pentavalent antimonials (SbV compounds: sodium stibogluconate, Pentostam®; meglumine antimoniate, Glucantime®) since the 1950s. These drugs are problematic due to toxicity/side effects [55, 56] and teratogenicity, as well as increasing resistance [57-60].

Pentamidine (Pentacarinat®) (see also 1.1.1.) is used as an alternative drug in cases resistant to antimonials, but severe side effects arising in the required long term treatment are reported [61, 62].

The antifungal polyene antibiotic amphotericin B (AmB) is used as a second-line antileishmanial drug in developing countries and as a first-line drug in industrialized countries [63]. Cure rates of up to 90% are achieved, but successive relapses possibly leading to AmB resistance in HIV/*Leishmania infantum* co-infection have been reported [64].

Serious acute side effects following infusion have been reported [65]. Alternatively, liposomal amphotericin B (L-AmB) can be used [65]. L-AmB leads to greater drug accumulation in infected cells, thereby increasing the therapeutic index and becoming more efficient, both in immunocompetent and immune compromised patients [66]. L-AmB is less toxic than conventional AmB, but in rare cases anaphylaxis has also been reported here. However, liposomal applications are prohibitively expensive and it is therefore difficult to increase their use in developing countries [66].

Since 2002, the oral antineoplastic agent miltefosine (Impavido®), an alkylphosphocholine, is available on the market for the treatment of leishmaniasis [67]. Cure rates of up to 94% have been documented [68]. Nevertheless, there are concerns about developing resistance [69, 70], teratogenicity as well as side effects [66, 71].

Interferon-γ can be administered in combination with SbV compounds, especially in immunosuppressed patients. It triggers the phagocytic activity of macrophages in order to kill amastigotes that settle in them. However, this treatment is cost intensive and due to lack of clinical trials it remains limited today [72].

The triazole antimycotic posaconazole, currently under investigation by the Drugs for Neglected Diseases initiative (DNDi) against Chagas disease, was found active against *Lam* and *Ldon* in rodent models [73]. Further compounds investigated by the DNDi are oxaboroles [66] and nitroimidazole compounds. Both showed *in vivo* efficacy and are currently in pharmacokinetic trials.

The antimalarial compound tafenoquine was reported to be highly active against *Leishmania in vitro* and in a rodent model [73]. Another antimalarial, orally administered artemisinin, was shown to effectively reduce the parasite burden in a BALB/c model of VL [74].

A vast number of natural products have been reported to show antileishmanial activity, including among others naphthoquinones, neolignans, alkaloids and chalcones [75-77]. Given the activities of these agents and their diverse range of effects on parasite biology, natural products are a potentially rich source of drug candidates against leishmaniasis.

1.1.4. Malaria

Human malaria is caused by the apicomplexan parasites *Plasmodium falciparum (Pfc), P. vivax (Pviv), P. malariae, P. ovale* and *P. knowlesi*. The disease– in spite of still representing a major poverty-associated health risk in many tropical countries– is no longer considered neglected by the WHO. This is largely due to the number of different initiatives currently in existence ranging from drug discovery to vaccine development. Emerging resistance to existing standard therapies also in the case of malaria urgently call for the development of new drugs. It is, however, important to stress that malaria in many respects still remains a neglected tropical disease: It is still most seriously affecting populations in

poor economic and hygienic conditions and the death toll from this disease in Africa constitutes over 90% of the total number of global deaths. Direct/indirect economic impact results in an estimated US $ 12 billion annual income loss in Sub-Saharan Africa [78]. Each year there are between 300 and 500 million clinical cases of malaria world-wide; estimates of the number who die from the disease range between one million and 2.5 million annually. A disproportionate number of deaths from malaria occur among the poor, and about half of those who die are children and pregnant women [78]. Currently, there is no vaccine for malaria and the parasite has already developed resistance against almost all currently available antimalarials. Unfortunately, new reports indicate the emergence of resistance against artemisinin derivatives as well [79].

Moreover, the efforts in drug development mainly focus on the most lethal parasite species, *P. falciparum*, neglecting the very serious problem of malaria caused by *Plasmodium vivax (Pviv)*. Although *Pviv* malaria is often referred to as 'benign', this form of malaria is a debilitating, life-threatening and economically repressive disease that is endemic to many tropical and temperate countries [80, 81]. According to one estimate, this parasite is responsible for an annual burden of US$ 1.4 to 4.0 billion worldwide [82]. *Pviv* malaria accounts for an estimated 80 million cases annually worldwide. More people worldwide live at risk from *Pviv* than *Pfc* [82]; 80 to 90% of all *Pviv* infections occur in the Middle East, Asia and the Western Pacific, the remaining 10-20% in Central and South America [83]. Due to the development of hypnozoites (dormant parasites) in the liver, this species may lead to frequent relapses. For radical treatment of *Pviv* infections, only the 8-aminoquinoline primaquine is commercially available. Despite the burden of *Pviv* malaria, relatively little effort is devoted to the development of new antimalarials against blood asexual stages or hypnozoites [84], which is partly due to the concept of a more benign disease. Moreover, no robust reproducible *in vitro* assaying system is available to cultivate this parasite's blood stage, which invades almost exclusively reticulocytes. Furthermore, only two *Pviv* vaccine candidates have reached phase I clinical trials which is paltry when compared to the more than 20 *Pfc* candidates, one of which is in phase III trials [85]. For these reasons the control of *Pviv* is refractory to the many existing tools available for *Pfc* control, and these associated gaps of knowledge [86] are considered to be a bane to renewed efforts to eradicate malaria [87].

Chloroquine is still the drug of choice in many developing countries for the treatment of *Pviv* malaria. However, chloroquine-resistant *Pviv* was reported frequently from various affected regions [88-91]. It is hence probable that the policy will change in the near future, with the use of artemisinin combination therapy (ACT) [92]. For treatment of dormant liver stages of *Pviv* and prevention of relapses, only primaquine in a 14-day regimen is effective. Its use is accompanied by poor compliance and is counter indicated for pregnancy, infants, and G6PD deficient populations, in which there is an increased risk of severe hemolysis [93].

NIH/NIAID ''Research Agenda for Malaria'' [94] and Roll Back Malaria ''Global Malaria Action Plan'' [95] have called for the development of a new class of anti-malarial drug with better safety and pharmacodynamic profiles while retaining anti-hypnozoite (to eliminate relapses) and anti-gametocyte (to combat disease transmission) activities. As with the natural product derived quinolines and artemisinin-based drugs, plant natural products offer promise as leads towards the development of new drugs for the treatment of *Pviv* (and *Pfc*) malaria.

2. THE POTENTIAL OF SECONDARY METABOLITES FROM PLANTS AS ANTIPROTOZOAL AGENTS

Natural products in the era of rational drug design still play an important role in the search for new active drugs. Besides the traditional role that many natural products have played and still play as drugs, they often represent valuable lead structures that can be developed further into useful therapeutics. A review by Newman und Cragg [96] shows in a very impressive manner that a large fraction of drugs newly approved within the last 25 years directly or indirectly (i.e. after chemical modification or synthesis of natural products or their analogs) have their origin in nature. Even more pertinent is their consideration of all approved anti-tumour drugs since the 1940s, of which more than 50% are of natural origin [96]. In a similar way, many drugs used against infectious diseases are natural products or derivatives. Perhaps the most prominent example in history of a remedy used successfully since ancient days against a protozoan infection would be the natural alkaloid quinine which is still used successfully and increasingly against malaria today. A likely reason why so many natural products are biologically active is the way in which these products have evolved. Natural products are the results of an evolutive process in which many such chemicals have been optimized under the selective forces of coevolution of the organisms producing them with their predators. For a thorough discussion of a plausible explanation for the relationship between chemical and biological diversity of secondary metabolites see [97]. Such natural chemicals can be considered pre-optimized to be potentially bioactive and therefore to possess "drug-like properties". Many chemoinformatics studies exist which show that a large fraction of natural products are especially „drug-like" or at least „lead-like" with structural and physicochemical properties rendering them potential drugs or leads (e.g. [98, 99]). Some authors have even suggested a "natural-product-likeness score" as a means to filter large chemical databases to find new active entities suitable for testing [99].

An exemplary study on one large class of plant secondary metabolites with a huge diversity of chemical structures and biological activities, sesquiterpene lactones (see section 4.2.1) has shown for the majority of this assembly of natural products with about 5000 different known chemical structures that they are very "drug-like" from a physicochemical point of view [100]. This statement is illustrated nicely for the sesquiterpene lactones as well as for another class of structurally and biologically diverse natural products, namely, lignans (see part II [1]) by comparing their structural properties with those of large collections of clinical drug candidates as well as available chemicals (Fig. **1**). In this example, a set of descriptors of structural features considered relevant for biological activity (so-called BCUT descriptors [101, 102], was generated for each of the mentioned groups of molecules and the resulting data matrix treated by a principal component analysis (PCA) [103]. The coordinate axes of the resulting chemical feature space are the first three principal components so that similar positions in this space correspond to an overall similarity of properties [103]. It is quite obvious that sesquiterpene lactones as well as lignans cluster in a very similar region of this space as the clinical candidates, while the properties of a randomly chosen sample of chemicals are much more widespread.

Cordell's often cited statement that "natural products offer a diversity of structure which simply cannot be matched through even the most active imaginations of the synthetic organic chemists" [107] certainly holds true. However, with respect to structural properties, "available chemicals" appear to be the more diverse set, at least when compared to the two groups of natural products just mentioned. The true advantage of natural products in comparison with an unfocused assembly of chemicals is that their diversity is focused to a region of chemical property space that apparently represents compounds with particular "bioactive" and "drug-like" structural and physicochemical features.

The huge efforts of numerous groups performing research in the field of natural products in order to find new molecules with activity against protozoans causing neglected diseases in this light deserve special attention. Section 4 of the present part I as well as

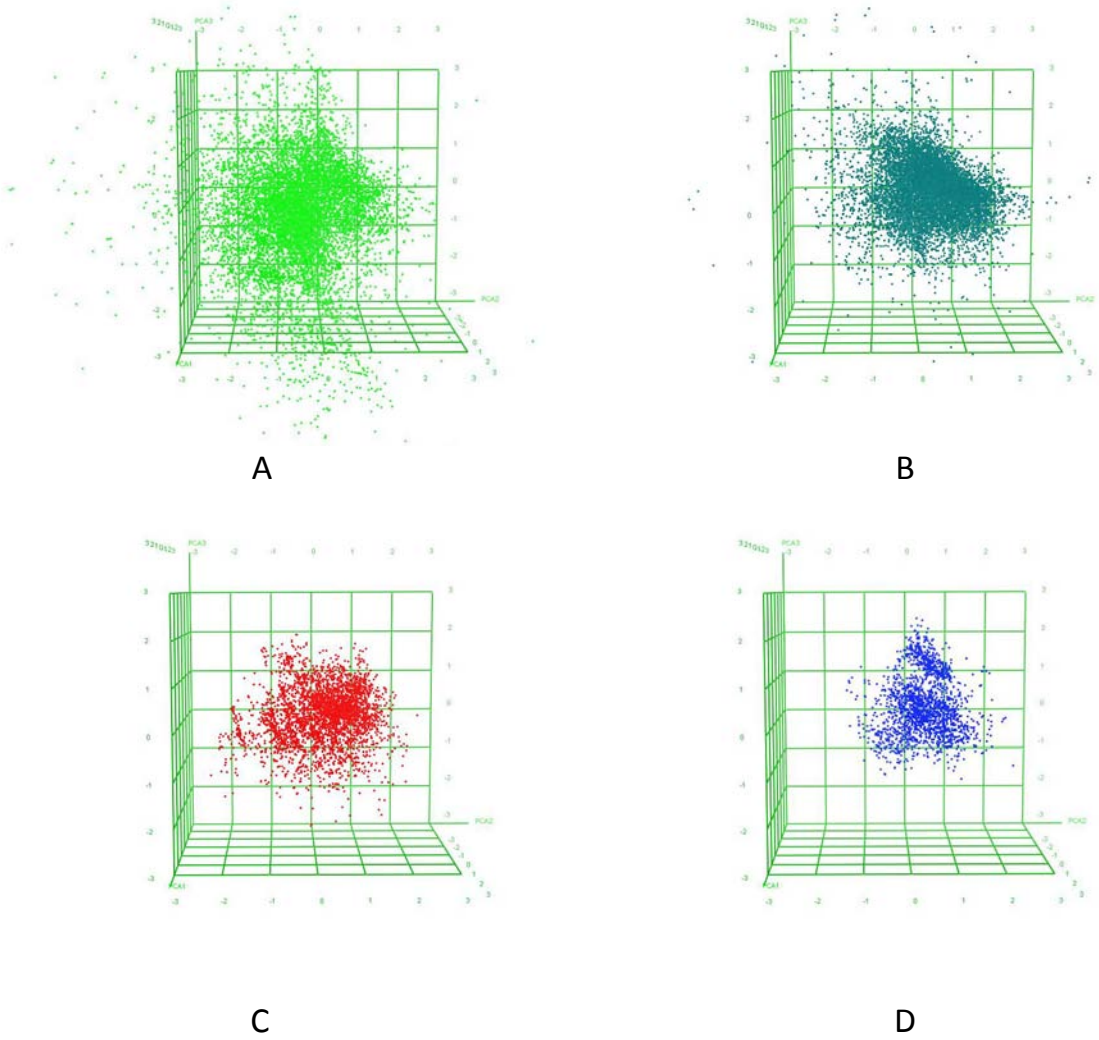


Fig. (1). Chemical feature space as result of a principal component analysis [103] of 24 BCUT-descriptors [102], calculated for the chemical structures of (**A**) 10125 commercially available chemicals [104], (**B**) 10297 clinical drug candidates [105], (**C**) 4861 sesquiterpene lactones and (**D**) 2575 lignans extracted from [106]. The feature space is defined by the first three principal component axes. Each point represents a single chemical entity so that similar position relates to chemical similarity. The regions occupied by the sesquiterpene lactones and lignans are very close to that occupied by "drug candidates " whereas „chemicals" distribute over a much larger volume, i.e. a much broader range of structural/chemical properties.

part II of this review [1] attempt to summarize these efforts. From this summary, the vast diversity of antiprotozoal natural products will become obvious.

3. DETECTION OF BIOACTIVE NATURAL PRODUCTS

3.1. Special Aspects Related to Screening and Testing of Extracts

A crude extract prepared from a plant or its parts typically contains from dozens to hundreds of different chemical compounds belonging to diverse biosynthetic classes of natural products and thus represents a natural library of potentially bioactive molecules. Therefore, when testing crude extracts or fractions thereof, it is important to bear in mind that a whole compound library is tested at once.

Bioassays for anti-infective chemicals can be performed with entire infected organisms (*in vivo*), cell based (*ex vivo*) or biomolecular target (*in vitro*) based assays. The latter two, often both referred to as *in vitro* tests, are widely used to initially detect anti-protozoal activity of new chemical entities such as natural products since only small amounts in the order of a few milligrams are needed of a crude extract or pure compound, whereas substantial amounts in the range of 100 milligrams to grams are needed for thorough *in vivo* studies.

Anti-protozoan drug discovery and development requires natural products with well defined purity and identification, a laborious and time consuming process [107, 108]. Some aspects related to the complex nature of the material must be kept in mind when setting out to test crude extracts or even fractions. Firstly, the composition of a particular extract represents only a "sub-library" of the total extractable chemistry of the extracted material. Hence, the choice of solvent naturally determines which part of the potentially extractable material is present. There is certainly no rule directing the choice of extract in the search for compounds with a particular bioactivity. However, since most "drug-like" molecules are of medium or low polarity and small molecular size, a good choice are usually extracts obtained with dichloromethane, ethyl acetate or alcohols. It is in any case a matter of trial and error to find the opimal extraction method for a particular plant that is to be investigated.

An example from one of the authors' experience is depicted in Fig. (**2**). The diagrams show antitrypanosomal activity data (IC_{50}, µg/mL, against *Tbr* bloodstream forms and *Tcr*, intracellular forms)

for various extracts obtained from aerial parts of two plant species, *Xanthium brasilicum* and *Ageratum conyzoides* (both Asteraceae). In both cases, it was very obvious that the dichloromethane extract would be the best choice to start an in-depth investigation on anti-*Tbr* constituents, the hexane and ethyl acetate extracts being already significantly less active; in both cases, a methanol- or even aqueous extract would not have been useful [109]. Certainly, such studies cannot be performed if a large number of extracts is to be screened. However, it is recommendable to test at least one non-polar and one medium polar extract.

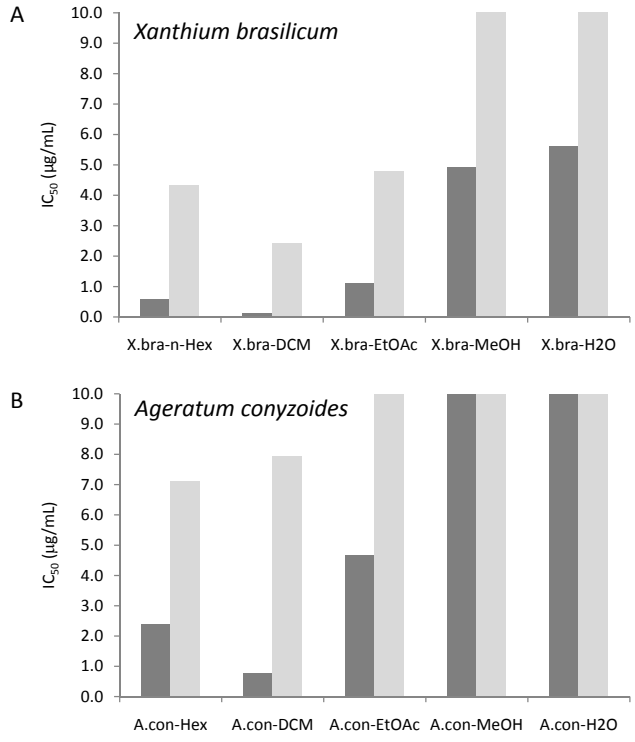


Fig. (2). Antitrypanosomal activity of crude extracts obtained with various solvents of different polarity from aerial parts of *Xanthium brasilicum* (**A**) and *Ageratum conyzoides* (**B**), both Asteraceae. Dark grey bars: *Tbr* (bloodstream forms), Leight grey bars: *Tcr* (intracellular amastigotes cultivated in L6 rat skeletal myoblasts). Data are from Ref. [109].

To continue the above mentioned example, in case of *X. brasilicum*, bioactivity-guided fractionation/isolation readily afforded sesquiterpene lactones with high *in vitro* antitrypanosomal activity [109], while *A. conyzoides* did not yield any compounds that could be held responsible for the high activity of the crude extract. This latter example may remind the reader that phenomena such as synergistic effects or unstable constituents may be at work in case of such complex mixtures which can lead to a loss of activity during workup.

Another important issue in enzyme-based assays is the fact that polar extracts almost always contain some polyphenolic constituents such as tannins that will unspecifically inhibit the function of many, if not all, proteins. Crude extracts obtained with alcohols and water may therefore not be suitable in such enzyme assays without prior removal of polyphenols.

Efficient screening of larger collections of crude extracts allowing dereplication and priorization of plant extracts for isolation of new bioactive natural products is typically based on chromatography hyphenated to UV, MS, MS/MS and NMR techniques [110-112]. For examples applying such approaches to detect and identify natural products with antiprotozoal activity see, e.g., [112-117].

3.2. *In-vitro* Enzyme Assays

Screening for potential antiparasitic agents by targeting specific enzyme targets has the advantage in identifying selective enzyme inhibitors that might be otherwise overlooked because of drug delivery or drug bioavailability shortcomings in *in-vitro* or *in-vivo* antiprotozoal screening methods. Many potential protozoal parasite protein targets have been identified and validated in the last two decades (Table **1**), and numerous hypothetical targets have been suggested through genomic analyses [118-122].

Screening of plant extracts and/or phytochemical agents against protozoal biochemical targets has the advantage of identifying hits based on target inhibition that *in-vitro* or *in-vivo* assays against the living parasites would have missed due to problems of drug transport, bioavailability, or membrane permeability. However, only a few screening studies have been undertaken with plant extracts/phytochemicals on parasite target proteins (Table **2** and Fig. **3**).

Bloodstream forms of trypanosomatids lack functional tricarboxylic acid cycles and are therefore highly dependent on glycolysis for ATP production [123]. Glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) has been identified as a key target for antitrypanosomal chemotherapy. Tomazela and co-workers [124] have identified two pyranochalcones and three flavonoids from *Neoraputia magnifica* that inhibit *Tcr* gGAPDH (Table **2**). A number of coumarins from various plant sources has been screened for *Tcr* gGAPDH inhibitory activity (Table **2**), with chalepin showing notable activity (IC_{50} = 64 µM) [125]. Indeed, *Tcr* gGAPDH has been co-crystallized with chalepin and the structure refined to 1.95 Å resolution [126]. Monomethyl kolavate, isolated from the stem bark of *Entada abyssinica*, was found to inhibit *Tb* gGAPDH with an IC_{50} of 12 µM [127]. The indolosesquiterpene alkaloids polysin, greenwayodendrin-3-one, 3-*O*-acetylgreenwayodendrin, and polyveoline, from the hexane bark extract of *Polyalthia suaveolens* showed weak to moderate inhibiton of *Tb* gGAPDH [128]. These compounds also inhibited *Tb* phosphofructokinase (TbPFK) while 3-*O*-acetylgreenwayodendrin and polyveoline inhibited *Tb* aldolase (TbALD) [128].

Cruzain is the major cysteine protease of *Tcr*, and has been the focus of much recent anti-chagasic drug discovery efforts [129]. A number of essential oils have shown cruzain inhibitory activity. Thus, for example, *Croton draco* bark essential oil inhibited cruzain with an IC_{50} of 15.8 µg/mL [130], and the leaf oil of an undescribed *Eugenia* sp. had an IC_{50} of 36.4 µg/mL [131]. Leaf essential oils from Neotropical members of the Lauraceae [132], particularly *Ocotea* spp. [133], showed promising cruzain inhibitory activity ($IC_{50} \leq 20$ µg/mL). Sesquiterpenoids are apparently responsible for the cruzain inhibition of these essential oils: β-caryophyllene (IC_{50} = 32.5 µg/mL), α-copaene (IC_{50} = 5.2 µg/mL), germacrene D (IC_{50} = 22.1 µg/mL), α -humulene (IC_{50} = 28.2 µg/mL) [132], and zingiberene (IC_{50} = 8.6 µg/mL) [131], but there was also notable synergistic as well as antagonistic effects in binary mixtures of essential oil components [132].

Triterpenoids isolated from crude bark extracts have shown cruzain inhibitory activity. Lupeol and the 3-hydroxytetradecanoic acid ester of lupeol inhibited cruzain with IC_{50} values of 40.8 and 53.0 µg/mL, respectively [134]. The chloroform bark extract of a *Salacia* sp. had promising activity (IC_{50} = 11.4 µg/mL), but the individual friedelane triterpenoid components were less active: canophyllol (IC_{50} = 87.4 µg/mL), 25,28-dihydroxyfriedelin (IC_{50} = 154 µg/mL), 29-hydroxyfriedelan-3-on-28-al (IC_{50} = 71.1 µg/mL), and tingenone (IC_{50} = 79.8 µg/mL) [135].

The major cathepsin L-like protease from *Tbr* is rhodesain [136]. Phytochemical inhibitors of this cysteine protease include

Table 1. Potential Biochemical Targets for Antiprotozoal Chemotherapy
Note that the references cited in this table are not part of the main manuscript. A list of these references ([S1]-[S107]) is available as supportive/supplementary material

Organism	Protein Target	Reference
Leishmania amazonensis	Oligopeptidase B (LaOPB)	[S1]
Leishmania donovani	Adenine phosphoribosyltransferase (LdAPRT)	[S2]
	Cyclophilin (LdCYP)	[S3]
	Glyoxalase II (LdGLOII)	[S4]
	N-Myristoyltransferase (LdNMT)	[S5]
	Nucleoside hydrolase (LdNH)	[S6]
	Oligopeptidase B (LdOPB)	[S7]
	Pteridine reductase (LdPTR)	[S8]
	S-Adenosylhomocysteine hydrolase (LdAdoHcyase)	[S9]
	Uridine 5'-monophosphate synthase (LdUMPS)	[S10]
Leishmania major	Deoxyuridine triphosphate nucleotidohydrolase (LmajdUTPase)	[S11]
	Dihydroorotate dehydrogenase (LmajDHODH)	[S12]
	Glyoxalase I (LmGLOI)	[S13]
	Methionyl-tRNA synthetase (LmajMetRS)	[S14]
	N-Myristoyltransferase (LmNMT)	[S15]
	Nucleoside diphosphate kinase b (LmajNDKb)	[S16]
	Nucleoside hydrolase (LmajNH)	[S17]
	Oligopeptidase B (LmajOPB)	[S18]
	Peroxidase (LmajAPX)	[S19]
	Phosphodiesterase B1 (LmajPDEB1)	[S20]
	Pteridine reductase 1 (LmajPTR1)	[S21]
	Trypanothione synthetase-amidase (LmajTrySA)	[S22]
	Tyrosyl-tRNA synthetase (LmajTyrRS)	[S23]
	UDP-glucose pyrophosphorylase (LmajUGP)	[S24]
Leishmania mexicana	Aldolase (Lmex ALD)	[S25]
	Glucose-6-phosphate isomerase (Lmex PGI)	[S26]
	Glyceraldehyde-3-phosphate dehydrogenase (Lmex GAPDH)	[S27]
	Phosphoglycerate mutase (Lmex iPGAM	[S28]
	Phosphomannomutase (LmexPMM	[S29]
	Pyruvate kinase (LmexPyK)	[S30]
	Triosephosphate isomerase (Lmex TIM)	[S31]
Plasmodium falciparum	Arginase	[S32]
	Calpain	[S33]
	Chorismate synthase (PfCS)	[S34]
	Cyclophilin (PfCYP19)	[S35]
	1-Deoxy-D-xylulose-5-phosphate reductoisomerase (PfDXR)	[S36]
	Deoxyuridine triphosphate nucleotidohydrolase (PfdUTPase)	[S37]
	Dihydrofolate synthase-folylpolyglutamate synthase (PfDHFS-FPGS)	[S38]
	Dihydroorotate dehydrogenase (PfDHOD)	[S39]
	Enoyl-acyl carrier protein reductase (PfENR)	[S40]
	Falcipain-2 (PfFP-2)	[S41]
	Ferredoxin-$NADP^+$ reductase (PfFNR)	[S42]
	Glutathione reductase (PfGR)	[S43]
	Glutathione *S*-transferase (PfGST)	[S44]
	Glyceraldehyde-3-phosphate dehydrogenase (PfGAPDH)	[S45]
	Glycerol kinase (PfGK)	[S46]
	Guanylate kinase (PfGK)	[S47]
	Hypoxanthine guanine phosphoribosyltransferase (PfHGPRT)	[S48]

(Table 1). Contd…..

