ANTILEISHMANIAL COMPOUNDS FROM NATURE

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ELUCIDATION OF THE ACTIVE PRINCIPLES OF AN EXTRACT FROM *VALERIANA WALLICHII* RHIZOMES

Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades
der Julius-Maximilians-Universität Würzburg

vorgelegt von

Jan Glaser

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Eingereicht am .....

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1. Gutachter Prof. Dr. Ulrike Holzgrabe

2. Gutachter .....

der Dissertation

1. Prüfer Prof. Dr. Ulrike Holzgrabe

2. Prüfer .....

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"Wer nichts als Chemie versteht, versteht auch die nicht recht."

Georg Christoph Lichtenberg (1742-1799)

DANKSAGUNG

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Meiner Familie

1. INTRODUCTION

1.1. The Genus *Valeriana*

1.1.1. History

Valeriana (V.) officinalis is a traditional medicinal plant in European folk medicine. The genus *Valeriana* is part of the honeysuckle family (*Caprifoliaceae)* with 150 to 250 subspecies distributed worldwide in the temperate and sub-tropical zone. The perennial herb *V. officinalis*, which is native in Europe, can reach a height of 150 cm, has a hollow stem and a yellow-brown rhizome with protruding roots. The blooming period lasts from Juni to August manifesting itself in white to reddish flowers (s. Fig. 1). *Valeriana* preferably grows by the wayside or along riverbanks. The dried plant and its extracts can be recognized by the characteristic and unpleasent smell of isovaleric acid. The name *Valeriana* may be derived from the latin verb '*valere*' meaning 'being-well' or 'being-strong' and hinting at the positive effects which are credited to the plant. *V. officinalis* is farmed for medicinal use in Belgium, England and Eastern Europe and, in smaller amounts, in Germany [1].

Fig.1. Flower of *Valeriana officinalis* cultivated in the Botanical Garden Würzburg. –Photo: Author.

The healing properties of the plant were already known to the ancient Greeks and Romans. The Greek physician Galen (2nd century AD) suggested a plant named 'phu' (the term may depict the unpleasant smell) as a sleeping aid [2]. The Romans used the same plant as a diuretic, analgetic and spasmolytic agent [3]. This knowledge was pursued during the Middle Ages by famous scholars like Hildegard von Bingen (1098-1179) [4], who recommended the plant against stitch and gout [5]. In written sources from this period the name *Valeriana* appears for the first time. The use of *Valeriana* was

Fig.2. Dried rhizomes of *Valeriana wallichii*. -Photo: Author.

brought forth to the modern era by Paracelsus (1493-1541) [6]. His contemporary Otto Brunfels (1488-1534) considered *Valeriana* to be diuretic, antihelminthic and effective against genital warts [7]. In the 19th century *Valerian* was recommended for the first time as a natural sedativum and sleeping aid by Christoph Wilhelm von Hufeland (1762-1836) [8]. This persists to be the main field of application to date. Aqueous or alcoholic extracts of the dried rhizomes are used almost exclusively. Today, *Valeriana* is widely commercially available in the form of capsules, tea, drops, tablets, pills or tinctures for self-medication of stress, anxiety, and sleeping problems. The active principle for the sedative effect has not been fully determined yet.

V. wallichii (syn. *V. jatamansi* Jones) (s. Fig. 2) is a subspecies which is endemic to India [9]. It grows in India, Nepal and China at higher altitudes of the northwest of the Himalayans. The Ayurvedic name is '*tagara*' [10]. *V. wallichii* is commonly used in Ayurvedic medicine as *inter alia* analgesic, anti-inflammatory, antioxidant, antispasmodic, sedative and antiviral agent [11].

1.1.2. Chemical Composition and Activity

Due to the broad application as a natural sedative, *V. officinalis* is monographed in pharmacopoeias worldwide. The chemical composition of the plant has often been subject of intensive research to find the active, sleep-enhancing ingredient(s). The following compound classes are characteristic constituents of *Valeriana* [12].