Organism	Protein Target	Reference
	Hexose transporter (PfHT)	[S49]
	Lactate dehydrogenase (PfLDH)	[S50]
	Ornithine δ-aminotransferase (PfOAT)	[S51]
	Ornithine decarboxylase (PfODC)	[S52]
	Orotidine 5′-monophosphate decarboxylase (PfODCase)	[S53]
	3-Oxoacyl-acyl carrier protein reductase (PfOAR)	[S54]
	Peptide deformylase (Pf PDF)	[S55]
	Peptidylprolyl cis-trans isomerase (PfPPIase = PfFKBD)	[S56]
	3-Phosphoglycerate kinase (PfPGK)	[S57]
	Plasmepsin 1 (PfPM1)	[S58]
	Protein Farnesyltransferase (Pf PFT)	[S59]
	Purine nucleoside phosphorylase (PfPNP)	[S60]
	S-Adenosylmethionine decarboxylase (PfAdoMetDC)	[S52]
	Spermidine synthase (PfSpdSyn)	[S61]
	Thioredoxin reductase (PfTrxR)	[S62]
	Thymidylate kinase (PfTMPK)	[S63]
	Triosephosphate isomerase (PfTIM)	[S64]
Trypanosoma brucei	Adenosine kinase (TbAK)	[S65]
	Aldolase (TbALD)	[S66]
	Alternative oxidase (TbAOX)	[S67]
	Cathepsin B (TbCatB)	[S68]
	Dihydrofolate reductase (TbDHFR)	[S69]
	Dihydroorotate dehydrogenase (TbDHODH)	[S70]
	Glyceraldehyde-3-phosphate dehydrogenase (TbGAPDH)	[S66]
	Glycogen synthase kinase 3 (TbGSK3)	[S71]
	Hexokinase 1 (TbHK1)	[S72]
	N-Myristoyltransferase (TbNMT)	[S73]
	Nucleoside 2-deoxyribosyltransferase (TbNDRT)	[S74]
	Nucleoside hydrolase (TbNH)	[S75]
	Oligopeptidase B (TbOP)	[S76]
	Ornithine decarboxylase (TbODC)	[S77]
	Pteridine reductase 1 (TbPTR1)	[S78]
	Pyruvate kinase (TbPyK)	[S79]
	Rhodesain	[S80]
	Sphingolipid synthase (TbSLS)	[S81]
	Sterol 14α-demethylase (TbCYP51)	[S82]
	Triosephosphate isomerase (TbTIM)	[S83]
	Trypanothione reductase (TbTryR)	[S84]
	Trypanothione synthetase (TbTryS)	[S85]
	UDP-galactose 4'-epimerase (TbUDPGE)	[S86]
Trypanosoma cruzi	Aldolase (TcALD)	[S87]
	Adenosine kinase (TcAK)	[S88]
	Cruzain	[S89]
	Cyclophilin (TcCYP19)	[S90]
	Deoxyuridine triphosphatase (Tc dUTPase)	[S91]
	Dihydrofolate reductase (TcDHFR)	[S92]
	Dihydroorotate dehydrogenase (TcDHODH)	[S93]
	Farnesyl diphosphate synthase (TcFPPS)	[S94]

(Table 1). Contd…..

Organism	Protein Target	Reference
	Glyceraldehyde-3-phosphate dehydrogenase (TcGAPDH)	[S95]
	Hexokinase (TcHK)	[S96]
	Hypoxanthine phosphoribosyltransferase (TcHPRT)	[S97]
	Nucleoside diphosphate kinase b (TcNDKb)	[S98]
	Old yellow enzyme (TcOYE)	[S99]
	Oxidosqualene cyclase (TcOSC)	[S100]
	Phosphofructokinase (TcPFK)	[S87]
	Pteridine reductase 2 (TcPTR2)	[S101]
	Pyruvate kinase (TcPYK)	[S30]
	Sterol 14α-demethylase (TcCYP51)	[S102]
	Squalene synthase (TcSQS)	[S103]
	Triosephosphate isomerase (TcTIM)	[S104]
	Trypanothione reductase (TcTryR)	[S105]
	Trypanothione synthetase (TcTryS)	[S106]
	UDP-galactose 4'-epimerase (TcUDPGE)	[S107]

Table 2. Inhibition of Protozoan Protein Targets By Plant Extracts and Isolated Phytochemicals. Structures See Fig. (3)

Material	Protein Target	IC_{50}	Reference
Polysin (**1**) + Greenwayodendrin-3-one (**2**)	TbGAPDH	1050 μM	[128]
3-*O*-Acetylgreenwayodendrin (**3**)	TbGAPDH	110 μM	[128]
Polyveoline (**4**)	TbGAPDH	310 μM	[128]
Polysin + Greenwayodendrin-3-one (**1**+**3**)	TbPFK	20 μM	[128]
3-*O*-Acetylgreenwayodendrin (**3**)	TbPFK	170 μM	[128]
Polyveoline (**4**)	TbPFK	30 μM	[128]
3-*O*-Acetylgreenwayodendrin (**3**)	TbALD	0.5 μM	[128]
Polyveoline (**4**)	TbALD	80 μM	[128]
Monomethyl kolavate (**5**)	TbGAPDH	12 μM	[127]
3,5-Dimethoxycitrunobin 4-methyl ether (**6**) + 3-methoxycitrunobin 4-methyl ether (**7**)	TcGAPDH	115 μg/mL	[124]
5,5′,6,7-Tetramethoxy-3′,4′-methylenedioxyflavone (**8**) + 5,6,7-trimethoxy-3′,4′-methylenedioxyflavone (**9**)	TcGAPDH	24 μg/mL	[124]
3′,4′,5′,5,7-pentamethoxyflavanone (**10**)	TcGAPDH	33 μg/mL	[124]
Angustifolin (**11**)	TcGAPDH	130 μM	[125]
Bergapten (**12**)	TcGAPDH	347 μM	[125]
Chalepin (**13**)	TcGAPDH	64 μM	[125]
Isoangenomalin (**14**)	TcGAPDH	145 μM	[125]
Osthol (**15**)	TcGAPDH	210 μM	[125]
Phebalosin (**16**)	TcGAPDH	190 μM	[125]
Rauianin (**17**)	TcGAPDH	93 μM	[125]
Scopoletin (**18**)	TcGAPDH	260 μM	[125]
Sesalin (**19**)	TcGAPDH	123 μM	[125]
Xanthyletin (**20**)	TcGAPDH	175 μM	[125]
Quercetin (**21**)	TbHK1	4.1 μM	[144]
Myricetin (**22**)	TbHK1	48.9 μM	[144]
Croton draco bark EO	Cruzain	16.0 μg/mL	[130]
Beilschmiedia tilaranensis leaf EO	Cruzain	23.6 μg/mL	[132]
Eugenia "San Bosco"	Cruzain	36.4 μg/mL	[131]
Ocotea "los llanos" leaf EO	Cruzain	17.1 μg/mL	[132]
Ocotea meziana leaf EO	Cruzain	14.9 μg/mL	[132]
Ocotea "small leaf" leaf EO	Cruzain	19.2 μg/mL	[132]
Ocotea whitei leaf EO	Cruzain	15.8 μg/mL	[132]
Salacia "liana" $CHCl_3$ bark extract	Cruzain	11.4 μg/mL	[134]
β-Caryophyllene (**23**)	Cruzain	32.5 μg/mL	[132]
α-Copaene (**24**)	Cruzain	5.2 μg/mL	[132]
Germacrene D (**25**)	Cruzain	22.1 μg/mL	[132]
α-Humulene (**26**)	Cruzain	28.2 μg/mL	[132]
Lupeol (**27**)	Cruzain	40.8 μg/mL	[134]
Zingiberene (**28**)	Cruzain	8.6 μg/mL	[131]
Bornyl coumarate (**29**)	Rhodesain	92.2 μM	[137]

(Table 2). Contd…..

Material	Protein Target	IC_{50}	Reference
Bornyl caffeate (**30**)	Rhodesain	14.9 μM	[137]
Bornyl ferulate (**31**)	Rhodesain	32.2 μM	[137]
Rhynedulin A (**32**)	Rhodesain	41.8 μM	[138]
Rhynedulin B (**33**)	Rhodesain	138 μM	[138]
Rhynedulin C (**34**)	Rhodesain	40.9 μM	[138]
Cyclochandalone (**35**)	Rhodesain	56.4 μM	[138]
Ulexin B (**36**)	Rhodesain	19.9 μM	[138]
Rhynedulinal (**37**)	Rhodesain	47.4 μM	[138]
Scandenal (**38**)	Rhodesain	16.0 μM	[138]
Cajanin (**39**)	Rhodesain	58.2 μM	[138]
Cajanone (**40**)	Rhodesain	39.9 μM	[138]
Butyl brevifolincarboxylate (**41**)	TcTIM	14 μM	[143]
Ethyl brevifolincarboxylate (**42**)	TcTIM	8.0 μM	[143]
Methyl brevifolincarboxylate (**43**)	TcTIM	6.5 μM	[143]

bornyl cinnamates from *Verbesina turbacensis* acetone bark extract (IC_{50} = 49 μg/mL), bornyl coumarate, bornyl caffeate, and bornyl ferulate, with IC_{50} values of 92.2, 14.9, and 32.2 μM, respectively [137]. The dichloromethane bark extract of *Rhynchosia edulis*, which had shown rhodesain inhibitory activity (IC_{50} = 0.77 μg/mL), provided nine prenylated isoflavonoids that inhibited rhodesain (see Table **2**) [138]. Allicin, one of the main bioactive components of garlic, showed antiprotozoal activity against *Tbb* (IC_{50} = 13.8 μM) and *Pfc* (IC_{50} = 5.21 μM), attributable to inhibition of the cysteine proteases rhodesain and falcipain [139].

Trypanothione reductase (TryR), structurally and mechanistically related to glutathione reductase, is a key flavoprotein in the defense mechanism of trypanosomatids against oxidative stress [140]. Phytochemicals showing activity against *Tcr* TryR are ajoene from garlic (*Allium sativum*) [141] and lunarine from *Lunaria* spp. [142].

Triosephosphate isomerase (TIM) catalyzes the conversion of dihydroxyacetone phosphate to glyceraldehyde-3-phosphate and thus plays an important role in glycolysis and energy production. Brevifolincarboxylate esters from *Geranium bellum* showed inhibitory activity on *Tcr* triosephosphate isomerase [143]. Enzyme inhibitory plant extracts and phytochemicals with their biochemical targets are listed in Table **2**.

3.3. *In-vitro* Cell Based Assays

3.3.1. African Trypanosomiasis

The relevant parasite form to use for *in vitro* assays is the trypomastigote bloodstream form which can be cultivated axenically in established culture media [145, 146]. The question what strain of *Tb* to use has to be extended to the question of subspecies: *T.b.gambiense* (*Tbg*), *T.b.rhodesiense* (*Tbr*) or *T.b.brucei* (*Tbb*)? *Tbg* would be the most relevant subspecies to use (the vast majority of sleeping sickness cases are due to this subspecies) but it is more difficult to cultivate it as compared to *Tbr* and *Tbb*. The latter two subspecies are genetically almost identical and can both be used for drug screening. Two strains which are widely used are STIB900 (*Tbr*) and S427 (*Tbb*), a rather old isolate readily available in many laboratories. There are, however, examples showing that compounds with high activity against *Tbb* are not necessarily highly active against *Tbr* and that compounds yielding selectivity indices >1 against the former can show inverse selectivity (SI<1) when it comes to the latter parasite; compare [117] with [147], see section 4.2.1. Therefore, *Tbr* is probably the better choice when searching for potential leads against the human pathogen.

The assay is run in 72 hours using Alamar blue for endpoint determination [148]. The trypanosome starting density has to be low enough to allow the trypanosome population to expand in logarithmic phase and not reaching stationary phase (~ $2x10^6$/mL) before the end of the assay duration. Alamar blue (resazurin 12.5 mg in 100 mL phosphate-buffered-saline) is added for the last 4 hours or longer if the signal to background ratio is not > 5. A more detailed assay description can be found in [149]. The assay can be performed in two platforms, a medium throughput format as a pre-screen with a single compound concentration, and a serial dilution format with IC_{50} determination. The single dose concentration for extracts can be set to 10 μg/mL or to two concentrations e.g. 20 and 5 μg/mL. For pure compounds lower concentrations should be considered. The serial drug dilution, either 2-fold or 3-fold, usually starts at 90 μg/mL as the highest concentration. Extracts are dissolved in pure DMSO as a 10 mg/mL stock solution and are further diluted with culture medium to a final DMSO concentration of ≤1%.

As reference drugs melarsoprol or pentamidine can be used. Both are highly active *in vitro* with IC_{50} values in the low nM range but also rather toxic. The expectation should not be that a new active compound displays a similarly high activity. On the other hand, the drugs most used to treat sleeping sickness are eflornithine and the combination of nifurtimox/eflornithine [150]. Both drugs cannot be used as reference since they are not really active *in vitro* and their IC_{50} values are > 1μg/mL. Suramin could be used as a reference with an intermediate activity (IC_{50} of ~ 60-150 μg/mL). All these drugs are not easily available.

At an advanced stage the question of testing against other strains with different geographic origin and different characteristics arises. Drug-resistant strains of human origin do not really exist. There are isolates from patients who relapsed after treatment, however, these parasite populations are not phenotypically resistant when tested *in vitro* [151]. There are lab populations which were made resistant under lab conditions [152] or genetically modified populations which lack e.g. an adenosine transporter responsible for the uptake of certain classes of drugs [153]. Also *Tbg* isolates should be included in such a panel of isolates, especially recent isolates from endemic foci in Africa.

3.3.2. American Trypanosomiasis

Considering the many problems arising in the search for new drugs against CD, the identification of a panel of the minimum steps and decision gates to assess the efficacy and selectivity of a lead compound and the minimum criteria to advance the transition of *in vitro* studies to pre-clinical trials was the main purpose of a Technical Note published by Romanha and co-workers [34] which will be briefly summarized here:

To perform *in vitro* cell based assays for drugs against *Tcr* taking into consideration the biological aspects of the parasite and the disease characteristics, primarily there is a need to define mammalian cell lineages, parasite strains and evolutive forms, automated procedures and a cut-off value in comparison with the reference drugs -benznidazole (Bz) and nifurtimox (Nf)- using as

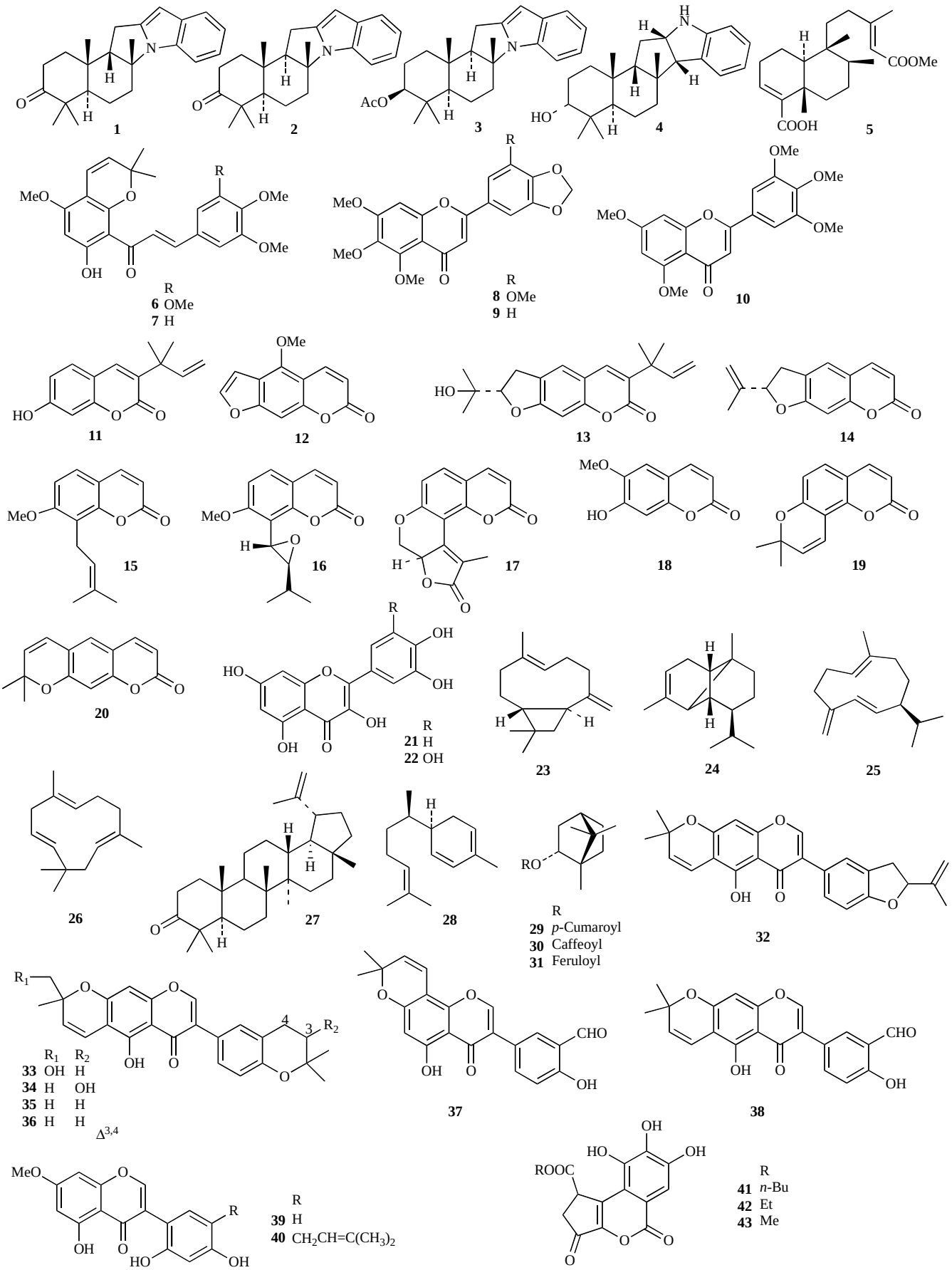


Fig. (3). Structures of natural products with inhibitory activity on protozoan protein targets (For targets and references, see Table **2**).

parameter the IC_{50} and the selectivity index (SI). Since trypomastigotes and intracellular amastigotes are the *Tcr* forms relevant to human infection, they should be used when searching lead compounds. Initially, the direct effect of a test compound on infected cell lines (e.g. Vero, HeLa, L929 or L6) is performed at a single concentration, allowing to check the effects on both amastigotes and trypomastigotes in one system. Based on the activity of Bz (IC_{50} about 1 μg/mL, 3.8 μM), the recommended concentrations for synthetic compounds and extracts of natural products are 1 and 10 μg/mL, respectively. Thus, to allow a large-scale screening, the use of an automated test is strongly recommended, establishing experimental conditions that lead to the parasitization of at least 50% of the host cells. A recommended and well-known parasite source is culture trypomastigotes from the β-galactosidase-transfected Tulahuen strain [154, 155]. Infected cells are exposed to the tested samples during four consecutive days and after the addition of chlorophenol red the enzymatic activity is measured at 570 nm. Compounds that lead to a similar or higher trypanocidal effect as compared to Bz will move on to the next step, which is the determination of the IC_{50} (the drug concentration that eliminates 50% of the parasites), using the same experimental model as above described. Since SI corresponds to the ratio of cytotoxicity:antiparasitic activity, i.e. IC_{50} host cell/IC_{50} parasite, it has been recommended for subsequent *in vivo* assays of *Tcr* infection that only compounds that attain SI $\geq$ 50 are further considered [156]. Next, to evaluate the toxicity to mammalian cells, a single drug concentration equivalent to 50 x the parasite IC_{50} of each compound is assayed using the vital stain alamarBlue.

Due to the broad spectrum of resistance/susceptibility to drugs displayed by different populations of *Tcr*, it is also recommended to analyse the activity of a new compound against parasites of different lineages, including those strains resistant to Bz and Nf, like Colombiana and YuYu strains [157, 158]. However, one of the main limitations to such an analysis is the difficulty of employing other parasite subpopulations, including the Y and Colombiana strains, e.g. transfected with β-galactosidase, or any other enzymes or green fluorescent markers. So, since *Tcr* Tulahuen strain transfected with β-galactosidase is a more easily available choice, its use is also recommended for *in vitro* assays. However, if other automated or alternative screening systems are available such as the one reported by McKerrow and co-workers [159], they may be used to evaluate and compare different parasite stocks from distinct geographical regions and diverse resistance profile to Bz and Nf and belonging to the six discrete typing units –*Tcr* I-VI [160].

It is important to note that, although high-stringency filters were considered in the flow chart proposed by Romanha and co-workers [34], *in vitro* and *in vivo* data do not always reveal a direct correlation since other pharmacological properties of the screened compounds and/or their metabolic products may obscure the distinction between efficacy and toxicity when experimental models (e.g. mice and dogs) are employed.

3.3.3. Leishmaniasis

Bioassay-guided fractionation of natural products usually yields high sample numbers for biological assays. Thus, the use of fast and low cost assays is mandatory; axenic promastigotes or amastigotes fulfill these criteria [161]. Classical methods for assessing activity use light microscopic analysis but are time-consuming, especially when one considers direct counting of parasites after drug incubation. The use of colorimetric tests such as MTT [162] or Alamar Blue [163] results in faster, more precise and less expensive assays requiring relatively inexpensive equipment such as a plate reader. More sophisticated methods using fluorescence-assisted cell sorting (FACS) analysis can easily evaluate the viability of *Leishmania* promastigotes by using SYBR-14, a membrane-permeable nuclear stain, in combination with propidium iodide (PI), a nucleic acid dye that is unable to penetrate intact living cells [164]. Alternatively, *Leishmania* viability can also be quantified by measuring cell division using 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) staining [165].

Considering the metabolic differences, axenic amastigotes present advantages when compared to promastigotes, as they are the clinically relevant stage of the parasite. The methods employ a cell counter [166], cell viability using an MTT-based method [162], ornithine decarboxylase activity [167] or labelling with a fluorescent dye, such as PI, followed by FACS analysis [168, 169]. Although axenic amastigotes are preferred over promastigotes, both are only semi-predictive, since neither provides useful information about penetration of the compound into the host cell nor for activity in the acidic environment of the macrophage phagolysosome [170].

The intracellular assay is hence the most adequate model for screening compounds [171]. However, this is a time-consuming assay that requires light microscopic analysis and previous cytotoxicity evaluations to avoid harmful concentrations to host cells. But with the introduction of reporter gene technology using fluorescent or colorimetry-based techniques, medium to high throughput screenings can easily be performed. A variety of reporter genes have been effectively used in biological screenings, including firefly luciferase, green fluorescent protein (GFP), β-galactosidase, β-lactamase, chloramphenicol acetyltransferase (CAT), and alkaline phosphatase [172]. These reporters facilitate sample screening with high sensitivity. The firefly luciferase gene is a common reporter and has been developed [173] to be used in screening studies. Green fluorescent protein (GFP) also allows imaging and quantification of parasites using a fluorimeter [174], a fluorescence microscope or by FACS analysis [173]. Colorimetric detection through a β-galactosidase reporter has been described for *Leishmania* [175] as a promising technique for drug screening with promastigotes and intracellular amastigotes. However, the use of fluorescent markers or enzymes for the screening of large libraries has many drawbacks. Typically, transfected parasites do not express a sufficient amount of fluorescent protein for microplate reader evaluation and thus require the use of FACS analysis, which decreases the throughput.

Very recently, a promising novel *ex vivo* splenic explant culture system from hamsters infected with luciferase-transfected Ldon has been described which allows an efficient screening of large compound libraries for anti-leishmanial activity [176].

Whatsoever, for an appropriate evaluation, drug susceptibility assays using field isolates are indispensable, since most studies include laboratory parasite strains.

3.3.4. Malaria

P. falciparum (Pfc), etiologic agent of Malaria tropica, can be readily cultured *in vitro* over long periods of time through replenishing of red blood cells and culture medium at routine intervals [177]. Synchronized [178] cultured *Pfc* is the basis for modern antimalarial screening of a broad range of samples including isolated natural products [179]. Many examples for successful testing of natural products against *Pfc* are presented in section 4. In contrast, under *in vitro* conditions, *P. vivax* (*Pviv*) undergoes limited culture due to the need for substantial amounts of reticulocytes. In maturation tests over 48 h of so-called "short-term" culture, *Pviv* growth involves the maturing of young parasite forms within infected red blood cells. Over *ca*. 48 h, about 40 % of parasites reach the schizont form, however, parasitemia decreases over time due to low or no re-invasion of new red blood cells by these growing parasites. As with *Pfc*, parasitemia of *Pviv* is commonly assessed during culture using optical microscopy on stained smears of blood from culture, but usually cultured *Pviv* exhibits lower parasitemias than *Pfc* and due to the lack of synchronization, results are not strictly reproducible. To complicate matters, these tests are supposedly more reliable if they are performed with fresh parasites, therefore they should be performed

only in endemic areas. At present, no evidence exists confirming that tests with frozen parasites lead to similar results. However, despite all the technical limitations, this approach has been used successfully to discriminate between populations with differing levels of clinical efficacy of chloroquine and specific polymorphisms [180]. Recently, a highly sensitive method for evaluating *Pviv* growth in short-term culture was introduced. This method is capable of detecting parasitemia levels as low as 10^{-8} and is based on a colorimetric double-site antigen capture enzyme-linked immunoabsorbent assay (called DELI), which detects lactate dehydrogenase generated by the parasites over 48 h. Ultimately a functional test based on the detection of parasite protein synthesis, this method has been applied to the generation of drug-response curves for known antimalarial compounds and allows for the calculation of 50% inhibitory concentrations (IC_{50}) in a less time-consuming manner [181], and may help in determining the sensitivity of *Pviv* to novel compounds including natural products. Recent *ex vivo Pviv* invasion assays have focused on the mixing of enriched umbilical cord blood reticulocytes with matured, trypsin-treated *Pviv* schizonts concentrated from clinical isolates, with reasonable reliability, but still with the disadvantage of requiring a continuous source of umbilical cord blood, what may limit a high-throughput screening of a large number of available antimalarial candidates [182].

If a *Pviv* sporozoite source is available in insectary facilities, hepatoma cell cultures permit the screening of activity against infective stages of the parasite (screening for transmission preventing or prophylactic drugs). A hepatoma stage blockage using such protocols was demonstrated for extracts of the Amazonian prophylactic plant *Ampelozizyphus amazonicus* (Rhamnaceae) [183], and in the near future testing efficacy against culture-generated hypnozoites should certainly pave the way in the search for drugs, which ultimately may contribute to the eradication of this parasite.

3.4. *In-vivo* Tests

3.4.1. African Trypanosomiasis

Compounds with *in vitro* activity (IC_{50} < 0.2 μg/mL) and a selectivity index > 200 or no overt toxicity when applied to mice can be considered for *in vivo* efficacy studies. In a first step an acute mouse model of infection will be used mimicking the 1st stage of sleeping sickness [184]. In a second step, and only for compounds which resulted in cure of the acute model, a CNS mouse model can be used which mimicks the 2nd stage of human disease [185]. The question of what subspecies and what strain to use comes up. Harmonization among laboratories is an important issue and led to a certain consensus to use either S427 (*Tbb*) or STIB 900 (*Tbr*) for the acute model and GVR35 (*Tbb*) for the CNS model of infection.

For the acute model S427 or STIB 900 can be used or any other *Tbb* or *Tbr* strain which grows well in laboratory mice. Inbred or outbred mice can be used, preferably females because they are easier to keep in small groups. The mice are kept in standard cages under standard conditions, and are given commercial pellets and water *ad libitum*. Groups of 4 mice are infected by the intraperitoneal route with a defined inoculum which depends on the virulence of the trypanosome strain and the susceptibility of the mouse breed used. An untreated group of mice serves as control; these mice should not die before treatment of the experimental groups is completed. Usually a 4 day treatment is applied on days 3 to 6 post-infection. Alternatively, treatment can be done on days 1 to 4 giving the compound a better chance to cure the mice. Parasitemia has to be checked twice per week starting 24 hours after the last compound application for a period of 60 days post-treatment. Primary endpoint is reduction of parasitemia, secondary endpoint can be survival time of mice or time to relapse after parasitemia clearance. Using survival time by allowing mice to die of the infection is not allowed any longer in many countries. Time to relapse can be used as an alternative to time to death. Relapsing mice have to be euthanized before they die of the infection.

Compounds which result in cure of mice after a 4 day treatment regime should be titrated to determine the minimal curative dose (dose response experiment). A single dose corresponding to the total dose applied in the 4 day treatment can give information on the half life of a compound. Standard drugs to be used as positive controls are suramin and pentamidine isethionate, both resulting in cure of the treated mice when dosed appropriately.

T.b.gambiense should only be considered for pure compounds that have demonstrated high efficacy in the primary *Tbb/Tbr* model. *Tbg* strains are difficult to propagate in rodents and usually require an adaptation phase in immuno-compromised or immuno-suppressed rodents [186, 187]. Adapted isolates exist which can be propagated in white mice but only in concert with immuno-suppression by cyclophosphamide.

The GVR35 CNS mouse model [188] uses a chronic *Tbb* isolate which is leading to a CNS infection within 15 days but not to fatalities during the first 30 days. Treatment is delayed to days 21 to 25 post-infection. Monitoring of parasitemia is up to day 180 post-treatment to make sure that no relapse from the CNS has taken place. Standard drugs are diminazene diaceturate at 40 mg/kg at day 21, a 1st stage drug which clears the bloodstream but not the CNS infection leading to a relapse parasitemia around day 20 post-treatment. The only standard drug which can cure this model is melarsoprol at 15 mg/kg given for 5 days. This model is only being used for compounds which are able to cure the acute mouse model after a 4-day treatment.

3.4.2. American Trypanosomiasis

As discussed in item 3.3.2, the establishment of stringent criteria and filters is fundamental to move compounds from *in vitro* to *in vivo* assays avoiding the use of a large unnecessary number of animals taking into consideration (i) ethical aspects related to the use of experimental models (3R principle: reduce, refine, and replace); (ii) infrastructure limitations found in many research laboratories for performing a high number of *in vivo* tests, (iii) the costs and time involved; besides (iv) the potential reduction of laboratory acquired infection due to the manipulation of high number of animals.

The *in vivo* acute toxicity determination is recommended as the first step to exclude toxic compounds/doses to vertebrate hosts. Thus, the use of acute toxicity studies through the determination of Maximum Tolerated Dose (MTD) or NOAEL (No observed adverse effect level) is recommended. An escalating dose study by using a single mouse (one female and one male) for each compound dose may be used fulfilling the 3R principle as suggested by guidelines of the Organisation for Economic Co-operation and Development (OECD) [189]. Then, the infected mice may be treated with three doses, setting the highest dose at the MTD value. *In vivo* efficacy studies are then approached through sequential protocols aiming to achieve reduced parasitemia levels and mortality rates, inducing parasitological cure in mice infected with (i) a moderately benznidazole (Bz)-resistant *Tcr* strain (e.g. Y strain) and (ii) a naturally Bz-resistant strain (like Colombiana strain). The criterion for a compound to progress through these steps is to display a trypanocidal activity similar/superior to that of Bz (100 mg/kg/day, *p.o.*). For the first set of *in vivo* experiments, the use of outbred genetic background animals like Swiss Webster mice acutely infected with Y strain is an interesting experimental model for CD drug screening since it displays an acute infection profile with high parasitemia levels and mortality rates (>70%), allowing a comparison between the effect of the compound/extract tested and that of Bz. As a negative control, the infected and non-treated group should receive the same vehicle used for the dilution

of the compound. It is important to point out that only animals displaying positive parasitemia (evaluated by Pizzi-Brener method [190]) should be used and that the treatment must be started at the parasitemia onset in order to try to reproduce an acute infection, always taking into consideration the search for the best activity under the treatment employing fewer doses (e.g. number of doses necessary to reach parasitological cure using Bz administration at the chosen experimental model). Y strain is a good choice for identification of novel compounds for CD since (i) it presents moderate resistance to Bz and Nifurtimox [158], (ii) it has been largely used in studies of drug activity *in vitro* and *in vivo*, allowing head-to-head comparison among the activity of different compounds and (iii) allows identification of the ideal dose for the subsequent steps of the screening process within a short time interval (the *in vivo* assay can be completed in less than 40 days). Importantly, the first step with Y strain before moving to other resistant parasite strains like Colombiana is also relevant regarding biosafety procedures, since only certified research teams and laboratories are able to assay resistant strain. Also, for drug screening, taking into consideration that the parasite biological characteristics as such are similar independent of the infection status (acute and chronic), the use of *in vivo* chronic models does not yield any fundamental contribution to the determination of the efficacy of a new trypanocidal drug besides requiring longer periods of parasitemia positivation and endpoints [34].

Finally, the establishment of parasitological cure rates using at least two reproducible protocols like hemocultive and polymerase chain reaction (PCR) analysis [191] after immunosuppression at 30 days post treatment is desirable. As reported by Romanha and co-workers [34], the employment of cyclophosphamide (three cycles with doses of 50 mg/kg of body weight for four consecutive days and intervals of three days between each cycle) enhances the sensitivity of the parasitological cure analysis [192-195]. Another aspect relevant is the health status of the experimental animals (maintained in "SPF" conditions), keeping an adequate maintenance and handling, hygiene conditions (chow changes and cage cleaning), weight standardization and parasite inoculums, in order to allow reproducible results.

3.4.3. Leishmaniasis

Animal models are expected to mimic the pathological features and immunological responses observed in humans when exposed to a variety of *Leishmania* spp. with different pathogenic characteristics. Many experimental models have been developed, each with specific features, but none accurately reproduces what happens in humans. Animal models enable drug activity to be determined in relation to absorption (route of administration), distribution (different sites of infection), metabolism (pro-drugs, immuno-modulators), and excretion and to give an early indication of the toxicity [170]. The definition of protocols to use in animal models to study leishmaniasis should take in account the *Leishmania* species, and the susceptibility/resistance of the animal lineage. Several models, using rodents, dogs and monkeys have been described. The majority of the experimental models use mice for the facility to handle these animals and the necessity of low concentrations of compounds to be tested. The use of isogenic strains presenting standard defined profiles of physiology, immunology and genetics, make them a prototype to study new drugs. On the other hand, wild mice would reflect better the genetic diversity found in the human host [54, 196]. In cutaneous leishmaniasis, BALB/c mice have a susceptibility profile to infection, while C57Bl/6 e CBA/J mice present a resistance profile, with the capacity to recover from the infection [197-199]. As demonstrated for *Leishmania major* (*Lmaj*) infection, BALB/c mice developed a Th2 response with predominant production of IL-4, IL-5 and IL-10 cytokines which are associated to susceptibility [200]. On the other hand, C57Bl/6 mice infected with the same parasite do not develop serious lesions once they present a protective Th1 response characterized by the production of IL2, IFN-γ and TNF-α associated to resistance [201]. In visceral leishmaniasis BALB/c mice represent a good model since in them, as in humans, the spleen and liver are the main affected organs [198, 202]. Syrian Golden Hamsters (*Mesocricetus auratus*) have been used as a model for visceral leishmaniasis since they also develop spleen and liver infections, turning into a chronic disease similar to human visceral leishmaniasis. Hamsters are also used in experiments with species presenting low infectivity to mice, such as *L. braziliensis* (*Lbra*) and *L. panamensis* (*Lpan*) [202-204].

The choice of the inoculation route to treat experimental leishmaniasis is important and depends on the animal model and the clinical manifestation developed. The most common routes are: intralesional, topical, subcutaneous, intradermal, intravenous and intraperitoneal [198, 199, 205]. Animals are preferentially infected with the amastigote forms and, depending on the *Leishmania* species, a period between one week and twelve weeks may be necessary to establish the infection. In general, the test compound is administered at a fixed dose (e.g. < 100 mg/kg), but the limiting issue would certainly be the toxicity [161]. Different regimens (1 to 20 days) and routes of administration could be initially performed, but it should be considered that experimental therapeutic failure occurs for the majority of tested compounds.

The development of the infection and treatment is followed according several criteria, such as: a) measurement of cutaneous lesion size, which should be done at different times after infection, i.e. when the footpad swelling is measured using a dial caliper. This value is expressed as the difference in the thicknesses in millimeters between the inoculated footpad and the median of the footpads in the control group. However, the determination of drug efficacy by measuring changes in lesion size (three dimensions) during and after the course of treatment can be misleading as much of the lesion is composed of inflammatory cells with amastigotes restricted to dermal layer of the skin [203]. Usually, parasite burden determination is carried out by the classical method of light microscopy, through the counting of the parasites in smears obtained by the imprint technique [206]. To evaluate the parasite load in mice with cutaneous leishmaniasis after the end of treatment, animals are euthanized and the draining lymph nodes and spleen are aseptically removed, weighed and homogenized. This evaluation could also be performed using a parasite limiting dilution assay [198, 199]. Culturing parasites from biopsies (micro dilution technique) provides an alternative measurement of activity and has been shown to give quantitative data [207]. Occasionally, a fragment of the infected tissue of footpad or ear is removed [208]. To evaluate the parasite load in mice with visceral leishmaniasis (VL), the spleen and liver are usually the target tissues, but investigation of the bone marrow could also provide important information [209]. The number of parasites per mg of tissue is estimated based on the tissue weight and the parasite load from the culture dilutions [210, 211]. Also, Leishman-Donovan Units are the most accepted and used technique, which consists in the number of amastigotes per 500 cell nuclei (liver and spleen) multiplied by the organ weight (mg) [212]. However, a first drawback is that classical methods are time-consuming and do not allow the evaluation of large libraries of previously *in vitro* tested compounds. Secondly, the accuracy of these techniques can be subjected to a high variation, especially when one considers counting errors from different observers during the light microscopy analysis. In contrast, the polymerase chain reaction (PCR) has been shown to overcome such problems, including the low sensitivity and time found in microscopic examination of tissue smears [212]. The development of quantitative real-time PCR (qPCR) to evaluate *Leishmania* in different tissues has been proposed [213-215], and it has recently proved to be a powerful technique for the evaluation of drug efficacy in experimental models of *Leishmania* [216, 217]. Based on the detection of the parasite DNA or RNA and the

respective standardized curves, qPCR allows the quantification of parasites in spleen, liver or any other infected tissue after drug treatment. Molecular techniques have become popular among parasitologists and easier to manipulate since the introduction of automated DNA/RNA extractors and high throughput thermocyclers.

Additional assays can also provide important information about the potential changes in the immune response, such as, the antibody profile (ELISA assay), cytokine measurement (IFN-γ, IL-4, IL-10) in the supernatant of cultures of lymph nodes and spleen (cutaneous leishmaniasis) or spleen visceral leishmaniasis (ELISA assay) and cell population in the blood (T CD4+/CD8+; T CD4+CD25+ and B cells). In order to evaluate the *in vivo* toxicity of the tested compounds it is recommended to evaluate potential hepatic toxicity by mensuration of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes and renal toxicity by the creatinine assay [198, 199].

4. PLANT NATURAL PRODUCTS WITH ANTIPROTOZOAL POTENTIAL: TERPENOIDS

The antiprotozoal potential of natural products has been reviewed in recent years, e.g. [218-223]. Usually, these reviews were on certain aspects of the topic with respect to a particular disease or class of natural products; the last *general* review on the potential of natural products against the major protozoan neglected diseases is that of Ioset, published in 2008 [223]. In this review, we attempt to give a comprehensive overview on the literature not contained in this previous work as well as the more recent reports published since then. It will certainly not be possible to avoid that

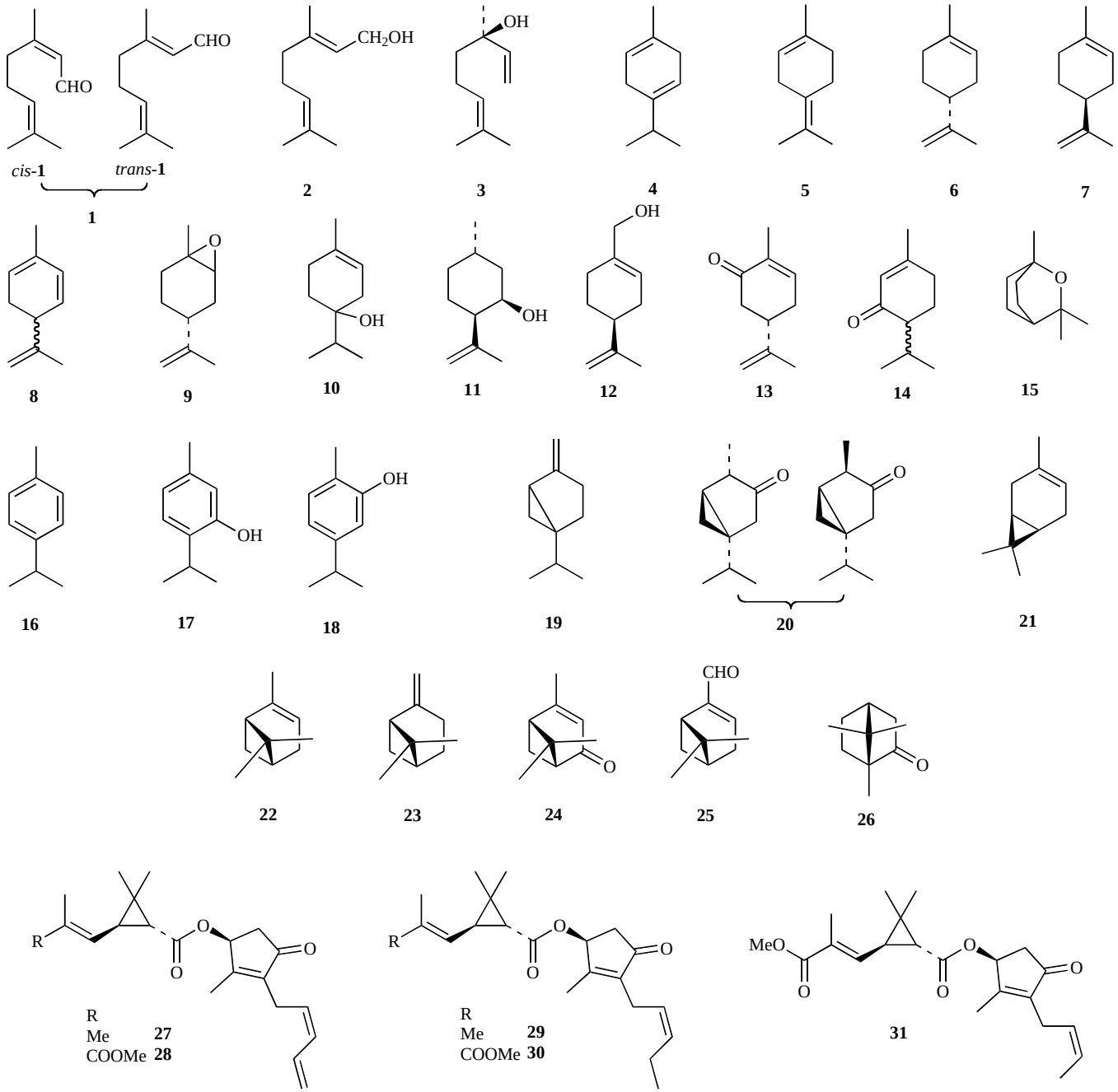


Fig. (4). Structures of monoterpenes with antiprotozoal activity.

particularly important reports already cited in previous reviews are mentioned again; on the other hand, it is impossible to cover all literature. The authors therefore apologize to all who feel that important contributions are missing.

It will become obvious that the view of the numerous authors often differs in terms of what is to be considered "promising", "highly active" etc.. The authors of the present review in most cases do not intend to classify certain levels of activity as "promising" or "less promising". Such judgement is up to the reader, who will be able to reach her/his own conclusions in this respect by careful reading and comparing the many results summarized here.

The discussion of the various classes of natural products roughly follows biosynthetic criteria. In the present part I, only natural products of terpenoid origin are included. Natural products of other biosynthetic classes will be covered in part II of this review [1].

Terpenoids are a very large class of natural products which may be formed in plants via two different biosynthetic routes, the classical Mevalonate pathway as well as the DOX/MEP or Rohmer pathway [224]. Both pathways lead to the basic building block for all terpenes, isopentenyldiphosphate, which is subsequently used to build up the various terpenoid subclasses, yielding a vast structural diversity with the many thousands of terpenoids known today.

This section is subdivided into the major biogenetic subclasses of terpenes occurring in plants, namely, the monoterpenes (C_{10}, section 4.1.), sesquiterpenes (C_{15}, section 4.2.), diterpenes (C_{20}, section 4.3.) and triterpenes (C_{30}, section 4.4.).

4.1. Monoterpenes

Monoterpenes are major constituents of numerous essential oils, and a number of essential oils have been reported to show antiparasitic activity against protozoal parasites [225]. The major monoterpenoid components of essential oils showing antiprotozoal activity are citral, linalool, terpinen-4-ol, thymol, carvacrol, camphor, 1,8-cineole, limonene, α -pinene, γ -terpinene, α -phellandrene, and *p*-cymene. The structures of these and further compounds are shown in Fig. (**4**) and their bioactivities are summarized in Table **3**.

Citral (**1**), a mixture of neral (*cis*-**1**) and geranial (*trans*-**1**), has shown antiprotozoal activity against *Tcr* trypomastigotes (IC_{50} = 24.5 µg/mL) [226] as well as *Lam* promastigotes and amastigotes (IC_{50} = 8.0 and 25.0 µg/mL, respectively) [227], while *trans*-**1** itself inhibited *Tcr* epimastigotes and *Lcha* promatigotes (IC_{50} = 3.1 and 40.3 µg/mL, respectively) [228]. Citral-rich essential oils have shown *in-vitro* antiparasitic activities. For example, *Cymbopogon citratus* essential oil was effective against *Tcr* [229] and *Linf* [230], and *Satureja punctata* against *Ldon* and *L. aethiopica* [231]. *C. citratus* essential oil has also exhibited *in vivo* antiplasmodial activity with mice infected with *Pber* [232].

Essential oils from *Croton cajucara* [233] and *Ocimum basilicum* [234] leaves, both rich in linalool (**3**), showed *in-vitro* antileishmanial activity against *Lam* and *L don* promastigotes, respectively. *O. basilicum* leaf oil was also active against *Giardia lamblia* trophozoites [235] and *Tcr* epimastigotes and trypomastigotes [236]. Similarly, *Antidesma laciniatum* [237] and *Salvia dolomitica* [238] essential oils were active against *Pfc*. **3** itself showed notable activity against *Tcr* epimastigotes and trypomastigotes (IC_{50} = 163 and 264 µg/mL, respectively) [236], *Tbb* bloodstream forms (IC_{50} = 2.5 µg/mL) [239], and *G. lamblia* trophozoites (100% kill at 300 µg/mL after 60 min) [235].

R-(+)-limonene (**6**) was shown to be active against *Tcr* epimastigotes and amastigotes (IC_{50} = 38.7 and 145.9 µg/mL, respectively), *Lcha* promastigotes (IC_{50} = 261 µg/mL) [228], *Lam* promastigotes and amastigotes (IC_{50} = 34.3 and 20.0 µg/mL, respectively) [240], and *Tbb* bloodstream forms (IC_{50} = 35.6 µg/mL) **[**241**].** Interestingly, *S*-(–)-limonene (**7**) was more active than its stereoisomer on *Tcr* (IC_{50} = 3.1 and 40.3 µg/mL, respectively, for epi- and amastigotes) and on *Lcha* promastigotes (IC_{50} = 129 µg/mL) [228]. **7** also inhibited *Pfc* with an IC_{50} of 9.0 µg/mL [242]. The essential oils of limonene-rich chemotypes (25-30% limonene) of *Lippia alba* were active against *Tcr* epimastigotes [228] and the essential oil of *Ambrosia scabra* (22.0% limonene) showed antiplasmodial activity against both chloroquine-sensitive and -resistant strains of *Pfc* [243].

Probably the most active monoterpene identified so far against African trypanosomes (*Tbr*) was terpinen-4-ol (**10**), for which an *in vitro* IC_{50} value of 0.02 µg/mL was described. The IC_{50} for cytotoxicity against HL60 human promyelocytic leukemia cells was 1025 times higher so that **10** may be considered exceptionally selective [244]. Apparently, however, it has never been tested in an *in vivo* model.

Commercial pharmaceutical grade *Eucalyptus* oil contains > 80% 1,8-cineole (**15**) and has been shown to inhibit the growth of both chloroquine-sensitive and -resistant strains of *Pfc* [245]. 1,8-Cineole itself inhibited *Pfc* with an IC_{50} of 120 µg/mL [245]. *Achillea millefolium* essential oil, which is generally rich in **15** (30-40%) [246], showed antileishmanial activity against *Lam* promastigotes [247]. Similarly, *Thymus capitelatus* oil (58.6% **15**) inhibited the growth of *Linf* promastigotes [230], while *Helichrysum cymosum* leaf oil (20.4% **15**) showed antiplasmodial activity [248].

Nigella sativa seed essential oil has been characterized as having a high *p*-cymene content [249] with as much as 60% *p*-cymene (**16**) [250], and *N. sativa* seed oil reduced parasitemia and extended life spans of rats infected with *Tb* [251]. Consistent with these observations, **16** has also shown moderate *in-vitro* inhibitory activities on *Tcr* epimastigotes and amastigotes (IC_{50} = 28.1 and 190.5 µg/mL, respectively) as well as *Lcha* promastigotes (IC_{50} = 149.1 µg/mL) [228].

Essential oils rich in the phenolic monoterpenes thymol (**17**) or carvacrol (**18**) generally show antiprotozoal activity. For example, *Lippia origanoides* essential oil (46.2% **18**) was active against *Tcr* epimastigotes and amastigotes as well as *Lcha* promastigotes [228]; *Origanum virens* and *Thymbra capitata* oils, both dominated by **18**, inhibited *G. lamblia* trophozoite proliferation [252]; *Chenopodium ambrosioides* essential oil (62.4% **18**) was active against *Lam* promastigotes [240]. **18** has been shown to inhibit *Tbb* bloodstream forms (IC_{50} = 11.25 µg/mL) [241] as well as *Tcr* epimastigotes and amastigotes (IC_{50} = 3.0 and 27.3 µg/mL, respectively) and *Lcha* promastigotes (IC_{50} = 28.0 µg/mL) [228]. The essential oils from *Lippia graveolens* [252], *Lippia origanoides* [228], and *Thymus vulgaris* [253], rich in **17**, showed antiprotozoal activity against *G. lamblia*, *Lcha*, and *Tcr*, respectively. **17** had IC_{50} values of 22.86, 0.33, 3.2, and 53 µg/mL on *Tbb* bloodstream forms [241], *Tcr* epimastigotes [228], *Tcr* amastigotes [228], and *Tcr* trypomastigotes [236], respectively. **17** was also effective against *Lcha* [228] (IC_{50} = 65.2 µg/mL) and *Lam* [254] (IC_{50} = 22.63 µg/mL) promastigotes. Activity of **17** against *Lpan* promastigotes was relatively low, however (IC_{50} = 194 µg/mL) [255].

α-Pinene (**22**) has shown antitrypanosomal activity against *Tb* (IC_{50} = 4.1 µg/mL) and *Lmaj* (IC_{50} = 55.3 µg/mL) [244] and essential oils with high concentrations of **22**, have also shown antiprotozoal activity. For example, *Virola surinamensis* leaf oil (49.7% **22**) inhibited *Pfc* trophozoites [256]. Although β -pinene (**23**) was shown to be less active (IC_{50} = 54.8 and 200.1 µg/mL, respectively) toward *Tb* and *Lmaj* than α-pinene, essential oils rich in **23** have shown antiprotozoal activities. Thus, for example, *Aframomum sceptrum* rhizome essential oil, composed of 12.7% **23**, inhibited *Tbb* [257]; *Xylopia aethiopica* bark oil (10.1% **23**) showed *Pfc*-inhibitory activity [237].

Table 3. Antiprotozoal Activities of Monoterpenoids. Structures see Fig. (4)

Compound	Organism	IC_{50} (µg/mL)	Reference
Citral (**1**)	*L. amazonensis* promastigotes	8.0	[227]
	L. amazonensis amastigotes	25.0	[227]
	L. donovani promastigotes	19	[234]
	T. cruzi epimastigotes	??	[236]
	T. cruzi trypomastigotes	24.5	[226]
Geranial (*trans*-**1**)	*T. cruzi* epimastigotes	3.1	[228]
	T. cruzi amastigotes	> 30	[228]
	L. chagasi promastigotes	40.3	[228]
Geraniol (**2**)	*P. falciparum*	8.0	[242]
Linalool (**3**)	*G. lamblia* trophozoites	100% kill at 300 µg/mL	[235]
	P. falciparum	43.2	[260]
	P. falciparum	80.1	[242]
	T. b. brucei bloodstream forms	2.5	[239]
	T. b. brucei bloodstream forms	39.32	[241]
	T. cruzi epimastigotes	162.5	[253]
	T. cruzi trypomastigotes	264	[253]
γ-Terpinene (**4**)	*T. cruzi* epimastigotes	162.9	[228]
	T. cruzi amastigotes	> 30	[228]
	L. chagasi promastigotes	145.1	[228]
Terpinolene (**5**)	*L. major* promastigotes	387.9	[244]
	T. brucei bloodstream forms	31.0	[244]
R-(+)-Limonene (**6**)	*T. b. brucei* bloodstream forms	35.55	[241]
	T. cruzi epimastigotes	38.7	[228]
	T. cruzi amastigotes	145.9	[228]
	L. chagasi promastigotes	261.0	[228]
	P. falciparum	166	[260]
	L. amazonensis promastigotes	34.3	[261]
	L. amazonensis amastigotes	20.0	[261]
S-(–)-Limonene (**7**)	*T. cruzi* epimastigotes	211.2	[228]
	T. cruzi amastigotes	> 30	[228]
	L. chagasi promastigotes	129.1	[228]
	P. falciparum	9.0	[242]
α-Phellandrene (**8**)	*L. major* promastigotes	32.8	[244]
	T. brucei bloodstream forms	9.2	[244]
Limonene oxide (**9**)	*T. b. brucei* bloodstream forms	22.58	[241]
Terpinen-4-ol (**10**)	*T. b. brucei* bloodstream forms	39.51	[241]
	L. major promastigotes	335.9	[244]
	T. brucei bloodstream forms	0.02	[244]
Isopulegol (**11**)	*P. falciparum*	43.5	[242]
Perilla alcohol (**12**)	*P. falciparum*	28.4	[242]
S-(–)-Carvone (**13**)	*T. b. brucei* bloodstream forms	12.94	[241]
	T. cruzi epimastigotes	139.2	[228]
	T. cruzi amastigotes	6.0	[228]
	L. chagasi promastigotes	> 300	[228]
Piperitone (**14**)	*T. b. brucei* bloodstream forms	41.12	[241]
1,8-Cineole (**15**)	*T. b. brucei* bloodstream forms	83.15	[241]
	P. falciparum	120	[245]
p-Cymene (**16**)	*T. cruzi* epimastigotes	28.1	[228]
	T. cruzi amastigotes	190.5	[228]
	L. chagasi promastigotes	149.1	[228]

(Table 3). Contd…..