Valepotriates

The most characteristic *valerian* compound class is a family of iridoid esters. Described in 1968 by Thies [13], the first isolated compounds valtrate (**1**) and didrovaltrate (**2**) were combined under the umbrella term valepotriates (short for valeriana-epoxy-triester) (s. Fig. 3). These compounds are mostly produced in the subsurface parts of the plant. They are unstable in alkaline or acidic environment, temperature sensitive and decompose easily in alcohol [14]. In the last decades a variety of new valepotriates and valtrates [15-17] have been isolated and identified including structures with unusual

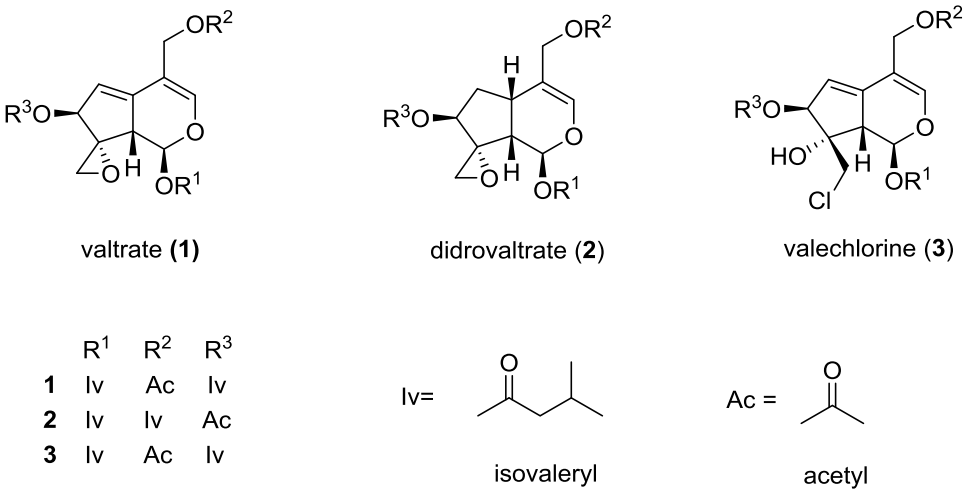

Fig.3. Some characteristic iridoids from *Valeriana*: valtrate (**1**), didrovaltrate (**2**) and valechlorine (**3**).

substituents e.g. valechlorine (**3**) [18]. This may be an artifact of the extraction process with chloroform [19]. Due to their variable esterification with a plethora of organic acids they possess a broad structural diversity.

Yellowish baldrinals (**4**) (s. Fig. 4) are the degradation products of the valepotriates [20]. The ability of valtrates of the diene type (**2**) to give a blue color under acidic conditions (halazuchrom reaction) is still used for the identification test in the United States Pharmacopoeia (USP). The iridoid esters have been under scrutiny for the last years due to their cytotoxicity [21] and their possible application in cancer therapy [22].

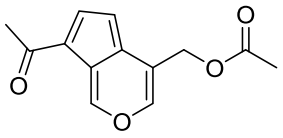

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Fig.4. Structure of baldrinal.

Constituents of the Volatile Oil

The ingredients of the *Valerian* essential oil can differ widely depending on species, climate, location and harvest time. The main monoterpene constituents are borneol (**5**) and esters thereof e.g. acetic acid bornyl ester (**6**). A plethora of sesquiterpenes and monoterpenes has been identified from diverse *Valeriana* species [23,24] e.g. the monoterpenes (-)-limonen (**7**), α-pinen (**8**), *p*-cymol (**9**) and the sesquiterpenes bisabolen (**10**) and maaliol (**11**) (s. Fig. 5). There are two ring systems only found in *Valerianacae*: the kessane and the valerenal type [25]. These characteristic carbon

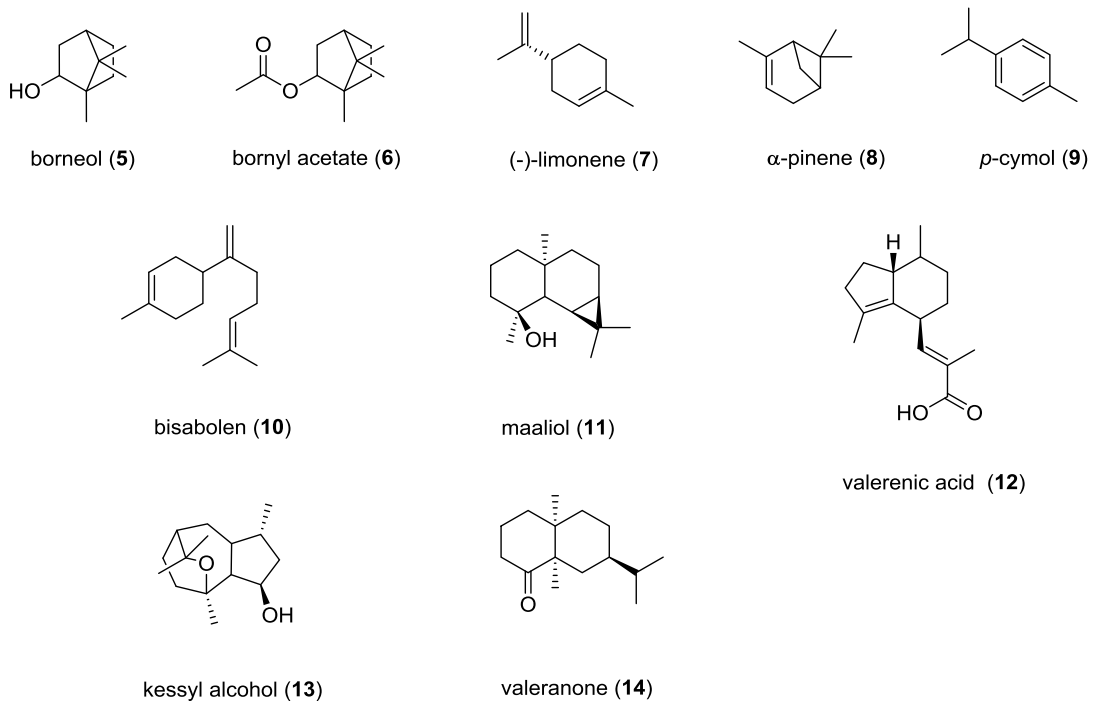


Fig.5. Some characteristic constituents of the volatile oil.

skeletons are part of the sesquiterpenes specific for *Valeriana* namely valerenic acid (**12**) and kessyl alcohol (**13**). Valeranone (**14**) is another hallmark compound for the *Valeriana* species.

Flavonoids

Only a few flavonoids and flavone glycosides are reported from *Valeriana* (s. Fig. 6). They are under investigation for their supposed sedative effect on the central nervous

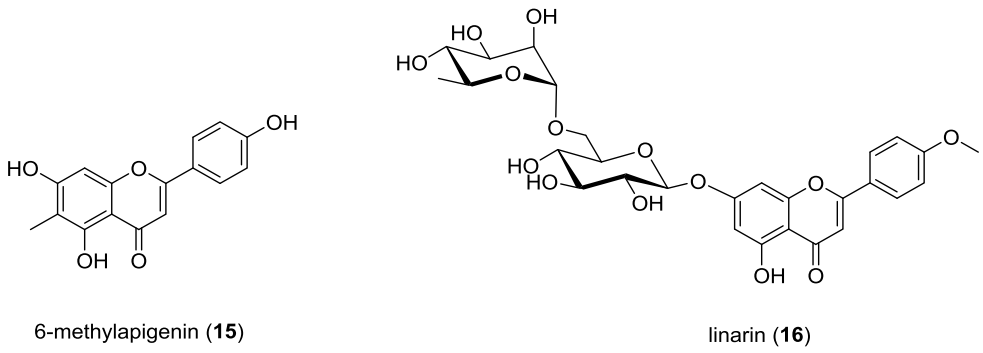


Fig.6. 6-Methylapigenin and linarin: flavonoids from *Valeriana*.

system. 6-Methylapigenin (**15**) from *V. wallichii* for example is reported to inhibit the GABA receptor in mice [26,27]. Linarin (**16**) from *V. officinalis* is likely to have sedative and sleep enhancing properties [28].