Compound	Organism	IC_{50} (µg/mL)	Reference
Thymol (**17**)	*T. b. brucei* bloodstream forms	22.86	[241]
	T. cruzi epimastigotes	62	[229]
	T. cruzi epimastigotes	0.33	[228]
	T. cruzi amastigotes	3.2	[229]
	T. cruzi trypomastigotes	53	[228]
	L. chagasi promastigotes	65.2	[228]
	L. amazonensis promastigotes	22.63	[254]
	L. panamensis promastigotes	194	[255]
Carvacrol (**18**)	*T. b. brucei* bloodstream forms	11.25	[241]
	T. cruzi epimastigotes	3.0	[228]
	T. cruzi amastigotes	27.3	[228]
	L. chagasi promastigotes	28.0	[228]
Sabinene (**19**)	*L. major* promastigotes	126.6	[244]
	T. brucei bloodstream forms	17.7	[244]
Thujone (**20**)	*T. b. brucei* bloodstream forms	38.79	[241]
δ-3-Carene (**21**)	*L. donovani* promastigotes	27	[234]
α-Pinene (**22**)	*L. major* promastigotes	55.3	[244]
	T. brucei bloodstream forms	4.1	[244]
β-Pinene (**23**)	*L. major* promastigotes	200.1	[244]
	T. brucei bloodstream forms	54.8	[244]
Verbenone (**24**)	*T. b. brucei* bloodstream forms	30.24	[241]
Myrtenal (**25**)	*T. b. brucei* bloodstream forms	17.24	[241]
Camphor (**26**)	*T. b. brucei* bloodstream forms	37.39	[241]
Pyrethroids			
Pyrethrin I (**27**)	*P. falciparum*	11.7	[262]
	T. b. rhodesiense	6.9	[262]
Pyrethrin II (**28**)	*P. falciparum*	4.0	[262]
	T. b. rhodesiense	10.6	[262]
Jasmolin I (**29**)	*P. falciparum*	9.3	[262]
	T. b. rhodesiense	30.9	[262]
Jasmolin II (**30**)	*P. falciparum*	5.0	[262]
	T. b. rhodesiense	12.0	[262]
Cinerin II (**31**)	*P. falciparum*	5.8	[262]
	T. b. rhodesiense	12.2	[262]

An essential oil from *Artemisia absinthium* rich in camphor (**26**) (27.4%) has shown notable *in-vitro* antileishmanial activity against *Ldon* and *Lam* amastigotes [258], consistent with the antiprotozoal activity of **26** (IC_{50} against *Tbb* = 37.39 µg/mL) [241]. Similarly, *Artemisia gorgonum* essential oil (28.7% **26**) inhibited the growth of *Pfc* [259].

A variety of further monoterpenoids that have shown antiparasitic activities are summarized in Table **3**.

4.2. Sesquiterpenes

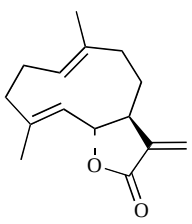
4.2.1. Sesquiterpene Lactones

Sesquiterpenes with over 10,000 known structures [106] constitute the largest class of terpenoids. Within this class, lactone structures are particularly widespread. Approximately 50% of the sesquiterpenes contain at least one lactone group. Their vast chemical diversity, mainly focused within the Asteraceae family, has been related to the wide range of biological activities and structure-activity relationships have been reviewed [100].

Artemisinin with its unusual *seco*-cadinane cyclic peroxide structure is the most prominent example for a sesquiterpene lactone (STL) with antiprotozoal activity. Its well known antimalarial activity and medicinal chemistry as well as clinical aspects have been extensively reviewed elsewhere [263-266]. It deserves mentioning that artemisinin as well as some derivatives have also been reported to show *in vitro* activity against *Tbr* and *Tcr* but their IC_{50} data in the range between 10 and 50 µM were far from being impressive [267]. Artemisininoids have also been demonstrated to show activity against *Ldon* [268, 269]. Artemisinin was described to trigger cell-cycle arrest and apoptosis but its parasiticidal activity was only moderate with IC_{50} values against pro- and amastigotes of 160 and 22 µM, respectively [268]. However, in an earlier QSAR study with a large series of artemisininoids against *Ldon*, several semi-synthetic derivatives where shown to be more potent than artemisinin itself [269].

A variety of STLs from various skeletal subclasses, mostly derived from the germacrane skeleton (germacranolides, guaianolides, xanthanolides, eudesmanolides and pseudoguaianolides) and a few others, have been reported to possess considerable antiprotozoal potential; structures see Fig. (**5A - 5C**)). Most of these compounds were found in Asteraceae species; plant families are therefore only mentioned in this section if otherwise. Some interesting STLs isolated from fungi are not mentioned here.

Germacranolides



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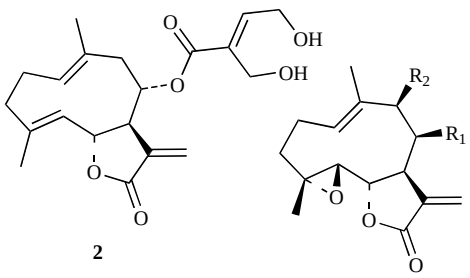
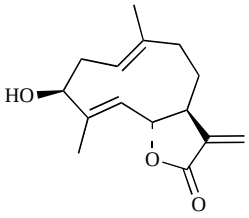


2

	R_1	R_2
3	H	H
3a	H	3,4-Epoxy-3-Me-hexanoate
3b	H	3-Hydroxy-2-methylbutyrate
3c	H	3-Hydroxyisovalerate
3d	H	3-Oxo-2-methylbutyrate
3e	OAc	H



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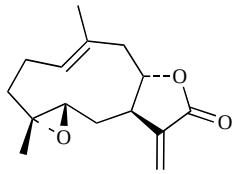

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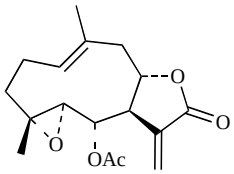

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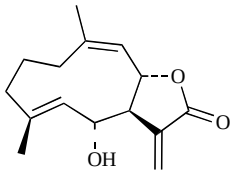

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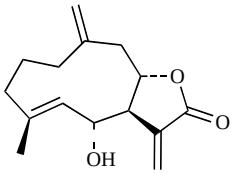

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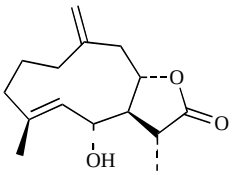

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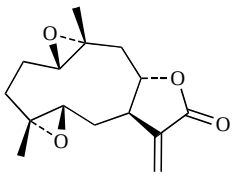

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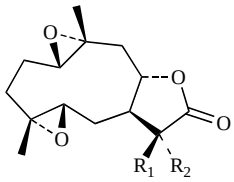
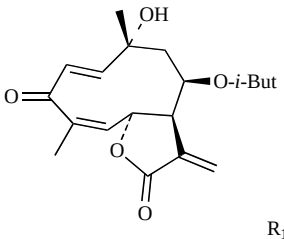


R_1	R_2	
Me	H	**11***
H	Me	**12***



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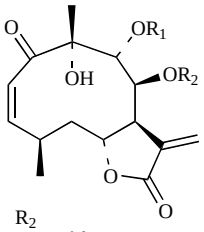
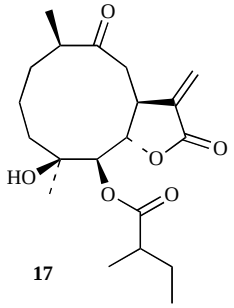


R_1	R_2	
Ac	*i*-Val	**14**
i-Val	H	**15**
H	*i*-Val	**16**



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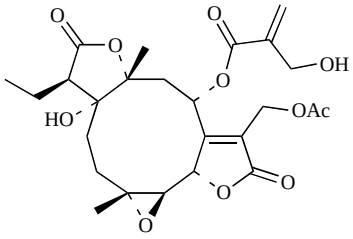

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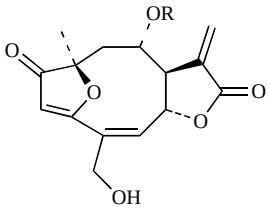
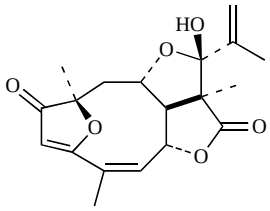


R		
Meac	**19**	
Ang	**20**	



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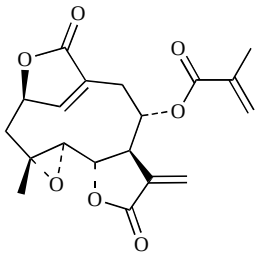

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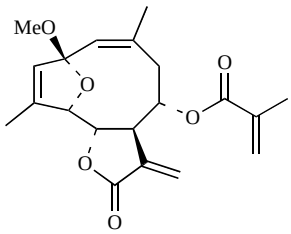

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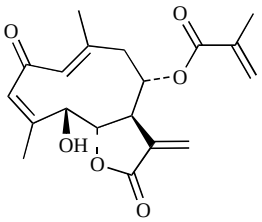

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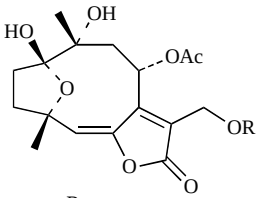
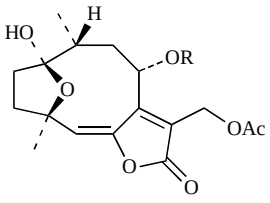


R	
Ac	**25**
Et	**26**



R	
27	Tig
28	Meac-3'-OH
29	Ang-4'-OH

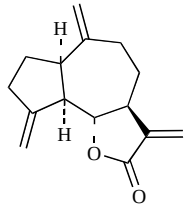
*Structures corrected according to CAS entries.

i-But: Isobutyrate
i-Val: Isovalerate
Meac: Methacrylate
Ang: Angelate
Tig: Tiglate

A

(Fig. 5). Contd…..

Guaianolides



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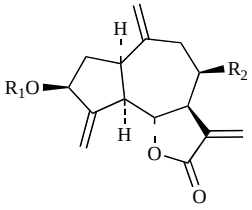
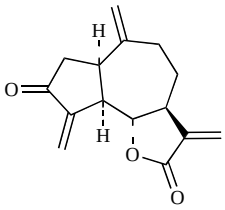


	R_1	R_2
31	OAc	H
32	OAc	OH
33	OH	$OOCC(CH_2)CH_2OH$



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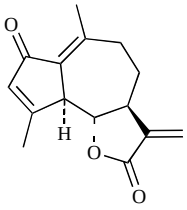

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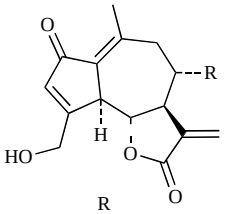
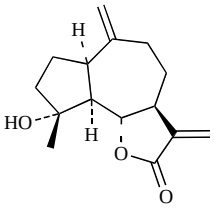


R	
36	OH
37	OOC-Bz



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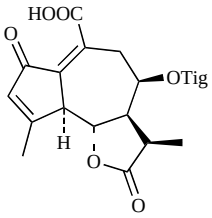

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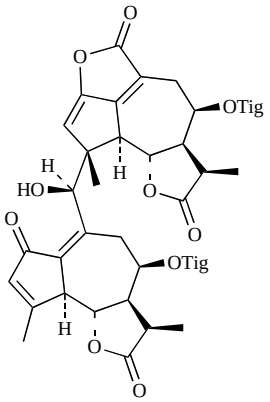

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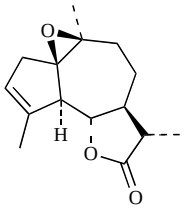
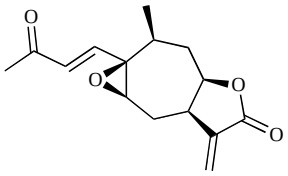


41

Xanthanolides and modified Xanthanolides



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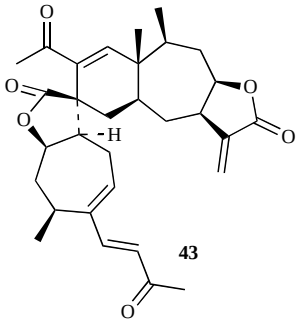

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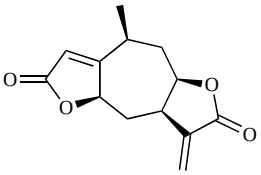

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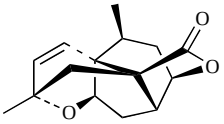

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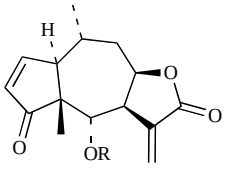
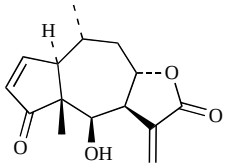
Pseudoguaianolides



R	
H	**46**
Ac	**47**



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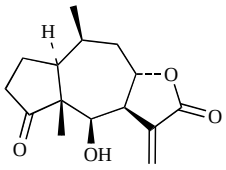

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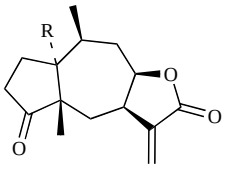
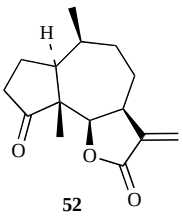


R	
H	**50**
OH	**51**



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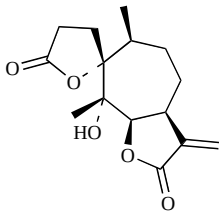

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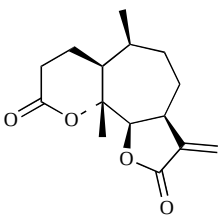
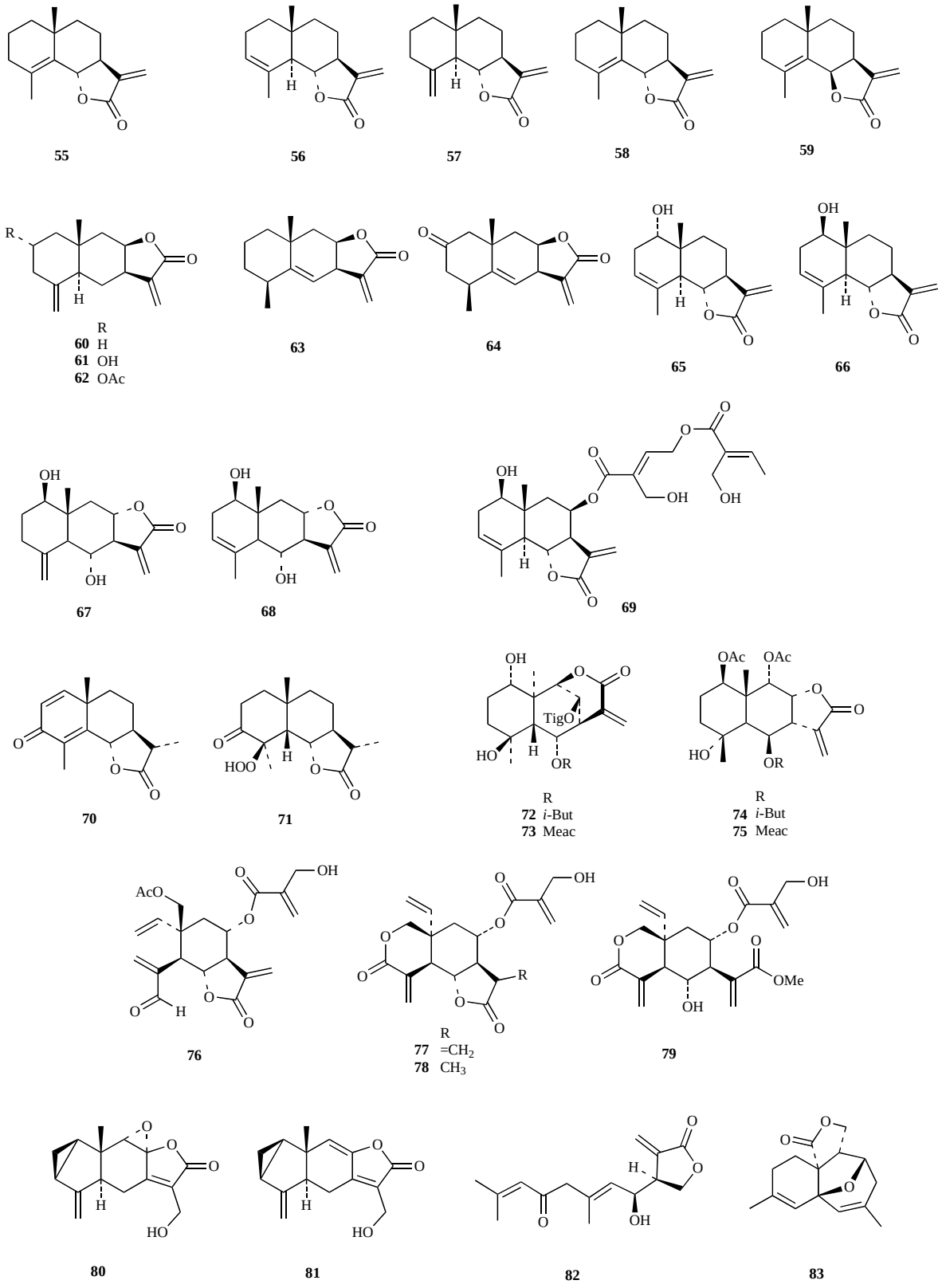


54

B

(Fig. 5). Contd…..



C

Fig. (5). Structures of sesquiterpene lactones with antiprotozoal activity.

A. Germacranolides.

B. Guaianolides, xanthanolides and pseudoguaianolides.

C. Eudesmanolides and modified eudesmanolides.

Germacranolides

The structures of compounds mentioned in this section are presented in Fig. (**5A**). Quite recently, the germacranolides costunolide **1** from *Saussurea costus*, eupatoriopicrin **2** from *Eupatorium cannabinum* and parthenolide **3** from *Tanacetum parthenium* were reported along with some guaianolides and eudesmanolides (see below) by Julianti *et al.* to show activity against *Tbr* trypomastigotes (STIB900 strain; IC_{50} 1.3, 1.2 and 0.8 μM, respectively) [117]. It appears noteworthy that the relatively polar dihydroxyacyl sidechain by which **2** differs from **1** apparently has no influence on antitrypanosomal activity. However, the former was less selective (SI=1.3 vs. 5.9 for costunolide, determined with L6 rat skeletal myoblast cells), i.e. the polar ester group increased cytotoxicity. Parthenolide **3**, whose antitrypanosomal, antileishmanial and antiplasmodial activity had also been reported by others [270-272] was found the most active compound within this study (IC_{50}= 0.8 μM) and also showed the highest selectivity (SI=6.5 in relation to L6 rat skeletal myoblast cells).

The germacranolide hanphyllin **4** closely related to costunolide (= 3β-hydroxycostunolide) from *Artemisia gorgonum* was reported active against *Pfc*, strain FcB1 (IC_{50}=9.3 μM, SI=11.5 (VERO cells) [273] but nothing so far seems to be known about antitrypanosomal or antileishmanial activity.

Very recently, Otoguro *et al*. included **1** and **3** in a study on various terpenoids against *Tbb* strain GUTat 3.1 where these compounds were found even more active and selective (IC_{50} 0.28 and 0.077 μg/mL, respectively; SI: 17 and 31, respectively, determined with the human MRC-5 embryonic cell line) [147] than against the *rhodesiense* form [117]. The *Tbb* parasite, infecting mainly cattle, is thus even more sensitive to STLs than the human pathogen. This difference in activity and selectivity of the same compounds against two so closely related parasites shows that it may be inappropriate to extrapolate results from one parasite to another even if so intimately related, and that the search for agents against the human pathogen must be conducted with this pathogen itself and not a close relative.

Quite interestingly, an analog of **3** with a 7(12)8-lactone ring, 4α,5β-epoxy-8-*epi*-inunolide **5**, was found more than 20 times less active than the 7(12)6 lactonized **3** in the last mentioned study (IC_{50}=1.73 μM) [147] so that the mode of lactonization appears to be very important for antitrypanosomal activity in this type of compounds.

Several hydroxyparthenolide esters have been isolated very recently in the present author's laboratory yielding some insight into structure-activity relationships in relation to the ester groups. Most prominently, a 9β-hydroxyparthenolide ester **3a** with a new C_6-epoxycarboxylic acid (3,4-epoxy-3-methylhexanoic acid) from *Inula montbretiana* [274] showed good *in vitro* activity against *Tbr* (IC_{50}= 0.69 μM, SI=9, determined with L6 rat skeletal myoblasts). The 3-hydroxy-2-methylbutyrate **3b** and 3-hydroxyisovalerate **3c** of 9β-hydroxyparthenolide were only slightly less active and showed selectivity in the same range. In contrast, the 3-oxo-2-methylbutyrate **3d** was less active against *Tbr* (IC_{50}=3.7 μM) but showed promising antiplasmodial activity against *Pfc* with an IC_{50} of 4.1 μM and an SI of 35. Neither **3** [270] nor the other three mentioned esters [274] were so active against the malaria parasite (IC_{50} of **3**: 11.9 μM [270]) and, most notably, all showed SI values <1 (more toxic to L6 cells than to *Pfc*).

A further hydroxyparthenolide ester, lipiferolide (8β-acetoxyparthenolide, **3e**), isolated from *Liriodendron tulipifera* (Magnoliaceae) is the most active and selective parthenolide derivative against *Tbr* found so far with an IC_{50} of 0.22 μM and SI = 21. It has hence also been subject to a preliminary *in vivo* test against *Tbr* in the acute mouse model where it showed a promising effect, however with few mice and only one concentration tested (Schmidt, T.J. *et al*., unpublished). Its antiplasmodial activity was not as impressive with IC_{50}= 7.3 μM [275]. The nature and position of the ester group, in any case, has a strong impact on antiprotozoal activity in this group of compounds **3**-**3e**.

Oncosiphon pilluliferum, a plant used in traditional medicine against fevers -in spite of an earlier report according to which it was inactive against experimental Malaria [276]- was more recently investigated for antiplamodial activity. Four germacranolides with 7(12),8-fused lactone moieties (**6**-**9**) were isolated from *Oncosiphon piluliferum* along with two eudesmanolides showing the same mode of lactonization. Three of the germacranolides with exomethylene lactone groups, 4,5α-epoxy-6α-acetoxy-1(10)*E*,11(13)-germacradien-12,8α-olide (**6**), tatridin A (**7**) and tanachin (**8**) were significantly more active *in vitro* against *Pfc* (D10 strain; IC_{50}= 0.5, 0.4, and 0.5 μg/mL, respectively) and also showed higher selectivity (SI= 4.4, 15.0, 12.8, respectively, determined with CHO cells) than the two eudesmanolides (see below). A fourth germacranolide, 11β,13-dihydrotanachin (**9**), with a saturated lactone ring was essentially inactive (IC_{50}= 70.0 μg/mL) [277].

A similar germacranolide from *Carpesium cernuum*, 11(13)-dehydroivaxillin (**10**), also showed significant *in vitro* activity against the D10 strain of *Pfc* (IC_{50}= 0.53 μg/mL= 2.0 μM; SI= 13.1; SK-OV-3 cells). This compound showed promising results *in vivo* (*Pber* mouse model) where 10 mg/kg/day over 4 days yielded a similar result as 5 mg chloroquine [278]. Two further related epoxygermacranolides, ivaxillin (**11**) and 11-*epi*-ivaxillin (**12**) (*in vitro* IC_{50}= 4.5, 2.5 μg/mL) and the pseudoguaianolide carpesiolin (see below) isolated from the same plant were less active [279].

The germacranolide tagitinin C (**13**) from *Tithonia diversifolia* displayed significant activity against *Pfc* strains FCA (chloroquine sensitive), FCB1 and W2 (chloroquine resistant) with IC_{50} values of 0.33, 0.24 and 0.25 μg/mL (0.95 and 0.69 and 0.72 μM), respectively, but was only moderately selective being cytotoxic against HRC-116 tumor cells with an IC_{50} of 2.03 μM [280].

The germacranolides neurolenin B (**14**) and a mixture of neurolenins C and D (**15** and **16**), isolated from *Neurolaena lobata* and found to be responsible for the antiplasmodial properties of this plant [281] were more recently also investigated for activity against *Lmex* and *Lbra* promastigotes as well as *Tcr* trypo- and epimastigotes [282]. *Lmex* (IC_{50} 3.4 and 5.5 μg/mL for **14** and **15**+**16**, respectively) was found to be more susceptible to the compounds than *Lbra* (99 and 188 μg/mL, resp.). In case of *Tcr*, **14** was more active than its congeners against both trypo- and epimastigotes (IC_{50} 6.3 vs. 4.9 μg/mL, respectively) where it even outmatched the positive controls nifurtimox and benznidazole.

Ineupatorolide A (**17**), a germacranolide with a saturated cyclodecane ring skeleton from *Carpesium rostulatum* was reported to be more active *in vitro* against *Pfc* (D10 strain) than artemisinin (IC_{50} 0.007 vs. 0.015 μg/mL/0.02 vs. 0.05 μM) [283] and was also proven to be effective *in vivo* in *Pber*-infected mice where its activity was comparable with that of chloroquine [284].

An unusual germacranolide dilactone, 16,17-dihydrobrachycalyxolide (**18**), was isolated from *Vernonia brachycalyx*. This compound was tested *in vitro* against *Lmaj* promastigotes and against four strains of *Pfc*. Against *Lmaj* it yielded an IC_{50} value of 34 μM and displayed cytotoxicity against human macrophages with an IC_{50} of 16 μM rendering it not interesting as antileishmanial lead. Its antiplasmodial activity was somewhat stronger with IC_{50} values of 5.9 and 8.3 μM against the V1/S and K39 strains, respectively [285].

In studies on constituents of *Lychnophora* species against *Tcr* trypomastigotes [286, 287], germacranolide STLs of the furanoheliangolide subtype, especially goyazensolide (**19**) (IC_{50} 67.6 μg/mL [286]/ 56.8 μM ([287])) showed some activity. 15-deoxygoyazensolide, bearing a methyl instead of the hydroxymethylene group (structure not shown) was also reported to

possess some activity against *Tcr* trypomastigotes [288]. It appears noteworthy that **19** was very recently tested *in vitro* against intracellular amastigotes of *Tcr* and displayed an IC_{50} of 1.08 μM and even higher activity against *Ldon* (axenic amastigotes) and *Pfc* (K1) with IC_{50}= 0.45 and 0.29 μM, respectively, but it presented only low selectivity with a cytotoxic IC_{50} of 0.49 μM (L6 cells). However, **19** in this same study was found to be exceptionally active *in vitro* against bloodstream forms of *Tbr* with an IC_{50} value of only 0.07 μM (SI= 6.8) (Schmidt, T.J., *et al.*, unpublished)**. 19** and a set of closely related compounds were also isolated from *Camchaya calcarea* [289] and tested for antiplasmodial activity (*Pfc*, K1) where **19** was reported less active than stated above (IC_{50}= 3.3 μM). In this latter study, centratherin (**20**), the angeloyloxy homolog of **19**, was the most active compound with IC_{50}= 0.3 μg/mL (0.8 μM). Centratherin and its congeners also displayed considerable cytotoxicity in a similar concentration range against four mammalian cell lines [289].

The furanoheliangolide derivative eremantholide C (**21**) from *Lychnophora trichocarpha*, structurally related to goyazensolide and centratherin, but with a modified lactone structure that does not have an exocyclic methylene group, was tested along with some semi-synthetic derivatives against *Tcr* trypomastigotes *in vitro*. The activities were quite low, the most active compound being the natural product itself with 100% growth inhibition at 3600 and 1800 μg/mL (Y and CL strain, respectively) [290].

A variety of germacranolides of different structural subclasses and some guaianolides isolated from *Elephantopus mollis* were reported to possess high activity against *Lmaj* promastigotes [291]. Here, elephantopin (**22**) and 2-deethoxy-2β-methoxyphantomolin (**23**) showed particularly interesting IC_{50} <0.1 μM. A variety of partial synthetic derivatives were prepared from molephantin (**24**) of which some were found as active as the two compounds already mentioned. Some simple structure-activity relationships could be established; most importantly the dependence of activity on the presence of an α,β-unsaturated exocyclic methylene lactone group, well known to be important for many other biological activities of STLs (overview see [100, 292]) was proven also for antileishmanial activity [291].

Very recently however, 8,13-diacetyl-piptocarphol (**25**) and 8-acetyl-13-*O*-ethylpiptocarphol (**26**) from *Pseudoelephantopus spicatus*, two STLs structurally related to the *Elephantopus*-lactones of the furanoheliangolide type but not containing an α,β-unsaturated exocyclic methylene lactone, were found to be highly active against axenic amastigotes of *Lam*. Their activity with IC_{50} values of 0.20 and 0.37 μM was in a similar range as that of the positive control amphothericin B (0.41 μM) and they were both found to be highly selective against *Leishmania* in relation to several human cell lines [293].

Three STLs structurally related to the piptocarphols but with sterically different furan ring fusion, namely, a tigloyloxy- and a 4-hydroxymethacryloyloxy-hirsutinolide derivative as well as vernolide D (**27**-**29**), were reported active against *Pfc in vitro*. The IC_{50} values of these compounds from *Vernonia cinerea* were in the same range (3.5-3.9 μM); no selectivity data were included in this study [294].

Guaianolides

The structures of guaianolides (**30**-**41**) mentioned in this section are depicted in Fig. (**5B**). An early study on the guaianolide dehydrozaluzanin C (**34**) from *Munnozia maronii* revealed quite promising effects against promastigotes of 12 strains of different *Leishmania* species (IC_{90} between 2.5 and 50 μg/mL, depending on strain and incubation time), along with *in-vivo* activity against *Lam* (PH8) in infected BALB/c mice where it appeared somewhat less potent than the reference drug, glucantime. Compound **34** was more active *in vitro* than benznidazole and nifurtimox against epimastigotes of 15 strains of *Tcr* (IC_{90} between 2.5 and 50 μg/mL, depending on strain and incubation time) [295] while it had previously been shown to be inactive against trypomastigotes of this parasite (ref. cited according to [295]).

Two guaianolides, dehydrocostuslactone (**30**) and zaluzanin D (**31**), were also tested in the study on *Saussurea costus* STL against *Tbr* already cited above [117]. The former yielded an IC_{50} of 4.4, the latter was among the less active compounds in this investigation with an IC_{50} of 10.8 μM [117]. The 8-hydroxylated congener, 8β-hydroxyzaluzanin D (**32**), isolated from *Mikania hoehnei* had also previously been reported to be of low activity against *Tcr* trypomastigotes with an IC_{50} > 250 g/mL [296]. Thus, such C-2β-acetoxy guaianolides are poor antitrypanosomal agents in contrast to their structural analogs without a 2β-*O*-acetyl function such as dehydrozaluzanin C (**34**; see above). This is confirmed by a recent report on the guaianolide cynaropicrin (**33**) from *Cynara* species (artichoke), bearing only an OH function at C-2β, which showed quite good activity against *Tbr* in-vitro (IC_{50} 0.3 μM) and also *in vivo*, where it decreased parasitemia in the acute mouse model by 98% after a 4-day treatment (10 mg/kg/d, *i.p.*) [297].

The guaianolide dehydroleucodine (**35**) from *Artemisia douglasiana*, structurally similar to **34**, was shown at 10 and 25 μg/mL (ca. 40-100 μM) to rapidly and irreversibly damage *Tcr* epimastigotes [298]. Quite importantly, the effect could be blocked with thiol agents such as dithiothreitol (DTT) and glutathione (GSH), which indicates that the antiparastitic activity is related to reaction with biological thiols in the protozoan cell in the same way as observed with many other biological effects of STLs (overviews see [100, 292]. Later on, **35** was also demonstrated to be active against *Lmex* [299].

Two constituents of lettuce (*Lactuca*) and cichory (*Cichorium*) species, the guaianolides lactucin (**36**) and lactucopicrin (**37**), were reported to possess antiplasmodial activity with inhibitory concentrations that completely prevented parasite growth of *Pfc* strain Honduras-1 *in vitro* of 10 and 50 μg/mL, respectively [300].

Quite recently, 11,13-dehydrocompressanolide (**38**), a guaianolide isolated from *Tanacetum parthenium* (feverfew), was demonstrated to be of interest as an antileishmanial agent. *In vitro* tests against promastigote forms of *Lam* revealed an IC_{50} value of 2.6 μg/mL. Although about 10 fold higher concentrations were needed to reduce the survival of intracellular amastigotes (90% reduction at 20 μg/mL), this compound may be of high interest since it also was quite selective being 385 times more toxic to the promastigotes than to murine macrophages. Although being less active than parthenolide (germacranolide **3**), the main constituent of this plant, this guaianolide was about 10 times more selective and may hence be considered an interesting lead [301].

A recent study on the constituents of *Eupatorium perfoliatum* from one of the authors' (TJS) laboratories led to isolation of the dimeric guaianolide diguaiaperfolin (**40**) as the most active of three STLs tested against *Pfc in vitro* (IC_{50}= 2.0 μM). This structurally unique STL is over 6 times more active than its monomeric biogenetic precursor, 2-oxo-8-tigloyloxyguaia-1(10),3-diene-6,12-olide-14-carboxylic acid (**39**), and displayed a selectivity index of antiplasmodial over cytotoxic activity (L6 cells) of 8.3. In contrast with most other guaianolides described here, it contains an 11,13-dihydro saturated lactone structure so that reaction with a biological target cannot be mediated by an exomethylene lactone group. Instead, and in contrast with the less active monomer, it contains an α,β,γ,δ-unsaturated carbonyl group in subunit A which represents an alternative Michael acceptor structure that might account for its antiplasmodial activity. It is noteworthy that **40** appears to be selective for the malaria parasite since it was considerably less active against *Trypanosoma* and *Leishmania* species [302].

A series of 11,13-dihydroguaianolides similar to the less active monomer just mentioned was isolated from *Artemisia gorgonum*

along with two guaianolides and two germacranolides containing exomethylene lactone structures. Within this series, tested against *Pfc* (FcB1 strain), the saturated lactones showed only low activity, the most active compound of this type being arborescin (**41**) with an IC_{50} value of 3.8 μg/mL (15.3 μM). Arborescin was considered the most promising compound in this study, probably due to its selectivity index of about 19 (VERO cells) although the compound with the highest (yet moderate) antiplasmodial activity was the germacranolide hanphyllin (**4**; see above) [273].

Xanthanolides (4,5-seco-Guaianolides)

The structures of xanthanolides and derivatives thereof (**42**-**54**) mentioned in this section are depicted in Fig. (**5B**). Interesting *in vitro* activity was reported for a series of xanthanolide type STLs isolated after activity-guided fractionation from *Xanthium brasilicum*. The most active compound was 8-epixanthatin-1β,5β-epoxide (**42**) with IC_{50} values of 0.33 and 0.60 μM against *Tbr* trypomastigotes and axenic amastigotes of *Ldon*, respectively, and very agreeable selectivity in relation to L6 cells (IC_{50}= 22.14 μM; SI = 67 and 37, respectively) [109]. This compound would definitely deserve to be tested *in vivo* which, however, was precluded so far due to a shortage of material. Of the dimeric xanthanolides isolated from this plant, pungiolide A (**43**) was also quite active against *Tbr* (IC_{50}= 0.64 μM; SI= 14.7). Less active but even more selective was a di-*nor*-xanthanolide (4,15-dinor-1,11(13)-xanthadiene-3,5β:12,8β-diolide, **44**) isolated in very small quantity from the same extract, with an IC_{50} of 8.2 μM against *Tbr* and no significant cytotoxicity (IC_{50} >385 μM, L6 cells) [109].

At this point it is also interesting to mention xanthipungolide (**45**), an unusual intramolecular Diels-Alder product formed from a xanthanolide precursor, which was isolated along with the mentioned xanthanolides from *X. brasilicum*. Xanthipungolide, although much less active than the mono- and dimeric xanthanolides just mentioned, showed weak activity against *Ldon* axenic amastigotes with an IC_{50} of 63 μM but was highly selective not only with respect to cytotoxicity (IC_{50} >365 μM (L6 cells)) but also in comparison with *Tbr* (IC_{50}= 162 μM) [109].

Pseudoguaianolides

Structures of the mentioned pseudoguaianolides (**46**-**54**) are reported in Fig. (**5B**). The pseudoguaianolide helenalin (**46**) isolated from *Arnica* and *Helenium* species was found particularly active *in vitro* against *Tbr* trypomastigotes as well as against intracellular amastigotes of *Tcr*. With IC_{50} values of 0.051 an 0.695 μM, respectively, it was more active than its diastereomer mexicanin I (**48**; 0.318 and 1.870 μM). Both also showed cytotoxicity against L6-rat skeletal myoblasts, but some selectivity was observed (SI: 19.5 and 7.7 for *Tbr* by helenalin and mexicanin I, respectively) which made especially helenalin interesting. Four further compounds tested in the same study (two dihydrohelenalin derivatives and two eudesmanolides) were far less active than **46** and **48** [303] indicating a strong dependence of activity on the presence of two reactive partial structures, which was later on confirmed in QSAR studies [270]. Further studies on **46** and **48** on effects against *Tcr* epi- and trypomastigotes by other authors later on revealed that the two compounds are quite active also against these extracellular stages. Interestingly, their effects were not reversible by addition of DTT and GSH so that their mechanism of action was postulated to differ from that of dehydroleucodine (see above) [304]. Moreover, antileishmanial [296] as well as antiplasmodial activity [305] was also demonstrated for **46** and a variety of its derivatives [270, 305]. With respect to its mechanism of action against the kinetoplastid parasites, **46** was initially expected to interfere with the trypanothione system [303] since STLs of this type are known to react readily with thiols and interfere with thiol metabolism in many cells [100, 292]. Tests for inhibitory activity against trypanothione reductase [L. Krauth-Siegel, pers. commun.] excluded this enzyme as a target. More recently, helenalin acetate (**47**), almost equipotent with **46** *in vitro* [270], was found inactive against Rhodesain, a vital cysteine protease of *Tbr* [W. Setzer, pers. commun.]. Mexicanin I (**48**), when tested against the *Tcr* cysteine protease cruzain showed negligibly low activity [A. T. Do Amaral, pers. commun.] so that cysteine proteases are not likely targets of these highly active molecules. Thus, a thiol-independent mechanism is at least conceivable although difficult to imagine since alkylation of thiols is usually involved in the bioactivities of such compounds [100, 270, 292].

Helenalin (**46**) was later on found inactive against *Tbr* in an *in vivo* mouse assay [R. Brun, pers. commun.]. However its exceptionally low IC_{50} against this parasite still makes it an interesting lead compound and studies on derivatives potentially active *in vivo* are still in progress.

Initiated by the interesting antitrypanosomal effects of **46** and **48**, Schmidt *et al.* undertook a structure-activity relationship study with 40 sesquiterpene lactones of various structural subclasses [270] in which besides the two major pathogenic *Trypanosoma* species also activity against *Ldon* and *Pfc* were included. Currently approximately 60 compounds have been included in this still ongoing investigation. Helenalin and its ester derivatives are still the most active compounds against *Tbr* within the whole series, if not the most active STLs in general.

A structural analog of mexicanin I, carpesiolin (= 2,3-dihydromexicanin I; **49**), from *Carpesium cernuum*, was reported to show some activity (IC_{50} 16.3 μg/mL) against *Pfc* (D10 strain) but was less active than the germacranolides (see above) tested in the same study [279] and also much less active than a variety of 2,3,11,13-doubly unsaturated pseudoguaianolides tested in [270].

Two further pseudoguaianolides, the isomers damsin (**50**) and confertin (**52**), were recently isolated from *Ambrosia peruviana*. They were tested *in vitro* against *Lam* (axenic amastigotes) as well as *Tcr* trypomastigotes. Both were reported to be active (IC_{50}= 3.3 and 1.9 μM, respectively), but **52** was much more selective (cytotoxicity against macrophages: IC_{50}= 216 μM vs. 23 μM in case of **50**). Hence **52** was also tested against macrophages infected with *Lam*, *Lbra* and *Lper*, where it showed activity with IC_{50}s ranging from 19 to 29 μM. On the other hand, **52** was inactive against *Tcr* while **50** showed moderate activity (IC_{50}= 13.2 μM) [306].

Besides the pseudoguaianolides already mentioned, two further compounds, psilostachyin (**53**) and peruvin (**51**) isolated from *Ambrosia tenuifolia* have been reported by Sülsen *et al.* to show activity against *Tcr* epi- and trypomastigotes as well as *Leishmania* spp. promastigotes [307]. The seco-pseudoguaianolide **53** (more precisely to be termed psilostachyin A [106]) was found more selective against *Tcr* and was later on studied in more detail at the cellular level [308]. This STL (*in vitro* IC_{50} 1.22 and 0.76 μg/mL against epi- and trypomastigotes, respectively) was chosen for *in-vivo* studies and reported to be active against *Tcr* in mice when administered intraperitoneally [307]. Quite noteworthy, **51**, reported to have shown IC_{50}s of 1.65 μg/mL on *Tcr* epimastigotes, displayed much less potent activity on trypomastigotes (52.8 μg/mL) [307]. It was also inactive when tested against intracellular amastigotes (IC_{50}= 28 μg/mL= 113 μM) in the present author's screening. Along the same lines, its effect on *Ldon* axenic amastigotes was 34 times less potent (IC_{50}= 13.4 μg/mL) (Schmidt, T.J. *et al.*, unpublished) than its reported activity on *Lmex* promastigotes (IC_{50}= 0.39 μg/mL) [307]. This is an excellent example to show that the various life forms of these parasites can differ dramatically in their sensitivity against a particular drug and that it is hence of crucial importance to investigate the relevant life stages.

More recently, psilostachyin C (**54**), a structural congener of psilostachyin A isolated from *Ambrosia scabra* was reported by the Sülsen group to be quite active against *Tcr* epi-, trypo- and amastigotes (IC_{50}= 0.6, 3.5 and 0.9 μg/mL) and to show some *in-*

vivo activity. The same study also reported *in-vitro* activity against *Lmex* and *Lam* promastigotes (IC_{50}= 1.2 and 1.5 μg/mL, respectively) and much lower cytotoxicity vs. mammalian cells (CC_{50}= 87.5 μg/mL) [309].

Eudesmanolides, Elemanolides, Lindenanolides

Structures of the mentioned eudesmanolides (**55**-**75**) and modified eudesmanolides (**76**-**81**) are shown in Fig. (**5C**).

In two studies already cited above, one against *T.b. rhodesiense*, the other against subspecies *brucei* ([117] and [147], respectively) STLs of the eudesmanolide type were included. In the former, the eudesmanolides arbusculin B (**55**) and α-cyclocostunolide (**56**) showed the weakest activity of the tested STLs (IC_{50}= 12.0 and 21.9 μM) and were the least selective with SI values <1 (i.e. they were more cytotoxic to L6 cells than to Trypanosomes) [117]. These findings are well in line with earlier observations of poor selectivity with other compounds of this structural subclass, namely, isoalantolactone, ivalin and ivalin acetate (**60**-**62**, respectively) [270, 303]. In the *Tbb* study [147], all investigated STLs showed SI values ≥1, in particular the eudesmanolides α- (**56**), β- (**57**), and γ-cyclocostunolide (**58**) as well as (−)-frullanolide (**59**), were quite selective, **56** yielding an SI of 29. Although some eudesmanolides such as alantolactone (**63**), 2-oxoalantolactone (**64**), douglanin (**65**) and santamarin (**66**) have shown SI >1 for *Tbr* / L6 cells [270] this discrepancy in selectivity clearly shows that selectivity indices of this type are to be treated with caution, especially when determined with different parasites (even though almost indistinguishably closely related) and mammalian cell lines. Eudesmanolides, whatsoever, do not appear the best of choices as potential anti-*Tbr* leads.

The eudesmanolides deacetyl-β-cyclopryethrosin (**67**) and sivasinolide (**68**), included in the above mentioned study on constituents of *Oncosiphon piluliferum* against *Pfc* were 5 to 10 times less active and less selective (IC_{50}= 4.4 and 2.6 μg/mL, SI= 2.3 and 1.5 (determined with CHO cells), respectively) than the germacranolides isolated from this plant (see above) [277].

Seven eudesmanolides with hydroxylated cyclocostunolide structures and esterified with complex hydroxylated and unsaturated acids were found in *Eupatorium semialatum* and tested *in vitro* against *Pfc*. They displayed moderate IC_{50} values between 8.9 and 31.7 μM. The most active eudesmanolide (**69**) was esterified with a similar dimeric hydroxytiglic acid as a guaianolide described in the same study. The latter (structure not shown here) was also reported to show antiplasmodial activity at a similar level (IC_{50}= 11.4 μM) [310].

Six eudesmanolides (α-santonin (**70**) and some congeners) with 11,13-dihydro saturated lactone ring structures were also included in the mentioned study against *Tbb* [147] five of which were found to be of only moderate activity (IC_{50} values ranging from 2->12 μg/mL). The only compound of this series with reasonably high activity was 4-peroxy-1,2,4,5-tetrahydro-α-santonin (**71**; IC_{50}= 0.4 μg/mL) whose activity can thus be attributed to the peroxy group. The low activity of the saturated lactones of this study once more demonstrates the necessity for the presence of reactive structure elements for high antiprotozoal activity.

Four eudesmanolides from *Wedelia trilobata* with quite unusual structures were found to display activity *in vitro* against *Pfc*. Wedelolides A (**72**) and B (**73**) yielded IC_{50} values of 1.9 and 4.1 μg/mL (μM), respectively, while the corresponding trilobolides (6-*O*-isobutyrate (**74**) and -methacrylate (**75**) were less active (14.7 and 8.9 μg/mL, respectively). No selectivity assessment was made [311].

Three elemanolides (2,3-*seco*-eudesmanolides) and one heliangolide (1,10-*trans*-4,5-*cis* germacanolide) from *Distephanus angustifolius*, were reported to be active against *Pfc* (chloroquine sensitive and resistant D10 and W2 strains, respectively) with IC_{50} values ranging from 1.55 to 4.94 μM. The elemanolides vernangulide B (**76**) and vernodalin (**77**) showed the best activity (IC_{50} 1.55 and 1.75, respectively, against the D10 strain). Both compounds were somewhat less active against the resistant strain. Their selectivity indices against *Pfc* (D10) in comparison with mammalian control cells (CHO cells) were 5.3 and 2.6 [312]. Two of the elemane derivatives isolated during this study, namely, vernodalol (**79**) and 11,13-dihydrovernodalin (**78**), had been described as antiplasmodial constituents of *Vernonia colorata* with IC_{50} values against PoW and Dd2 strains of 4.0 and 4.8 (vernodalol) and of 2.3 and 1.1 μg/mL (dihydrovernodalin) [313]. In this latter study, seven guaianolides (including two hydroperoxides) and one eudesmanolide were also isolated from another plant, *Artemisia afra*, and tested against *Pfc*. All of these compounds were found less active than the two elemanes already mentioned [313].

Two modified eudesmanolides of the lindenane type, oxyonoseriolide (**80**) and onoseriolide (**81**), isolated from *Hedyosmum angustifolium* (Chloranthaceae) were reported to be active against axenic amastigotes *Lam* and *Linf* as well as against intramacrophagic forms of the latter species. Against axenic cultures the lowest IC_{50} value was 19.8 μM (**81** vs. *Lam*); This compound was also more active than its congener against the intracellular parasites (IC_{50}= 24.3 μM vs. ≈140 μM at 72 and 96 h incubation) as well as against *Pfc* (IC_{50}= 12.5 μM, vs. 107.7 μM) and less toxic against two out of three mammalian cell lines [314].

STLs with Acyclic or other Carbon Skeleton

Three STLs with an acyclic carbon skeleton of the anthecotulide-type from *Anthemis auriculata* were tested against *Tbr*, *Tcr* and *Ldon*. 4-hydroxyanthecotulide (**82**, structure see Fig. **5C**) was the most active compound against all three parasites of this study, *Tbr* being the most sensitive (IC_{50}= 0.56 μg/mL, SI(L6 cells) 6.5) [315]. In this study, compound **82** was also the most active *in vitro* against *Pfc*, (IC_{50} 2.0 μg/mL). Activity against *Pfc* enoyl-ACP-reductase FabI (PfFabI), an enzyme of fatty acid synthesis, was reported but the IC_{50} value was in the range of 20 μg/mL [316] so that inhibition of this enzyme is not likely to be the sole mechanism responsible for the antiplasmodial activity of **82**.

Another compound form *A. auriculata*, anthecularin (**83** in Fig. **5C**), is noteworthy since it possesses an unusual structure with a previously unknown carbon skeleton. Anthecularin displayed only weak activity against *Tbr* (IC_{50}= 40.6 μM) and *Pfc* (K1 strain; IC_{50}= 94.7 μM) but was not cytotoxic against L6 cells [317].

4.2.2. Non-Lactonized Sesquiterpenes

A variety of studies have been published in which plant sesquiterpenes not containing lactone structures such as sesquiterpene hydrocarbons and -alcohols were tested for antiprotozoal activity and found active. The structures of compounds mentioned in this section are depicted in Fig. (**6**).

The activity of the dimeric naphthalenoid sesquiterpene gossypol (**1**) a constituent of *Gossypium* species (cotton, Malvaceae) as antitrypanosomal [318, 319] and antiplasmodial [223] agent was discovered in the early 1980s. Due to toxicity and a variety of other shortcomings, development of gossypol into an antiprotozoal drug was not possible so that it was abandoned [223]. Nevertheless it appears noteworthy, that a recent investigation on bioactivity of gossypol and derivatives has revealed that gossypol-6-methylether (**2**) as well as gossypol-6,6'-dimethylether (**3**) (compounds termed 6-methoxy- and 6,6'-dimethoxygossypol, respectively, in the original paper) have a significantly stronger *in vitro* activity against *Tb* trypomastigotes (subsp. not specified) than unsubstituted **1** (IC_{50} 3.1 μg/mL= 5.7 μM for **3**; vs. 7.8 μg/mL= 15.2 μM for **1**, respectively). At these concentrations, cytotoxicity against three human (cancer) cell lines was much lower so that substituted gossypol derivatives might yet regain interest as antiprotozal leads [320]. Surprisingly, it has also recently been

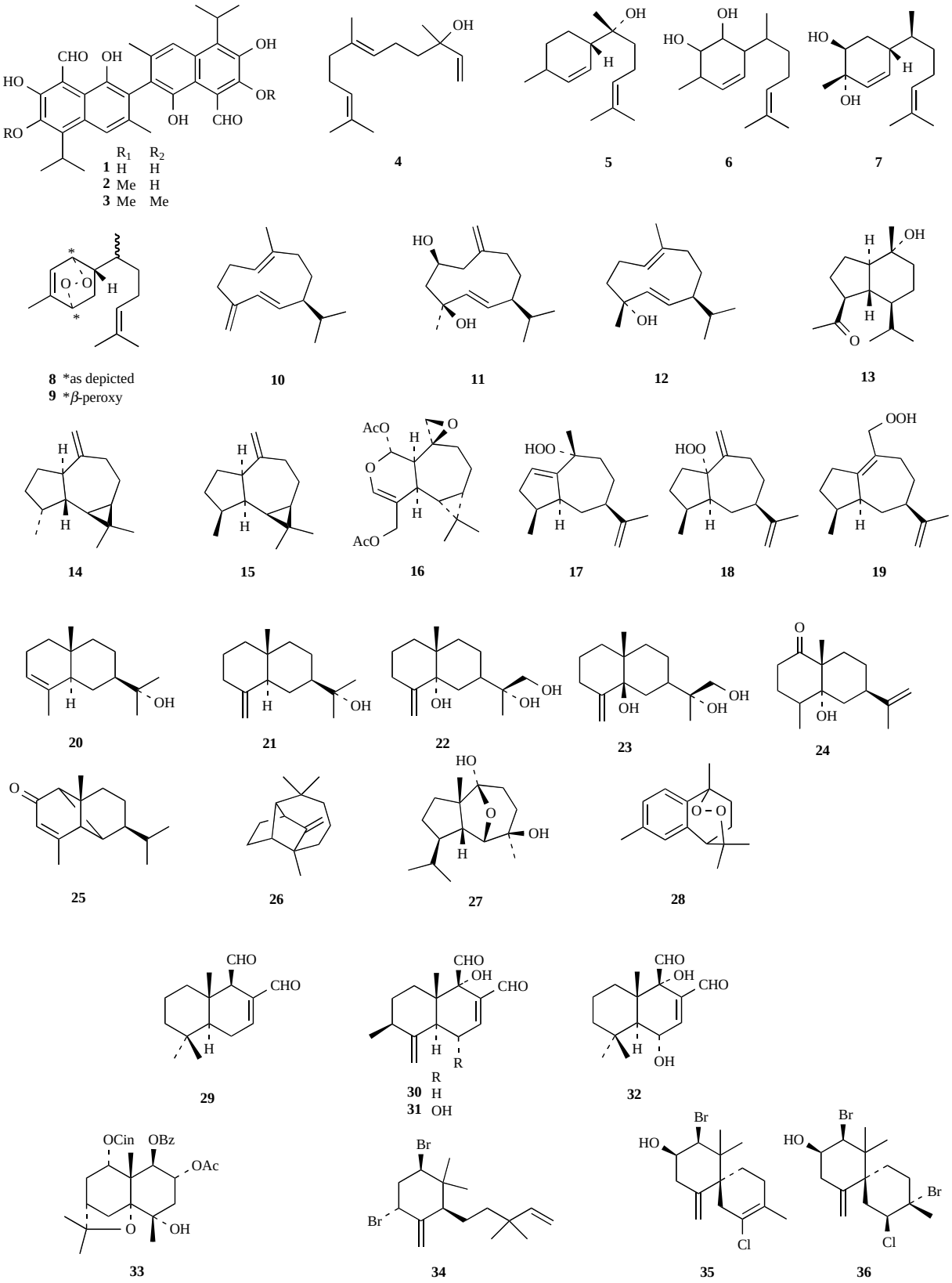


Fig. (6). Structures of non-lactonized sesquiterpenes with antiprotozoal activity.

demonstrated that optically pure R-(−)-gossypol is significantly more active against *Pfc* than its enantiomer/atropisomer or the racemate, so that its mechanism of action seems to be stereospecific [321].