Other Constituents

Some alkaloids have been reported as constituents of *V. officinalis* [29-31]. However, the sources are inconsistent in this matter. Polyphenols like caffeic acid derivatives and lignans [32] are described as well. Short-chained carbon acids, free fatty acids, amino acids and carbohydrates make up a smaller part of the ingredients [33,34].

1.2. Leishmaniasis

1.2.1. Historical Background

The human body has been host for protozoan parasites for millennia. Leishmaniasis, also called *kala-azar* or *dum-dum* fever, is widely distributed in the tropical and suptropical regions of the world and severely affects people in India, Africa and South America. In the 19th century explorer reported a disease which lead to unexplained fever. The British explorer Leishman discovered unknown bodies during obduction of the spleen of a dead british soldier in 1903 [35] and mistook them for degenerated trypanosoma. The Irish medical officer Charles Donovan reported similar unknown bodies in the spleen of patients with similar symptoms and made the connection to *kala-azar*. He had identified the pathogen of visceral Leishmaniasis. The Indian species of *Leishmania*, causing visceral Leishmaniasis, was called *L. donovani* to his honors [36]. Today, more than 20 Leishmania species are known.

1.2.2.Course of Disease and Pathology

The parasite is transmitted by the bite of an infected female sand fly which is native to many tropical and temperate countries around the world. The disease can appear in three different forms:

Cutaneous Leishmaniasis (CL)

Cutaneous Leishmaniasis, also known as Oriental sore, is mostly triggered by *L. major* and exclusively affects the skin. Shortly after the bite itching papules appear, developing into an ulcer. In most cases the lesions eventually heal spontaneously after month and years leaving disfiguring scars [37]. More than a million cases of cutaneous Leishmaniasis have been reported in the last 5 years (WHO 2015).

Mucocutaneous Leishmaniasis (ML)

The mucocutaneous form resembles the cutaneous form but additionally affects the mucosa of the respiratory system which leads to decomposition of the soft tissue of mouth and nose including perforation of the septum. This process induces severe disfigurements of the face of the patient. The mucocutaneous form is fatal if left untreated [38]. It is limited to South America and mostly prevalent in Andean countries [39] e.g in Colombia, Bolivia [40] and Ecuador [41]. Leishmania species of the new world genus (Vianna) are responsible (*L. Vianna (V.) braziliensis*, *L. (V.) amazonensis*, *L. (V.) panamensis* and *L. (V.) guyanensis*) [42]. However, only in 1-10% of the cases, the cutaneous develops into the mucocutaneous form.

Visceral Leishmaniasis (VL)

Visceral Leishmaniasis, mainly caused by *L. donovani* on the Indian subcontinent, affects the internal organs e.g. liver, kidney or spleen and is usually fatal if not treated in time. Symptoms are severe weight loss, intermittent fever, swelling of liver and spleen and possible dark coloring of the skin (therefore the name *kala azar*, black disease) [43]. The WHO (2015) estimates 300.000 cases of visceral Leishmaniasis worldwide and 20.000 annual deaths caused by this disease. A vast majority of cases of visceral leishmaniasis appears in the north of India.

1.2.3. Life Cycle

The parasite has a complex life cycle (s. Fig. 7). There are two main stages: the amastigote and the promastigote form. The sand fly injects promastigotes into the host during its blood meal (1). The parasites are phagocytozed by macrophages (2), differentiate into amastigotes without flagellum and then multiply (3). The amastigotes disrupt the membrane and are released into the blood stream (4) and infect other cells. When the sand fly ingests infected macrophages during a blood meal (5) the amastigotes are released (6) and transform into flagellated promastigotes (7), multiplying in the guts of the sand fly and are eventually transferred to another mammalian host (8 and 1).