The simple acyclic sesquiterpene alcohol nerolidol (**4**) was reported to have antileishmanial properties [322]. The growth of *Lam*, *Lbra*, and *Lcha* promastigotes as well as *Lam* amastigotes was inhibited by **4** at IC_{50} concentrations of 85, 74, 75, and 67 μM,

respectively. Although this activity level is not impressively high, **4** was even less cytotoxic (IC_{50} against human macrophages and foreskin fibroblasts were 125 and 136 μM). Nerolidol is an approved food additive which may make it interesting as an antiprotozoal lead compound [322].

In a similar way, (–)-α-bisabolol (**5**), well known as a major active constituent of chamomile oil may be of interest as antileishmanial lead since it was reported to inhibit the growth of *Linf* promastigotes at similar concentrations as the positive control drug pentamidine. Its IC_{50} value was determined at 10.99 μg/mL while pentamidine was only little more active (IC_{50}= 7.6 μg/mL). Bisabolol, known to be quite untoxic, was even somewhat more effective than pentamidine at higher concentrations [323] and may hence also be an interesting antileishmanial lead.

From *Cupania cinerea* (Sapindaceae) the common sesquiterpene caryophyllene epoxide and two bisabolane sesquiterpenes (**6** and **7**) were isolated along with a variety of other natural products. Tests against *Tbr* and *Pfc* revealed that all tree compounds were inactive against the latter and that only the bisabolanes showed weak activity against the former parasite (IC_{50} values 38 and 61 μM, respectively) [324]. Some antiplasmodial activity had been reported, somewhat contradictory, for caryophyllene oxide (IC_{50}= 2.8μg/mL, *Pfc* K1 strain) in a study on *Polyalthia cerasoides* (Annonaceae) [325]. Further active constituents from *Cupania* were two new linear diterpenoid glycosides found in this study (cupacinosides), see section 4.1.3. and triterpenes such as cupacinoxepin (see sect. 4.1.4) [324].

Two cyclic bisabolane type endoperoxides, zingiberene-3,6-alpha- (**8**) and –beta-peroxide (**9**), were isolated from *Senecio selloi* and *Eupatorium rufescens* (Asteraceae). Both were equally active against *Pfc* (strain FCH-5) with EC_{50} concentrations of 10 μg/mL [326], so that the activity in this case does not depend on stereochemistry but solely on the presence of the peroxide moiety.

Of three sesquiterpenes isolated from the Rutaceae *Raulinoa echinata*, only the simple hydrocarbon germacrene D (**10**) showed mentionable activity against *Tcr* trypomastigotes (61% growth inhibition at 100 μg/mL) [327].

Two germacradiene alcohols, a diol (**11**) and a monol (**12**) isolated from *Reneilmia cincinnata* (Zingiberaceae) were reported active *in vitro* against *Pfc*, strains D6 and W2. The former strain (resistant to mefloquine) was more susceptible to the compounds which showed IC_{50}s of 1.63 and 1.54 μg/mL, respectively. The bicyclic sesquiterpene ketone oplopanone (**13**), isolated in the same study, was less active but still showed some activity (IC_{50}= 4.17 μg/mL) [328].

Two volatile aromadendrane type sesquiterpene hydrocarbons, aromadendrene (**14**) and alloaromadendrene (**15**) were included in a study on the *in vitro* activity of essential oils and constituents against bloodstream forms of *Tb* (strain TC221) and *Lmaj* (MRHO/IR/76). Although they were much less active than the monoterpene alcohol terpinen-4-ol (see sect. 4.1.1.), it appears noteworthy that **15** was almost ten times more active against the trypanosomes (IC_{50}= 1.9 μg/mL) than its stereoisomer **14** (IC_{50}= 18 μg/mL) [244], which is an excellent example for the profound influence that stereochemistry may have on biological activity.

The oxygenated *seco*-aromadendrane type sesquiterpene plagiochilin A (**16**) from *Plagiochila disticha* (Plagiochilaceae) showed moderate *in vitro* activity against *Lam* amastigotes and *Tcr* trypomastigotes (IC_{50}= 7.1 and 14.8 μM, respectively); its cytotoxicity against human macrophages, however, was higher than the antiprotozoal effects [306].

Three guaiane type sesquiterpene hydroperoxides (**17**-**19**) were isolated from *Pogostemon cablin* (Lamiaceae) and found active against *Tcr* epimastigotes *in vitro* with minimum lethal concentrations (MLC) of 0.84, 1.7 and 1.7 μM; the corresponding alcohols did not show activity (MLC >200 μM) so that the activity can be attributed to the reactive peroxide structure [329].

Besides the lactones already mentioned in 4.1.2.1, the already cited most recent work by Otoguro *et al.* [147] comprises also non-lactonic sesquiterpenes and some diterpenes (see below) with activity against *Tbb*. Of six sesquiterpenes tested, α-eudesmol (**20**) showed the highest activity (IC_{50}= 0.1 μg/mL) and an SI of >1000 which makes this comparatively simple compound a very promising lead that should by all means be tested also against the human parasites, *Tbr* or *Tbg*. Very strikingly, the isomer β-eudesmol (**21**), differing only in the position of the double bond, was 55 times less active [147].

Two C-5 epimeric eudesmane triols, kudtriol (**22**) and 5-epikudtriol (**23**), isolated from *Jasonia glutinosa* (Asteraceae) and tested against *Ldon* promastigotes and *Pfc* differed in activity such that **22** inhibited both parasites' growth *in vitro* at 250 μg/mL while the epimer did not [330].

Another eudesmane type compound, corymbolone (**24**), was isolated with the chemically related mustakone (**25**) from *Cyperus articulatus* (Cyperaceae). Both inhibited the incorporation of ^3H hypoxanthine in two strains (NF54 and ENT 30) of *Pfc in vitro*. Mustakone was the more active compound with IC_{50}s of 0.14 and 0.25 μg/mL, respectively, while corymbolone yielded values of 1.07 and 1.92 μg/mL [331].

The tricyclic sesquiterpene hydrocarbon longifolene (**26**), isolated from the oleoresin of *Pinus oocarpa* (Pinaceae) along with diterpenes, was among the active compounds against *Tcr* (Y strain) epimastigotes. Longifolene showed an IC_{50} of 44 μg/mL, [332] corresponding to 216 μM (not 0.21 as reported in the original article).

From a variety of sesquiterpenes isolated from *Teucrium ramosissimum* (Lamiaceae) and tested *in vitro* against *Pfc*, homalomenol C (**27**) showed the highest activity with an IC_{50} of 4.7 μM while not being toxic to MRC-5 embryonic lung cells up to 100 μg/mL [333].

Of four sesquiterpenoid compounds isolated from *Cyperus rotundus* (Cyperaceae), one of relatively few examples of monocotyledonous plants with antiprotozoal sesquiterpenes, 10,12-peroxycalamenene (**28**) with a cyclic endoperoxide structure was found the most active constituent *in vitro* against *Pfc* with an EC_{50} of 2.33 μM [334].

The drimane sesquiterpene dialdehyde polygodial (**29**) from *Drimys* (Winteraceae) species, known for its cytotoxic and other activities, was reported to possess antileishmanial and anti-chagasic activity. It was tested against promastigotes of *Lch*, *Lam* and *Lbra* where it yielded IC_{50} values of 62, 32 and 35 μg/mL, respectively. It was shown, however, that no particular selectivity exists since the IC_{50} for cytotoxicity against rhesus monkey kidney cells (LLC-MK2) was 39 μg/mL. No activity was found against *Lcha* amastigotes. *Tcr* trypomastigotes were more sensitive against this dialdehyde with an IC_{50} of 2.03 μg/mL. In this test, benznidazole was used as positive control, which was less active than polygodial by factor 19 [335] so that polygodial may be an interesting lead for new antichagasic compounds.

A series of drimane- and coloratane type sesquiterpenes from *Warburgia ugandensis* (Canellaceae) were tested *in vitro* against *Tbr* and two strains (3D7 and K1, chloroquine sensitive and –resistant, respectively) of *Pfc*. 8,9-Dialdehyde structures were the most active against *Tbr* with IC_{50} values in the range between 0.56 and 6.4 μM. The three most active derivatives were muzigadial (**30**), 6α,9α-dihydroxy-4(13),7-coloratadiene-11,12-dial (**31**) and mukaadial (**32**) with IC_{50} values of 0.56, 1.25 and 0.64 μM, respectively. The last mentioned compound was also among the more active ones against the two mentioned strains of *Pfc* (IC_{50}= 6.4 and 7.9 μM). Some structure-activity relationships were

discussed by the authors, the most important one being that the presence of dialdehyde structures enhances activity. Determination of cytotoxicity against mammalian cells in order to assess the selectivity of these reactive dialdehyde structures was not performed [336].

A variety of polyacylated β-dihydroagarofuran derivatives such as **33**, known as characteristic metabolites from Celastraceae such as *Maytenus* and *Crossopetalum* species, have been demonstrated to possess an interesting type of activity in that they are able to reverse multidrug resistance, not only in cancer cells, but also in *Leishmania* parasites [337-339]. These compounds appear to interact with the transmembrane domain of P-glycoproteins in a very specific way as has been demonstrated in mechanistic studies with the human protein [340] as well as 3D QSAR analysis using the ComSIA method [341].

Although not isolated from *green plants*, some halogenated sesquiterpenes from red algae of the genus *Laurencia* should not remain unmentioned. 8-bromo-10-*epi*-β-snyderol (**34**) from *Laurencia obtusa* was reported to possess antimalarial activity *in vitro* against the D6 and W2 clones of *Pfc* which it inhibited at IC_{50} values of 2.7 and 4.0 μg/mL, respectively [342]. More recently, antileishmanial activity was reported for the spiro-derivatives elatol (**35**) and obtusol (**36**) as well as some related compounds isolated from *Laurencia dendroidea*. Elatol was reported to be active against *Lam* promastigotes and intracellular amastigotes at IC_{50} concentrations of 4.0 and 0.45 μM, respectively, the latter with an SI of 3, determined with J774G8 macrophages [343]. Even more recently, somewhat lower activity (IC_{50} 29.6 and 13.7 μM) was reported for elatol against the respective stages of the same parasite. In this study, obtusol was found slightly more active than elatol (IC_{50} 15.2 and 9.5 μM, respectively) and fairly selective with an SI of ≈35 [344].

4.3. Diterpenes

Diterpenes with many skeletal types are found in different plant families [106, 223, 345-348], marine organisms [149, 347, 349] as well as microorganisms [350]. Many of them have been tested against different protozoan parasites in the last years. Polycyclic antiprotozoal diterpenes such as labdanes, abietanes, clerodanes, kauranes and cassanes comprise the most common plant-derived compounds within this class. Other biologically active minor groups such as acyclic diterpenes, nor-diterpenes, pimaranes, phorbol esters and cembranes among others have also been reported. In addition, several semi-synthetic derivatives have been obtained, e.g. for application in studies of structure-activity relationships [350-352]. Most of these antiprotozoal diterpene classes comprising 53 chemical structures that have been reported in the last years are described herein (Fig. **7A**).

Nine promising diterpenes (**1**-**9**, Fig. **7A**) active against human African trypanosomiasis have been reported recently. Some of them were also tested against other parasites as follows. An *in vitro* screen to detect active compounds against *Tbb* indicated the following diterpenes from different sources: the kaurane *ent*-11α-hydroxy-16-kauren-15-one (**1**) from *Porella densiflora* (Porellaceae, a fungus), the labdanes labda-8(17),12-diene-15,16-dial (**2**) and labda-12,14-dien-7α,8α-diol (**3**) from *Curcuma aromatica* (Zingiberaceae) and *Phaleria perrottetiana* (Thymelaeaceae), respectively, as well as the sacculatane **4** from the liverwort *Pellia endiviifolia* (Pelliaceae) [147]. Among them, **1**, **2** and **4** showed potent antitrypanosomal activities, with EC_{50} values of 0.23, 0.45 and 0.37 μg/mL, respectively, whereas **3** was less active than the others (EC_{50}= 3.56 μg/mL). *In vitro* cytotoxicity assays of these 4 diterpenes against human diploid embryonic cells (MRC-5) indicate that **1** is highly cytotoxic (EC_{50}= 0.97 μg/mL), while **2**, **3** and **4** are only slightly cytotoxic with EC_{50} values of 24.8, 23.19 and 5.15 μg/mL, respectively.

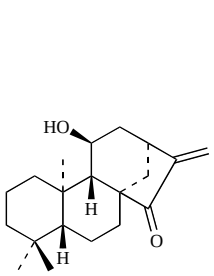
The genus *Aframonum* (Zingiberaceae) afforded 4 new antiparasitic diterpenes. The 2 labdanes 15ξ-methoxy-labdan-8(17),11(*E*),13(14)-trien-15,16-olide (**5**) and 12(*S*)-hydroxy-15ξ-methoxy-labdan-8(17),13(14)-dien-15,16-olide (**6**) isolated from the methanolic extract of the rhizomes of *Aframonum sceptrum* were tested *in vitro* against bloodstream forms of *Tbb* as well as promastigotes of *Ldon* [353]. Compound **5** showed moderate activity against *Ldon* (IC_{50}= 25.0 μM) and was poorly active against *Tbb* (IC_{50}= 204.0 μM), while **6** was reasonably active against *Tbb* (IC_{50}= 35.7 μM) and strongly active against *Ldon* (IC_{50}= 5.7 μM). The 2 labdanes identified as aulacocarpin C (**7**) and D (**8**) from the acetone extract of the seeds of *A. aulacocarpos* were active against 3 strains of *Tbr* and displayed IC_{50} values in the range of 15-29 μg/mL [354]. The labdane **7** was weakly active against the strains Lab 110 EATRO (IC_{50}= 15.5 μg/mL), KETRI 243 (IC_{50}= 29.0 μg/mL), and KETRI 243 As 103 (IC_{50}= 24.0 μg/mL), while **8** showed IC_{50} values of 17.5, 21.0, and 24 μg/mL, respectively. None of the compounds was active against a fourth strain tested, KETRI 269.

The cembrene serratol (**9**) isolated from the dichloromethane extract of the gum resin of *Boswellia serrata* (Burseraceae) showed significant activity against *Tbr* (IC_{50}= 1.1 μg/mL, 3.8μM) and *Pfc* (IC_{50}= 0.72 μg/mL, 2.5 μM) but was much less active against *Tcr* and *Ldon* (IC_{50}= 44 and 12 μM, respectively). Serratol displayed cytotoxicity against the L6 rat skeletal myoblast cell line at a higher concentration (IC_{50}= 11.4 μg/mL, 39 μM) [355].

Several diterpenes were screened for potential activity against Chagas disease and exhibited promising activities (**10**-**24**, Fig. (**7A**)). The compound *ent*-kaur-16-en-19-oic acid (**10**) isolated from the aerial parts of *Mikania obtusata* [356] as well as from the roots of *Viguiera aspilioides* (Asteraceae) [351] was the first kaurane diterpene active *in vitro* against trypomastigote forms of *Tcr* (Y strain, IC_{50}= 500 μg/mL, 1.66 mM) [351, 356]. Based on this finding, further natural [350] or semi-synthetic kaurane derivatives [351, 352, 357] have been evaluated against this parasite, therefore comprising a rich source of biologically active diterpenes.

A further promising class of trypanocidal diterpenes comprises the pimaranes. Five known natural *ent*-pimaranes (**11**-**15**) isolated from the dichloromethane extract of the roots of *Viguiera arenaria* (Asteraceae) as well as a C-13 acetoxy semi-synthetic derivative (**16**) were reported to show moderate *in vitro* activity against trypomastigote forms of *Tcr* (Y strain), with IC_{50} values in the range of 116.5 to 1454.5 μM [358]. The most active compounds were *ent*-15-pimarene-8β,19-diol (**14**) and *ent*-8(14),15-pimaradiene-3β-acetoxy (**16**), with IC_{50} values of 116.5 and 149.3 μM, respectively. The pimarane acanthoic acid (**17**), which was isolated from the *n*-hexane extract of the stems of *Annona amazonica* (Annonaceae), showed anti-proliferative *in vitro* activity against epimastigote forms (Y strain) of *Tcr* displaying an IC_{50} value of 59.0 μM [359]. The 50% cytotoxic concentration (CC_{50}) of **17** on monkey kidney cells ($LLCMK_2$) was of 347.0 μM.

Four new natural abietanes identified as 14-hydroxy-6,12-dione-7,9(11),13-abietatriene (**18**), 6,12,14-trihydroxyabieta-5,8,11,13-tetraen-7-one (**20**), ar-abietatriene-12-ol-6,7-dione-14,16-oxide (**21**), ar-abietatriene-12,16-diol-14,16-oxide (**22**) as well as the known compound ferruginol (**23**), which were obtained from the chloroform extract from the roots of *Craniolaria annua* (Martyniaceae), as well as the semi-synthetic derivative 14-hydroxy-6-oxoferruginol (**19**), displayed *in vitro* activity against trypomastigotes and epimastigotes of *Tcr* [360]. Compounds **18**-**22** showed good activity against trypomastigotes with IC_{50} values of 5.6, 0.85, 25, 20 and 5.8 μM, respectively, while the activity of ferruginol (**23**) was lower (IC_{50}= 52 μM). Compounds **21**-**23** showed lower activity against the epimastigote forms of *Tcr* with IC_{50} values in the range of 48 and 90 μM, while **19** and **20** were more active (IC_{50}= 5.7 and 2.1 μM, respectively). The cytotoxicity



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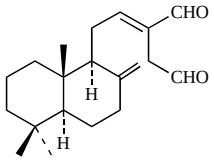

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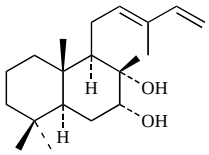

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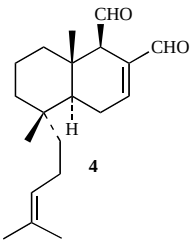

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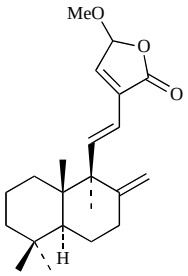

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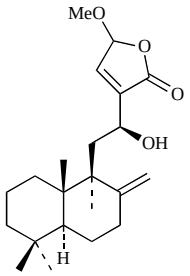

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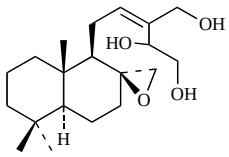

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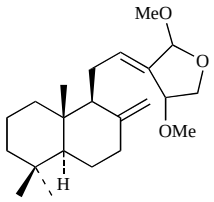

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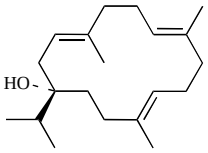

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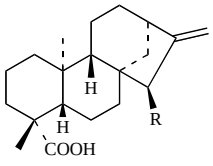

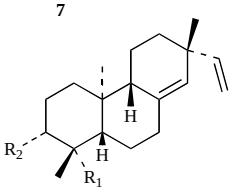

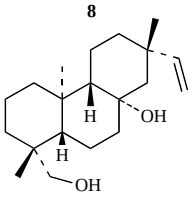

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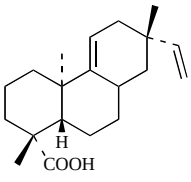

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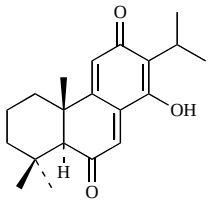
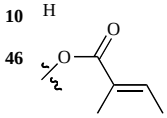


18

	R_1	R_2
10	R	H
11	COOH	H
12	Me	OH
13	CH_2OH	OH
14	COOH	H
15	Me	OAc



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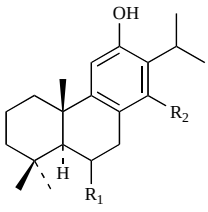

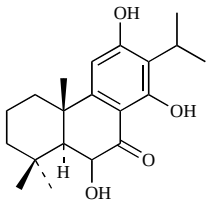

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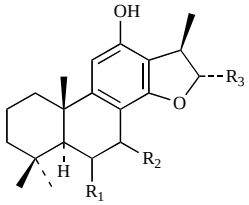

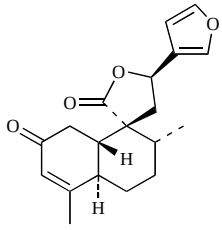

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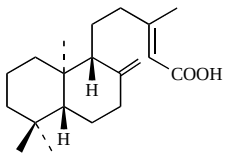
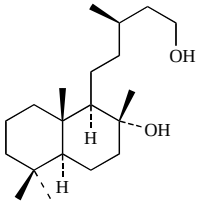


25

	R_1	R_2
19	=O	OH
23	H_2	H

	R_1	R_2	R_3
21	=O	=O	H
22	H_2	H_2	OH



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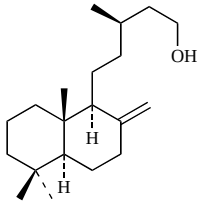

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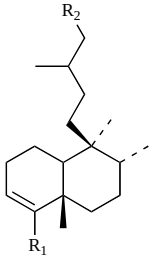

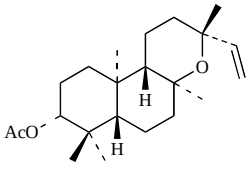

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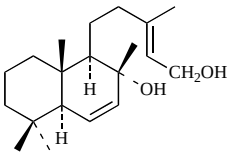


36

	R_1	R_2
28	CH_2OH	CH_2OH
29	CH_2OAc	CH_2OH
30	CH_2OAc	CH_2OAc
31	CH_2OH	CH_2OAc
32	CH_2OAc	CO_2H
33	CHO	CH_2OAc
34	CHO	CH_2OH

A

(Fig. 7). Contd…..

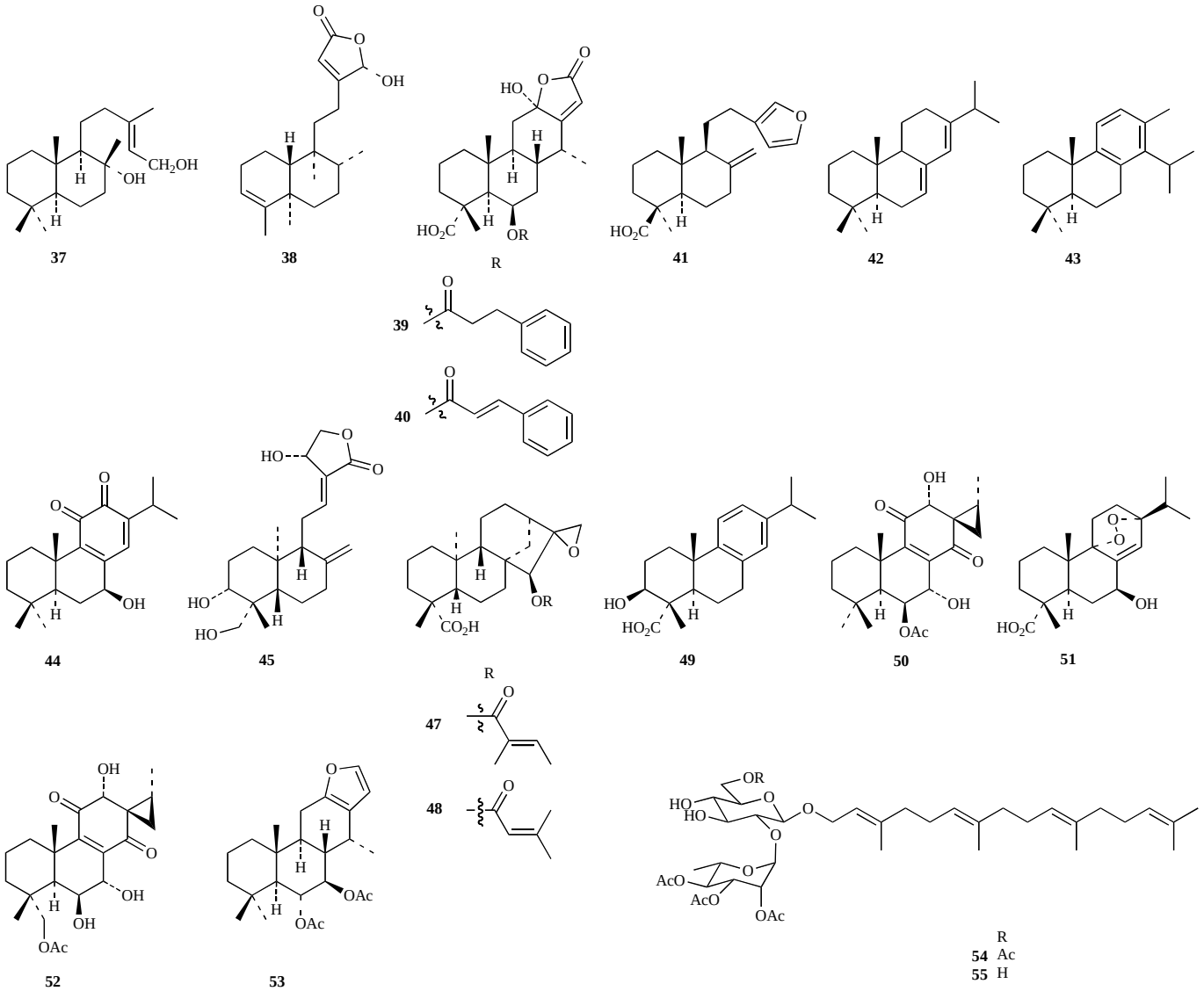


Fig. (7). Structures of Diterpenes with antiprotozoal activity.

on Vero cells was also low for all compounds (IC_{50} values from 28.3 to 200 μM) except for **19** (IC_{50} = 5.6 μM).

The known clerodane *trans*-dehydrocrotonin (**24**) from the stem bark of *Croton cajucara* (Euphorbiaceae) showed activity against trypomastigote forms of *Tcr* [361]. The effects of **24** on the 4 strains Dm28C, Y, GLT 291 and C45 were evaluated and the compound showed IC_{50} values of 109.5, 187.2, 181.9 and 144.4 μg/mL, respectively.

The known labdane copalic acid (**25**) isolated from the leaves of *Aristolochia cymbifera* (Aristolochiaceae) [362] displayed *in vitro* activity against trypomastigotes of *Tcr* (IC_{50}= 39.57 μM), low activity against amastigotes (IC_{50} > 164.5 μM) and lower toxicity on THP-1 mammalian cells (IC_{50}= 319.50 μM).

Several leishmanicidal diterpenes have been reported recently (**23** and **26**-**44**, Fig. (**7A** and **7B**)). The labdanes labdan-8α,15-diol (**26**) and labd-8(17)-en-3β,15-diol (**27**), isolated from the ethanolic extract from the leaves of *Aeonium lindleyi* (Crassulaceae), showed activity *in vitro* against promastigotes of *Ltro* (IC_{50} = 77.0 μM) and *Lbra* (IC_{50} = 68.0 μM), respectively [363]. Both compounds did not show significant activity against axenic amastigote forms of *Lam* up to 200 μg/mL.

Seven clerodanes (**28**-**34**) isolated from the aerial parts of *Cistus monspeliensis* and 3 labdanes (**35**-**37**) from the resin of *C. creticus* subsp. *creticus* (Cistaceae) were evaluated *in vitro* against promastigote cultures of *Ldon* [364]. Among the clerodanes, 18-acetoxy-*cis*-clerod-3-en-15-ol (**29**), 15,18-diacetoxy-*cis*-clerod-3-ene (**30**) and 15-acetoxy-*cis*-clerod-3-en-18-al (**33**) were the most potent and displayed IC_{50} values of 3.3, 3.4 and 5 μg/mL, respectively. The clerodanes 15,18-dihydroxy-*cis*-clerod-3-ene or cistadiol (**28**), 15-acetoxy-*cis*-clerod-3-en-18-ol (**31**), 18-acetoxy-*cis*-clerod-3-en-15-oic acid (**32**) and 15-hydroxy-cisclerod-3-en-18-al (**34**) exhibited higher IC_{50} values (18, 15 and 13 μg/mL, respectively). The labdanes *ent*-3β-acetoxy-13-*epi*-manoyl oxide (**35**), 13(*E*)-labda-7,13-diene-15-ol (**36**) and 13(*E*)-labd-13-en-8α,15-diol (**37**) showed IC_{50} values in the same range (17, 18 and 17 μg/mL, respectively).

The clerodane 16α-hydroxycleroda-3,13(14)*Z*-dien-15,16-olide (**38**), which was isolated from the ethanolic extract of the leaves of

Polyalthia longifolia var. *pendula* (Annonaceae), was found to be a potent and safe compound against *Ldon* [365]. This diterpene showed potent *in vitro* leishmanicidal effect on amastigote forms of the parasite (IC_{50}= 5.79 μg/mL). The *in vivo* effect has been carried out orally in hamsters infected with *Ldon* at 4 doses (25, 50, 100 and 250 mg/kg, *n*= 6) for 5 days showing efficacy up to 50 mg/kg. Survival studies *in vivo* (6 months) as well as cytotoxicity *in vitro* on J774A.1 macrophages indicated that compound **38** is safe. It was also shown that this clerodane inhibits DNA topoisomerases I of *Ldon*.

Two novel (**39** and **40**) and a known cassane (**41**) were isolated from the ethanolic extract from the stems of *Caesalpinia echinata* (Fabaceae/Caesalpinioideae) and displayed moderate activity against *Lam* [366]. The compounds 6β-*O*-2',3'-dihydrocinnamoyl-12-hydroxy-(13)15-en-16,12-olide-18-cassaneoic acid (**39**), 6β-*O*-cinnamoyl-12-hydroxy-(13)15-en-16,12-olide-18-cassaneoic acid (**40**) and lambertianic acid (**41**) inhibited *in vitro* amastigote-like forms of *Lam*. The cassanes **39**, **40** and **41** at 20 μg/mL showed 56, 69 and 62% leishmanicidal activity, respectively. These three compounds were not toxic *in vitro* on mononuclear cells obtained from human peripheral blood (PBMCs) at 20 μg/mL.

Four abietanes isolated from *Juniperus procera* (Cupressaceae) berries were active against *Pfc* (**42**) and *Ldon* (**23**, **43** and **44**) [367]. Abieta-7,13-diene (**42**) showed *in vitro* antimalarial activity against *Pfc* D6 and W2 strains (IC_{50}= 1.9 and 2.0 μg/mL, respectively). The compounds ferruginol (**23**), totarol (**43**) and 7β-hydroxyabieta-8,13-diene-11,12-dione (**44**) were effective against *Ldon* promastigotes showing IC_{50} values of 3.5, 3.5 and 4.6 μg/mL, respectively. The 4 compounds were not toxic on the mammalian kidney epithelial cell line at 4.76 μg/mL.

Besides the compounds already discussed above, additional four different classes of antimalarial diterpenes have been reported (**10** and **45**-**53**, Fig. (**7B**)). The labdane andrographolide (**45**) was isolated from a methanolic fraction from the barks of *Andrographis paniculata* (Acanthaceae) and showed *in vitro* activity on *Pfc* (IC_{50}= 9.1 μM) [368]. The combination of **45** with the anti-malarial compounds curcumin and artesunate indicated that the former was the better partner at different ratios. *In vivo* toxicity tests with **45** were carried out intraperitoneally in mice and neither mortality nor any other abnormality was observed during the treatment period.

The kauranes **10** as well as **46**-**48** isolated from the chloroform extract of the aerial parts of *Aspilia pruliseta* (Asteraceae) were evaluated *in vitro* against *Pfc* [369]. The 3 known *ent*-kaur-16-en-19-oic acid (**10**), 15-(2*Z*)-[(2-methyl-1-oxo-2-butenyl)oxy]-4α,15β-kaur-16-en-18-oic acid (**46**), 16,17-epoxy-15-[(2-methyl-1-oxo-2-butenyl)oxy]-4α,15β(*Z*)-kauran-18-oic acid (**47**) as well as the new compound *ent*-15β-senecioyloxy-16,17-epoxy-kauran-18-oic acid (**48**) displayed moderate activity. The IC_{50} values of **10**, **46**, **47** and **48** for the clone D6 of *Pfc* were 18.0, 14.3, 23.4 and 23.4 μM, respectively; for the clone W2 the values of **10**, **46** and **47** were 17.6, 18.4 and 17.5 μM, respectively, while compound **48** was inactive. Cytotoxicity was determined on the KB (human nasopharyngeal carcinoma) cell line and the 4 kauranes were not toxic at 20 μg/mL.

Four antimalarial abietanes (**49**-**52**) were isolated from the aerial parts of *Anisochilus harmandii* (Lamiaceae) [370]. Two new compounds were identified as 4-*epi*-triptobenzene L (**49**) and 12-*O*-deacetyl-6-*O*-acetyl-19-acetyloxycoleon Q (**50**) along with the 2 known 9α-13α-epidioxyabiet-8(14)-en-18-oic acid (**51**) and 12-*O*-deacetyl-6-*O*-acetyl-18-acetyloxycoleon Q (**52**). Compounds **49**, **50**, **51** and **52** exhibited strong *in vitro* antiplasmodial activity against *Pfc* with IC_{50} values of 4.70, 2.90, 3.03 and 7.20 μg/mL, respectively. The cytotoxicity of the 4 abietanes was tested against KB cells, human small lung cancer (NCI-H187) and human breast cancer (MCF-7) cells. The compounds **49**-**51** were not toxic, while **52** exhibited significant cytotoxicity against NCI-H187 cells (IC_{50}= 4.43 μg/mL).

A new cassane identified as 6α,7β-diacetoxyvouacapane (**53**), isolated from the seeds of *Bowdichia nitida* (Fabaceae), showed promising *in vitro* activity against *Pfc* 3D7 (IC_{50}= 0.39 μg/mL) and did not show cytotoxicity against human colon carcinoma COLO201 cells (IC_{50} > 100 μg/mL) [371].

Quite recently, two new acyclic diterpenes, namely acetylated geraniol glycosides termed cupacinosides (**54**, **55**), were isolated from *Cupania cinerea* (Sapindaceae) along with sesqui- and triterpenes (sections 4.2 and 4.4.) as well as some other compounds. Both compounds showed activity against *Tbr* and *Pfc in vitro*: IC_{50} *Tbr*: 4.6 and 15.8, *Pfc*: 1.3 and 2.3 μM, respectively, and lower cytotoxicity against L6 cells (IC_{50}= 11.6 and 8.7, respectively) [324].

4.4. Triterpenes Including Limonoids and Quassinoids

Triterpenes are C_{30} terpenoids widely distributed over the plant kingdom. These compounds, showing a broad range of biological activities, can be found in their free form in a multitude of cyclic skeletal types, or linked to sugar moieties (triterpene saponins). Frequently, triterpenes also occur in modified forms due to ring cleavage (*seco*-forms), oxidations and loss of carbon atoms (nor-triterpenoids, limonoids, quassinoids). These latter classes, often particularly interesting with respect to biological activity, are restricted to some plant families, mainly of the order Sapindales, such as Rutaceae, Simaroubaceae, Meliaceae or Cneoraceae.

The structures of the triterpenes mentioned here are shown in Fig. (**8A** (**1**-**27**), **8B** (**28**-**54**) and **8C** (**55**-**64**)).

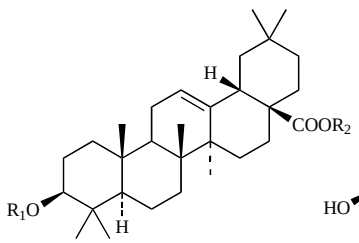
The most commonly distributed triterpenes in vegetal species are oleanolic (**1**) and ursolic (**8**) acids, which have been described as potent agents against *Leishmania* sp. [372]. Aiming to establish some structure/antileishmanial activity relationships, some derivatives of oleanolic acid (compounds **2 – 6**) were prepared [372]. Comparing the IC_{50} values determined for compounds **1** (360.3 μM), **8** (439.5 μM), **2** (406.0 μM), **3** (340.4 μM), **4** (240.4 μM), and **5** (174.9 μM), it was possible to suggest that an increase in lipophilicity at C-17 is more influential on antileishmanial activity than enhanced lipophilicity at C-3. The potassium salt **6** of **1** showed a loss of antileishmanial activity (IC_{50} 458.7 μM).

Compounds **1** and **8** also displayed activity against trypomastigote forms of *Tcr* (Y strain) with reported *in vitro* ED_{100} values of 1.6 and 0.4 μg/mL (3.5 and 0.9 μM), respectively [373]. Somewhat contrastingly, IC_{50} values of 12.8 μM and 17.1 μM were reported in another study for the same compounds against the same life stage and strain, respectively [374]. It is, however, interesting to note that the trypanocidal activity was reported to increase when ursolic acid was administered as its potassium salt derivative **6** (IC_{50} = 8.9 μM) [374] (compare previous paragraph).

The 3-epimer of **1**, *epi*-oleanolic acid (**9**) was isolated from *Celaenodendron mexicanum* (Euphorbiaceae) and reported to have antiplasmodial activity (*Pfc*) *in vitro* with an IC_{50} = 28.3 μM [375].

In another report, the phytochemical analysis of *Miconia fallax* and *M. stenostachya* (Melastomataceae) was described. Five pure triterpenes and two mixtures were obtained and tested against the Y strain of *Tcr* [376]. The most active compounds were **1, 8** and **10** (IC_{50} = 21.3, 80.4 and 56.6 μM) followed by **11** and **12** whose activity was very weak (IC_{50} = 295 and 402 μM). The mixtures of triterpenes did not display significant trypanocidal activity.

Takahashi *et al.* evaluated the *in vitro* leishmanicidal activity against *Lmaj* of several natural products (including sesqui-, di- and triterpenes as well as some phenylpropanoids) isolated from Ferns and *Betula* (Betulaceae) species, including some triterpenoids with oleanane, lupane, ursane, and dammarane skeleton [377]. Among



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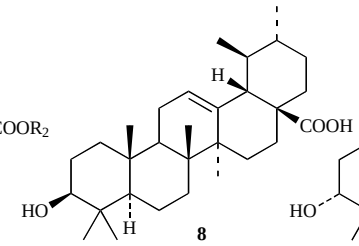
2	Ac	H
3	Laur	H
4	Ste	H

	R_1	R_2
5	H	Me
6	H	K
7	Caf	H



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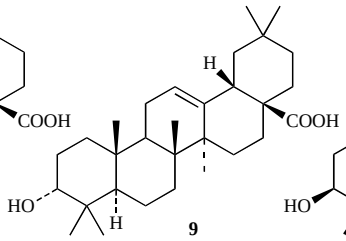

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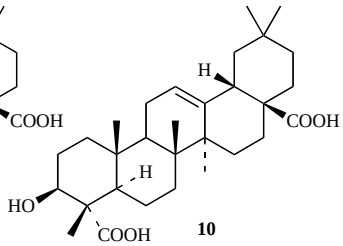

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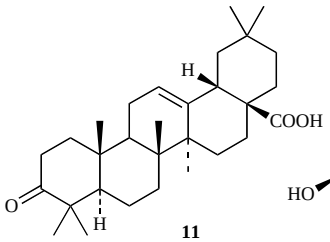

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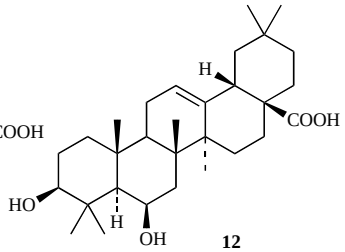

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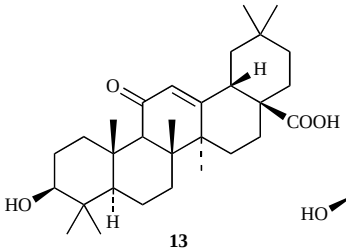

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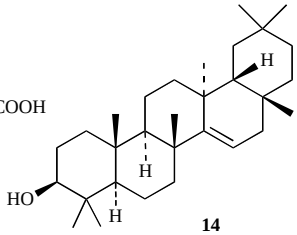

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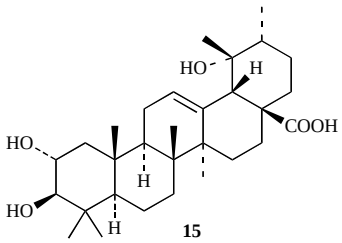

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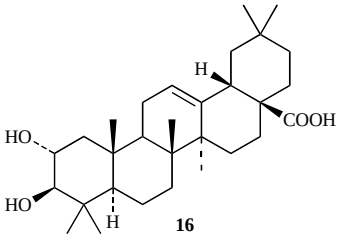

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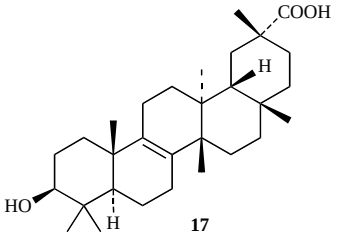

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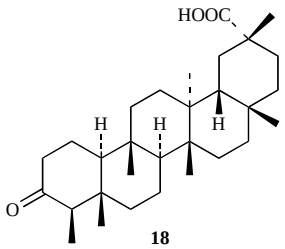

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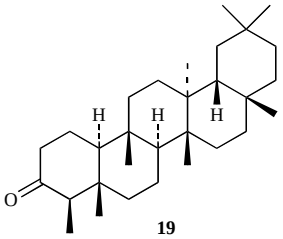

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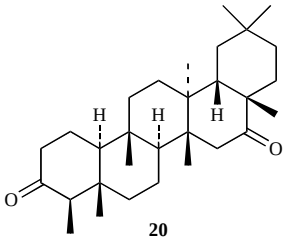

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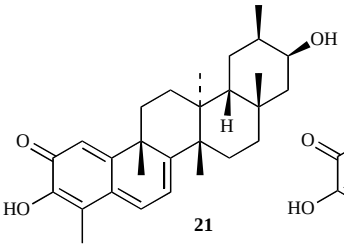

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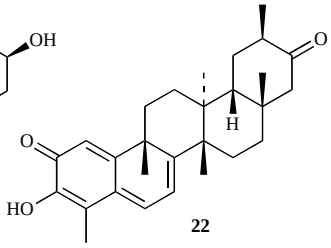

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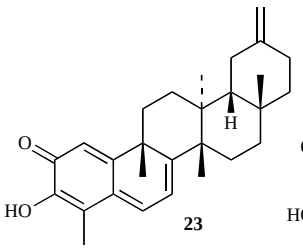

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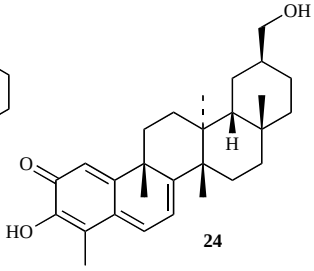

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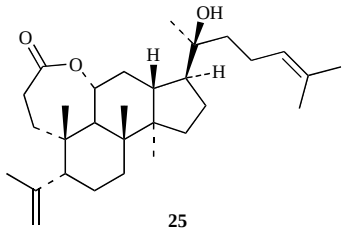

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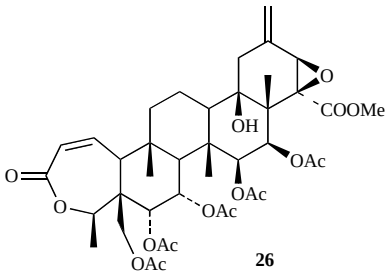

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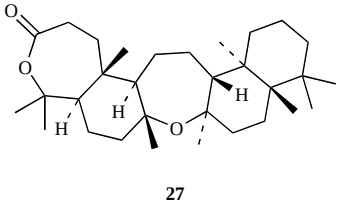

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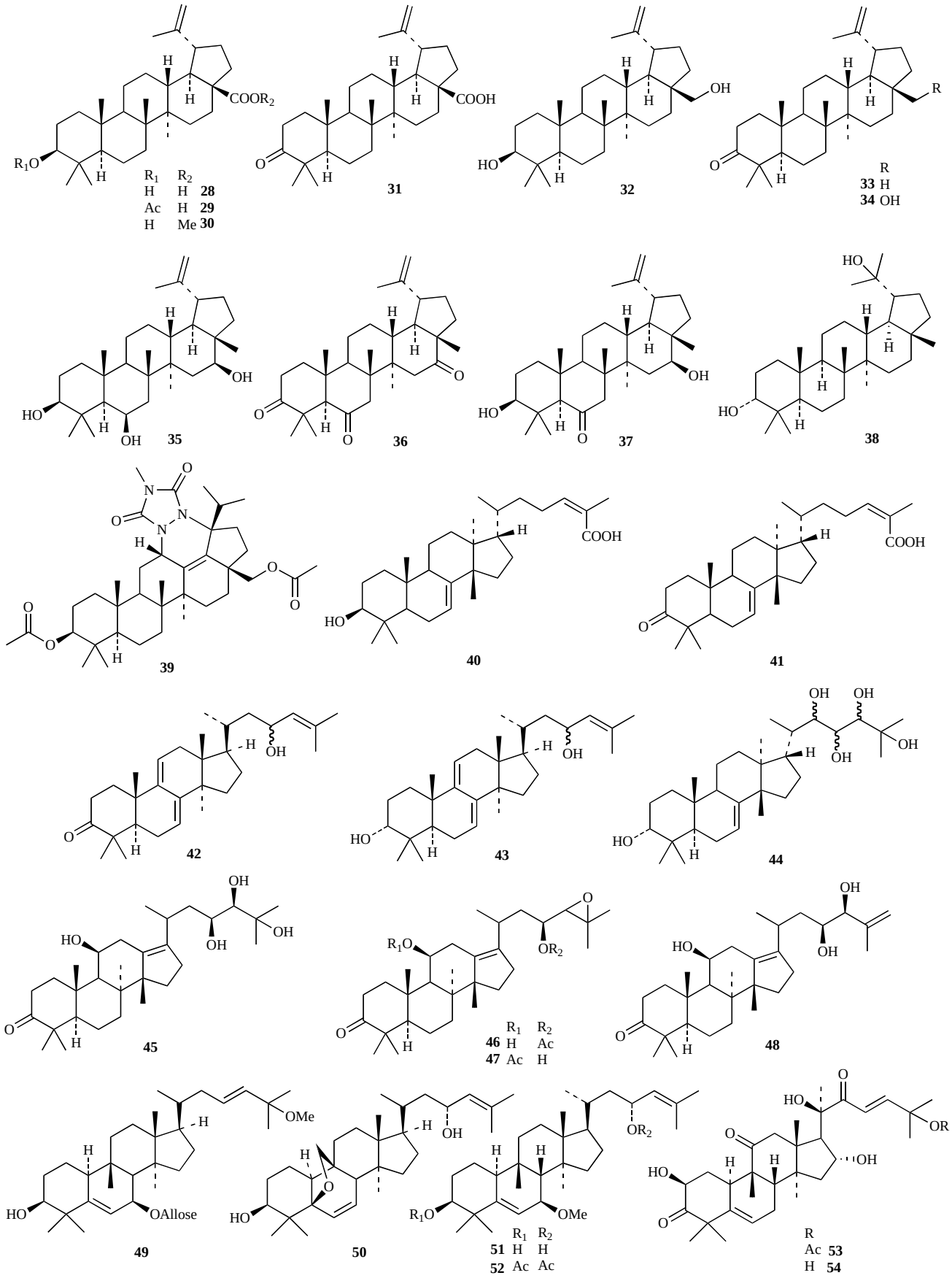
A

(Fig. 8). Contd…..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H H 28
Ac H 29
H Me 30
31
32
R
33 H
34 OH
35
36
37
38
39
40
41
42
43
44
45
R1 R2
46 H Ac
47 Ac H
48
49
50
R1 R2
51 H H
52 Ac Ac
R
Ac 53
H 54

B

(Fig. 8). Contd…..

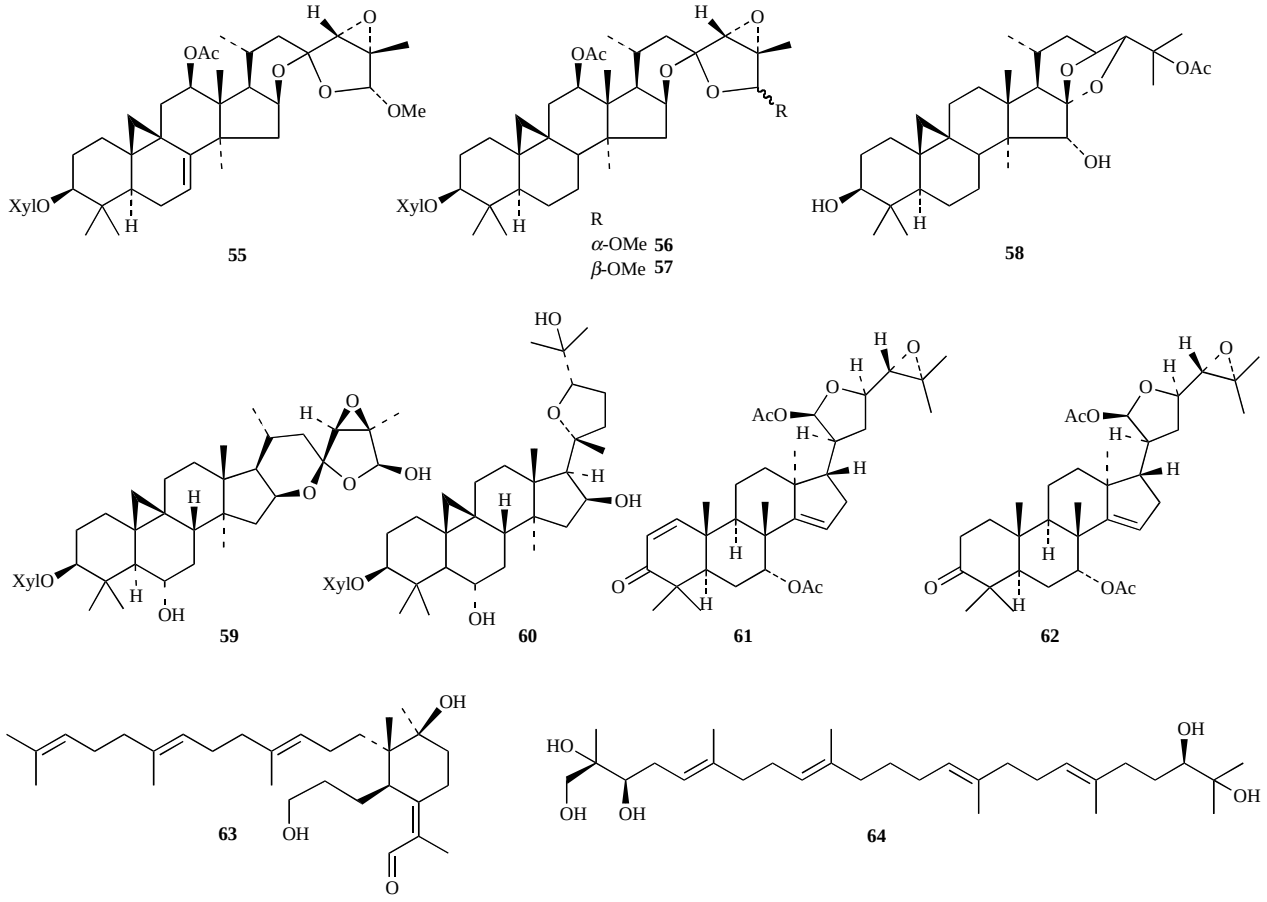

Fig. (8). Structures of Triterpenes with antiprotozoal activity.

the tested triterpenoids, seven showed antileishmanial activity – oleanolic acid (**1**), oleanolic acid acetate (**2**), oleanolic acid caffeate (**7**), ursolic acid (**8**), glycyrrhetic acid (**13**), betulinic acid (**28**) and ovalifoliolide A (**25**), with IC_{50} values ranging from 7.5 to 40 μg/mL. Based on their results, the authors suggest that a carboxylic moiety is important for the antileishmanial activity of most of these derivatives.

A pentacyclic triterpenoid, tormentic acid (**15**), was isolated from *Cecropia pachystachya* (Urticaceae) which displayed an *in vitro* IC_{50} of 15 μg/mL against chloroquine-resistant *Pfc* (W2 strain) and suppressed parasitemia in *Pber*-infected mice by 35 and 58 % when administered in 10 and 15 mg/kg for 4 days (chloroquine: 76 and 100%, resp.) [378].

Populnoic acid (**18**), isolated from *Austroplenckia populnea* (Celastraceae), displayed potential against *Ldon* (IC_{50} = 18 μg/mL), while its methyl ester was inactive [379]. The reported data indicate that the presence of a free carboxyl group in the molecular structure of this triterpene is crucial to antileishmanial activity.

Phytochemical analysis of a trypanocidal extract from *Cleiloclinum cognatum* (Hippocrateaceae) afforded two quinonemethide type triterpenoids, **21** and **22**, with relatively moderate IC_{50} values against trypomastigote forms of *Tcr* (Y strain) of 306.9 and 486.6 μM, respectively [380].

Two bisnortriterpene quinonemethides, isoiguesterin (**23**) and 20-*epi*-isoiguesterinol (**24**), were isolated from *Salacia madagascariensis* (Celastraceae) and showed potent activity against *Pfc* (D6 and W2 clones; IC_{50}=0.2 and 0.17 (**23**), 0.068 and 0.068 (**24**) μg/mL, resp.) as well as against *Ldon* (life stage not reported) IC_{50} = 0.032 and 0.027 μg/mL for **23** and **24** while being quite selective (Vero cells; IC_{50}=1.6 and 2.1 μg/mL) [381].

In vitro antileishmanial activity of 6α,7α,15β,16β,24-pentacetoxy-22α-carbomethoxy-21β,22β-epoxy-18β-hydroxy-27,30-bisnor-3,4-secofriedela-1,20(29)-dien-3,4R-olide (**26**), isolated from *Lophanthera lactescens* (Malpighiaceae), was determined against intramacrophage amastigote forms of *Lam* [382]. This compound displayed an IC_{50} = 0.41 μg/mL and did not show cytotoxicity to mouse cells. It was furthermore unable to affect proliferation of naïve or activated B and T cells, as well as B cell immunoglobulin synthesis.

Alakurtti *et al.* described the preparation of several derivatives of the widespread lupane triterpene betulinic acid (**28**), which were evaluated against amastigote forms of *Ldon* [383]. Among the twenty-three synthetic derivatives, four showed activities with GI_{50} (concentration for 50% growth inhibition) lower than 30 μM. Compound **39**, formed by the coupling of 3,28-di-O-acetyllupa-12,18-diene with 4-methylurazine was the most effective derivative with a GI_{50} = 8.9 μM against *Ldon* amastigotes. Additionally, these authors described also the effect of betulonic acid (**31**) against *Ldon*, whose GI_{50} was determined as 14.6 μM [383].

Phytochemical analysis of a stem bark extract from *Bertholletia excelsa* (Brazil nut, Lecythidaceae) [384] afforded several fractions with weak *in vitro* activity. A fraction showing trypanocidal (*Tcr* trypomastigotes) activity with 75.4% growth inhibition at 500 μg/mL was shown to consist exclusively of **28**.

Phytochemical analysis of *Pentalinon andrieuxii* (Apocynaceae) afforded betulinic acid (**28**), betulinic acid acetate

(**29**), betulinic acid methyl ester (**30**), betulonic acid (**31**), and betulin (**32**) which were evaluated for their antiprotozoal activity against *Leishmania* sp. and *Tcr*. The results showed that modifying the C-3 position increases leishmanicidal activity while modification of the C-3 and C-28 positions decreases trypanocidal activity [385].

Using an activity-guided isolation protocol, extracts from *Cupania cinerea* (Sapindaceae) afforded -besides diterpenes (section 4.3.) and sesquiterpenes (section 4.2.) as further active compounds- six triterpenes (**14**, **27, 33, 34**) which displayed IC_{50} against *Pfc* ranging from 3.0 and 4.7 (**34**, **33**) to >10 μM and against *Tbr* from 10.5 (**14**) to >90 μM. A new *seco*-triterpene lactone, cupacinoxepin (**27**) showed only moderate activity (8.7 (*Pfc*) and 71.6 (*Tbr*) μM) [324].

Teles *et al.* described the fractionation of an EtOH extract obtained from the fruits of *Combretum leprosum* (Combretaceae), which displayed leishmanicidal activity against promastigote forms of *Lam* (IC_{50} = 24.8 μg/mL). Chromatographic procedures afforded the triterpene 3β,6β,16β-trihydroxylup-20(29)-ene (**35**), which showed a potent inhibitory activity on promastigote proliferation (IC_{50} = 3.3 μg/mL) [386]. Aiming to discover some structure/activity relationships, this compound was subjected to some structural modifications. While acetylated derivatives were inactive, the ketones **36** and **37** were active against promastigotes (IC_{50} = 3.48 μg/mL and 5.8 μg/mL, respectively). Notably, none of the tested compounds showed cytotoxicity against mouse peritoneal macrophages [386].

Bioguided fractionation of the crude extract from *Grewia bilamellata* (Malvaceae/Grewioideae) that showed antiplasmodial to *Pfc*, D6 (IC_{50} = 2.2 μg/mL) and W2 strains (IC_{50} = 1.7 μg/mL), afforded two triterpenoids, 3α,20-lupandiol (**38**, IC_{50} = 19.8 and 19.1 μM, resp.) and 2α,3β-dihydroxyolean-12-en-28-oic acid (**16**, IC_{50} = 21.1 and 8.6 μM, resp.). None of the other isolated compounds were as active as the crude extract either [387].

Camacho *et al.* described the bioguided fractionation of an active extract from *Celaenodendron mexicanum* (Euphorbiaceae) against promastigote forms of *Ldon* and against *Pfc* [375]. Chromatographic separation afforded six triterpenes, three of which were reported to exhibit antileishmanial potential (IC_{50} in μM): **9** (18.8), **40** (50.0) and **41** (13.7), and five to possess antiplasmodial activity (IC_{50} in μM): **40** (139.4), **41** (122), **9** (28.3), **19** (765.5), and **34** (138.8). Despite the reported *in vitro* activity of compounds **9**, **40** and **41** against promastigotes, comparatively strong in relation to the positive control sodium stibogluconate (500 ± 1 μM), none of the compounds was able to inhibit the growth of amastigote forms of *Ldon*.

The same authors, working with *Guarea rhophalocarpa* (Meliaceae) [388] isolated two lanostane triterpenes (**42** and **43**) which showed some antiprotozoal activity against promastigote ($IC_{95\%}$ = 7.2 and 20.0 μM) and amastigote ($IC_{95\%}$ = 74.4 and 20.0 μM) forms of *Ldon* and against *Tbr* trypomastigotes ($IC_{95\%}$ = 5 and 1.75 μM). Both were not very active against *Pfc* and also less cytotoxic against KB cells [388].

Leite *et al.* described the isolation of eighteen triterpenes/limonoids from several parts of *Cedrela fissilis* (Meliaceae) [389]. All isolated compounds were tested against trypomastigote form of *Tcr* and eleven compounds showed activity in an IC_{50} range from 105 – 636 μM. The most active compounds were oleanolic acid (**1**) and the pentaol (**44**) with not very impressive IC_{50} values of 80.4 and 98.6 μM, respectively.

In a screening of plants used as antimalarials, Adams *et al.* found the ethyl acetate extract of *Alisma plantago-aquatica* (Alismataceae) to exhibit strong activity. Using bioguided fractionation, four active prostane triterpenoids (**45 – 48**) were isolated, whose IC_{50} against *Pfc* ranged from 5.4 to 13.8 μM [390].

Ramalhete *et al.* described the bioguided fractionation of MeOH extract from *Momordica balsamina* (Cucurbitaceae) to afford three curcubitane-type triterpenes and ten curcubitacins [391]. These compounds were evaluated against two strains of *Pfc*. Compounds **49** and **50** showed IC_{50} values of 4.6 and 7.4 μM (3D7 strain) and of 4.0 and 8.2 μM (Dd2 strain), respectively. All other compounds showed higher IC_{50}s > 10 μM.

The curcubitane-type triterpene karavilagenin C (**51)** was also isolated along with a hydroxylated derivative from *Momordica balsamina* (Cucurbitaceae). Moreover, fifteen semi-synthetic derivatives were prepared from **51** [392]. All compounds were evaluated against two different strains of *Pfc*. The natural product **51** displayed IC_{50} values of 10.4 and 11.2 μM against the 3D7 and Dd2 strains, respectively, and an IC_{50} of 16.7 μM for cytotoxicity against MCF-7 cells. The most active compound with fairly high selectivity was the diacetyl ester (**52**) (IC_{50}s of 0.5, 0.5 μM, SI=151, 126 for 3D7 and Dd2, resp.).

Two curcubitacin derivatives, cucurbitacins B (**53**) and D (**54**) along with 20-*epi*-bryonolic acid (**17**), were isolated from *Cogniauxia podolaena* (Cucurbitaceae), which is used in traditional medicine as antimalarial [393]. They were assayed for antiplasmodial activity (on FcM29, a chloroquine-resistant strain of *Pfc*) and the IC_{50} values of **53, 54** and **17** were determined as 1.6, 4.0 and 2.0 μg/mL, respectively. The cytotoxicity of **53** and **54** against KB and Vero cells, however, was also quite high (ca 90% growth inhibition at 10 μg/mL) while that of **17** was lower (ca 20%).

Triterpenes of the cycloartane type were extensively studied by Takahara *et al.* [394], who isolated a large set of this type from *Cimicifuga* spp. (Ranunculaceae) and evaluated 24 of these against *Pfc*, with EC_{50} values ranging from 1.0 to 13.0 μM. Compounds **55**, **56**, **57** and **58** were the most active (IC_{50}=1.3, 1.0, 1.8 and 1.7 μM, respectively) but showed little selectivity (FM3A cells).

Phytochemical analysis of *Astragalus bicuspis* (Fabaceae) afforded five cycloartane triterpenoids [395], two of which (**59** and **60**) showed modest leishmanicidal activity (IC_{50} 64.35 μM for **59** and 56.51 μM for **60**, against *Lmaj* promastigotes). However, both compounds were also screened for cytotoxicicty against 3T3 fibroblast cells. Compound **60** (IC_{50} 19.51 ± 5.3 μM) was more cytotoxic than active against the parasites.

Kitagawa *et al.* described the phyochemical analysis from *Brucea javanica* (Simaroubaceae), used in traditional medicine for treatment of malaria [396]. The isolated compounds were tested against a chloroquine-resistant strain (K1) of *Pfc* and two compounds, bruceajavanin A (**61)** and its 1,2-dihydro derivative (**62)** were found to exhibit *in vitro* inhibitory activities (IC_{50} and IC_{90} =1.1, 4.4 μM for **61** and 2.5, 4.3 μM for **62**).

Iridal (**63**), a monocyclic triterpene obtained from *Iris germanica* showed IC_{50} values ranging from 1.8 to 26.0 μg/mL against *Pfc* during three incubation times (72, 48 and 32 h) [397].

Limonoids

Limonoids are a class of degraded triterpene metabolites typically found in plant families formerly included in the order Rutales (now treated as part of Sapindales). These compounds generally have a large number of oxygen atoms and often show conspicuous biological effects, including antiprotozoal activity. The structures of limonoids mentioned in this review are shown in (Fig. **9**)**.**

In particular, limonoids are known for their high activity against *Plasmodium* spp. One of the most important antimalarial limonoids is gedunin (**1**) which has been isolated from the Meliaceae *e.g.* the fruit of *Azadirachta indica* A. Juss. (Neem tree) [398], the seeds of *Carapa guianensis* Aublet (crapwood) [399], *Khaya anthotheca*

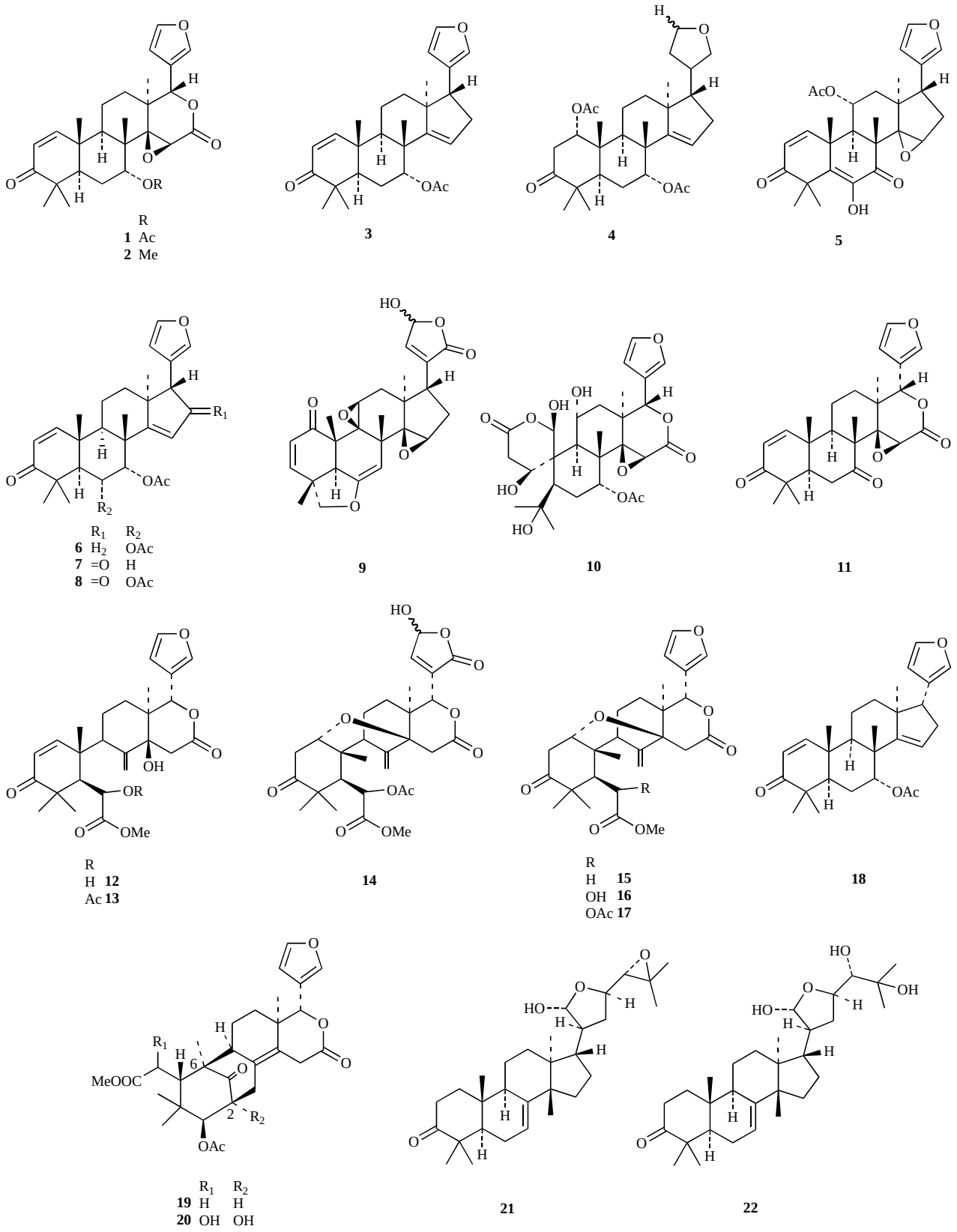

Fig. (9). Structures of limonoids and proto-limonoids with antiprotozoal activity.

[400], *Cedrela odorata* L. [401] (Meliaceae) among other species. Many of these plants are used as antimalarials and for fever in different regions of the world.

Gedunin (**1**) showed an *in vitro* IC_{50} value of 0.8 μg/mL against *Pfc* after a 48-h exposure (0.3 μg/mL after 96 h), roughly equivalent to quinine under the same experimental conditions [402]. A number of derivatives of **1** were prepared and screened *in vitro*

against *Pfc* D6 and W2 strains. All derivatives were less active than **1** (D6 and W2, IC_{50}=39 and 20 ng/mL, respectively) by 50 % or more. This study pointed to the importance of the α,β-unsaturated ketone and 7-acetoxy moiety and relative lack of importance of the furan ring to the activity of **1** against *Pfc* [403]. These qualitative structure activity relationships seem to apply to many limonoids which are analogously substituted on the A and B rings, as will be seen in the examples below.

Despite the promising *in vitro* activity, gedunin is not active *in vivo* in *Pber*-infected rodents at less than 50 mg/kg/day (44.6 % parasite inhibition). A derivative of **1** having a 7-methoxy group instead of the 7-acetoxy group (**2**) had better stability and exhibited slightly better inhibition of *Pber* (50 mg/kg/day) and synergism with the phenylpropanoid dillapiol (25 mg/kg/day) was also observed for **1** and the derivative **2**. More work is needed on derivatives of **1** to optimize their *in vivo* antimalarial activity [401].

Recently, a number of active antimalarial limonoids have been isolated. Azadirone (**3**) and neemfruitin A (**4**) were isolated from the fruit of *A. indica* and exhibited *in vitro* activities against chloroquine-sensitive and resistant strains (D10, W2, respectively) of *Pfc* (IC_{50}=5.96 and 3.40 μM (**3**); 2.82 and 1.74 μM (**4**)) which were comparable to those of **1** (1.66 and 1.31 μM) [398]. Also, anthothecol (**5**) was isolated from *K. anthotheca* and was shown to be as active as **1** against the W2 strain of *Pfc* using two *in vitro* test methods (micromolar and submicromolar IC_{50} values were obtained) [400].

From the seeds of *Chisocheton siamensis*, dysobinin (**6**), azadiradione (**7**) and mahonin (**8**) were isolated and exhibited *in vitro* activity (IC_{50}=2-3 μg/mL) against *Pfc* K1 multidrug resistant strain [404]. Wild chimpanzees in Uganda consume the leaves of *Trichilia rubescens* from which limonoids were isolated and one of these, trichirubine A (**9**), was found to have *in vitro* activity against chloroquine-resistant *Pfc* which was roughly equivalent to **1** [405].

The limonoid raputiolide (**10**), isolated from *Raputia heptaphylla* Pittier (Rutaceae), was shown to have good leishmanicidal activity against intracellular amastigotes of *Lpan* (IC_{50}=8.7 μg/mL) [406].

Murata *et al*. reported the antiplasmodial activities of limonoids and some acyclic triterpenoids from *Ekebergia capensis* (Meliaceae). The limonoid **11** inhibited growth of chloroquine-sensititve *Pfc* (FCR-3) at an IC_{50} of 6 μM. The acyclic triterpenoid **64** (Fig. **8C**) was active against this strain at 18 μM and against the chloroquine-resistant strain K-1 with an IC_{50} of 7.0 μM [407].

A variety of limonoid-type compounds (**12-17**) were isolated from *Lansium domesticum* (Meliaceae) [408] along with some triterpenes and investigated for antiplasmodial activity (*Pfc*, K1). Comparison of the activities indicated that replacement of a hydroxy group (**12**, IC_{50} > 20 μg/mL) by an acetoxy group at C-6 resulted in higher activity (**13**, IC_{50} = 3.2 μg/mL). At the same time, the hydroxy function at C-6 in **16** considerably decreases the antimalarial activity in comparison with the C-6 unsubstituted compound **15** (IC_{50} = 5.9 vs. >20 μg/mL in **16**) while acetylation as in **17** once more leads to an increase (IC_{50} = 3.8 μg/mL). The most active compound in this study was azadiaradione (**18**) with an IC_{50} of 2.9 μg/mL.

The chloroform extract of the bark of *Khaya senegalensis* (Meliaceae) yielded three tetranortriterpenoids of the mexicanolide group. Of these, fissinolide (**19**) was slightly active against chloroquine-resistant *Pfc* and *Lmaj* promastigotes (IC_{50}= 48 and 69 μM, resp.). The 2,6-dihydroxy derivative (**20**) was much less active

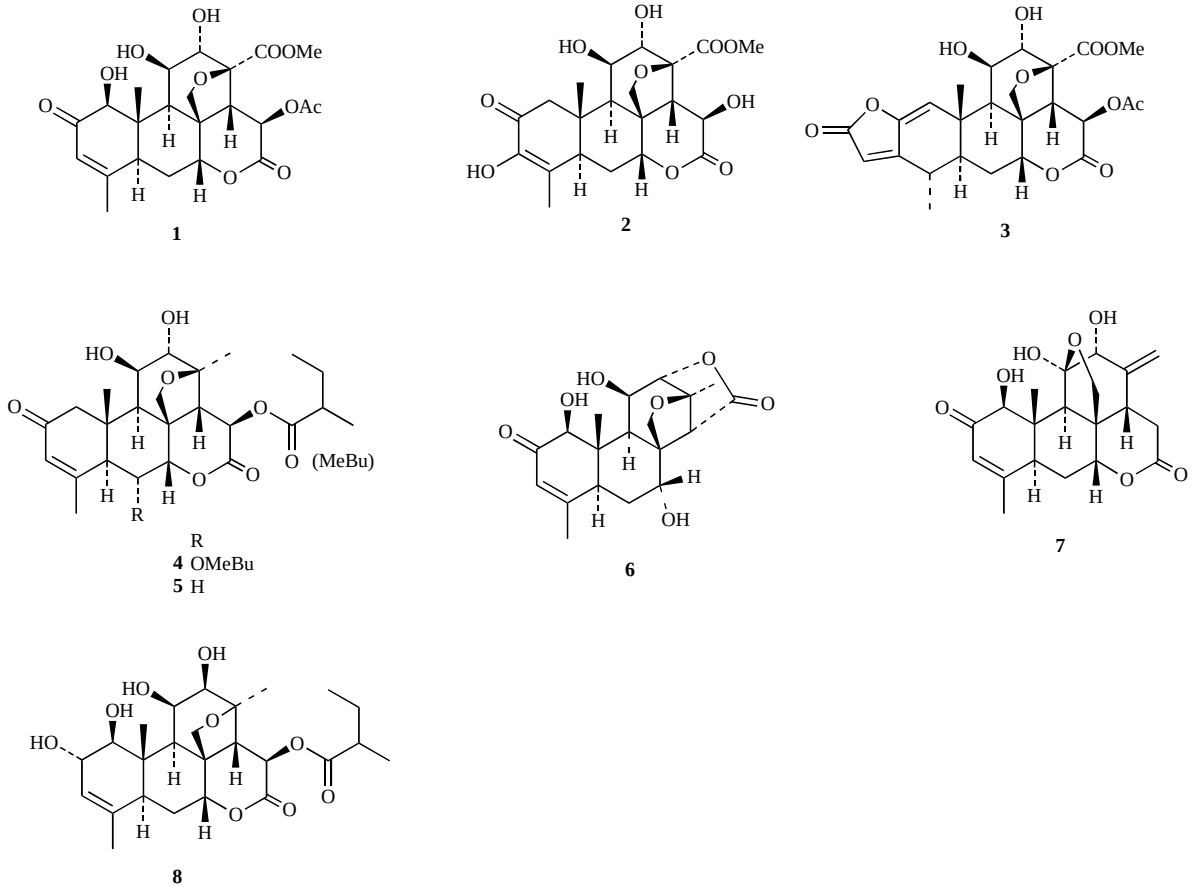

Fig. (10). Structures of quassinoids with antiprotozoal activity.

with IC_{50}s of 122 μM and >200 μM respectively, the third derivative (not shown) was inactive against both mentioned parasites [409].

Biavatti *et al*. described that the hexane extract from *Raulinoa echinata* (Rutaceae) displayed activity against trypomastigote forms of *Tcr* [327]. Phytochemical analysis, along with sesquiterpenes, a coumarin and some common triterpenes yielded the *proto-limonoids* melianone (**21**) and melianodiol (**22**) which inhibited the growth of *Tcr* trypomastigotes by 57% and 33%, respectively, when tested at 100 μg/mL; they were less active than the coumarin and germacrene D (see sections 4.3.2 and 4.1.2.2).

Quassinoids

Quassinoids are highly-oxygenated, degraded triterpenes found in plants of the Simaroubaceae family which exhibit a number of conspicuous biological properties, especially antimalarial activity. Structures of quassinoids mentioned in this review are displayed in Fig. (**10**).

In the last five decades, hundreds of quassinoids have been tested *in vitro* and *in vivo* against several strains of *Plasmodium*, many of these resistant to quinine and chloroquine, and generally present IC_{50} values in the nano or micromolar range. The mode of action of quassinoids is in general linked to inhibition of protein synthesis [410, 411].

The antimalarial activity of quassinoids is related to specific structural features of these compounds. Thus, the presence of an α,β-unsaturated ketone in A ring, the presence of an ether bridge between C-8 and C-11, or C-8 and C-13, fluorine-containing substituents on a C-15 oxygen atom significantly enhance the antimalarial activity of quassinoids as observed for isobrucein B (**1**) and the bruceanol series with *in vitro* activity in the nanomolar range [410, 411].

A number of quassinoids and related compounds having potent *in vivo* antimalarial activity have been identified. Bruceolide (**2**) displayed a strong antiplasmodial effect *in vivo* (ED_{50}= 1.1 mg/kg/day) against *Pber* in rodents and semi-synthetic acetyl and carbonate derivatives on the 3 and 15 positions of bruceolide exhibited enhanced stability *in vivo* [412]. Also, compound **1** together with sergeolide (**3**) was found to be highly active *in vivo* against *Pber* in rodents although these compounds exhibit toxicity [413]. *In vivo* studies of quassinoids against *Pvin* showed the potential of simalikalactone E (**4**) which inhibited parasite grown by 50 % after administration of doses of approximately 0.5 mg/kg/day *p.o*. [414]. Simalikalactone D (**5**) was identified as the active compound in the leaves of *Quassia amara* against FcB1 *Pfc* chloroquine-resistant strain *in vitro* (IC_{50}=10 nM). This compound inhibited 50 % of *Pyoe* rodent malaria parasites *in vivo* at a dose of 3.7 mg/kg/day *p.o*. [415]. Another highly active quassinoid, cedronin (**6**), was active *in vitro* against chloroquine-sensitive and resistant strains of *Pfc* (FCC2 and FZR8) with IC_{50} values of 0.63 and 0.69 μM, respectively. Its cytotoxicity vs. KB cells was much lower with IC_{50}= 10.4 μM. Cedronin was also found to be active *in vivo* (IC_{50}= 1.8 mg/kg/day) against *Pvin* in the 4-day suppressive test [416]. Similarly, ailanthone (**7**), isolated from *Ailanthus altissima*, was active against *Pfc in vitro* and *Pber in vivo* (IC_{50}=15 ng/mL and ED_{50} 0.76 mg/kg/day, respectively) [417]. Oricinolide (**8**) and simalikalactone D (**5**) were potent against *Pfc* clones D6 and W2 with IC_{50} in the nanogram/mL range, while only **5** was active against *Ldon* (IC_{50}=0.2 μg/mL) [418]. These and several earlier *in vivo* studies point to the potential of quassinoids, a class of antiprotozoan natural products largely unexploited mainly due to concerns in general with toxicity.

CONCLUSIONS

This consideration of only one major class of secondary plant metabolites presented in section 4 already shows the immense wealth of interesting natural compounds with activity against the protozoan parasites under study and the high potential of such secondary metabolites for the discovery of new leads and the development of drugs against neglected diseases. Part II of this review [1] will summarize the current knowledge on such activity in other classes of natural products and end with a more extensive conclusions section.

CONFLICT OF INTEREST

None declared.

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SUPPLEMENTARY MATERIALS

Supplementary material is available on the publishers web site along with the published article.

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