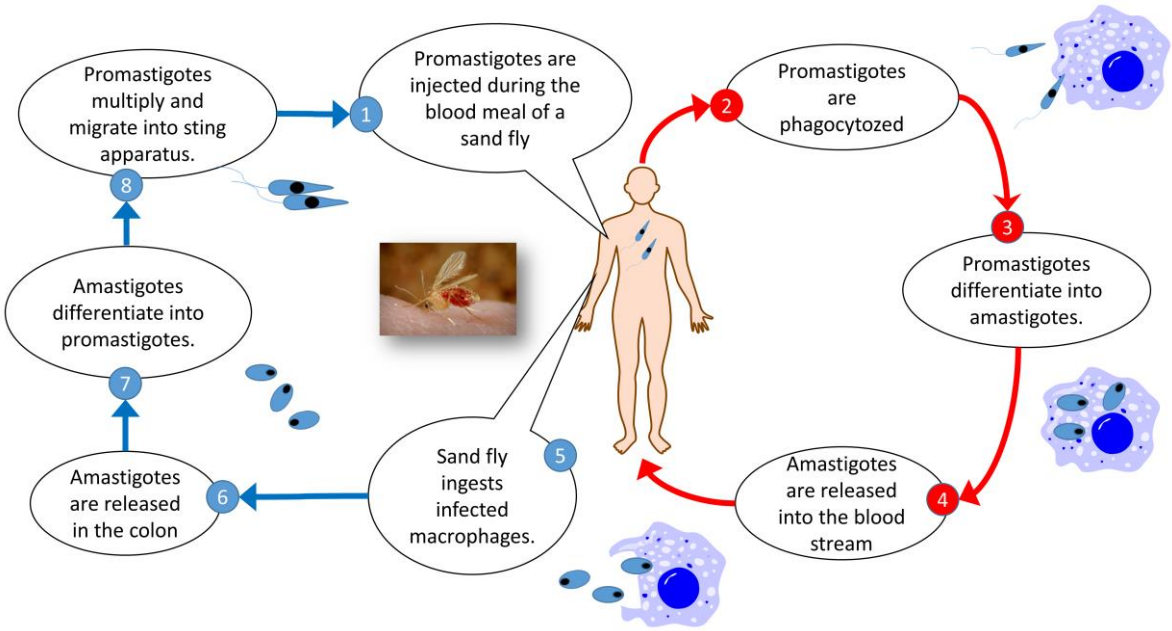


Fig.7. Life cycle of the Leishmanian parasite. (Photo of Sandfly: Public domain, CDC/ Frank Collins.).

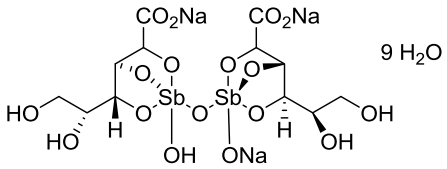
1.2.4. Therapy and Pipeline

There are no approved vaccines available yet. Other ways to fight the disease are vector control and bite prophylaxes. However, to treat the actual infection only a few drugs are disposable.

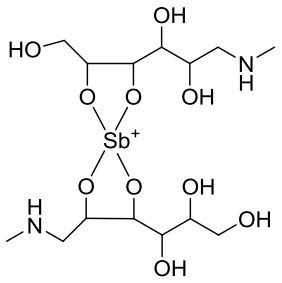
The first drug family effective against Leishmaniasis was of the pentavalent antimonials. At the beginning of the 20th century Brasilian pathologist Gaspar Vianna reported that tartar emetic (german: ‘Brechweinstein’) helped against cutaneous Leishmaniasis [44].

Later reports confirmed this activity in visceral Leishmaniasis as well [45]. Due to severe side-effects the clinical use was later stopped.

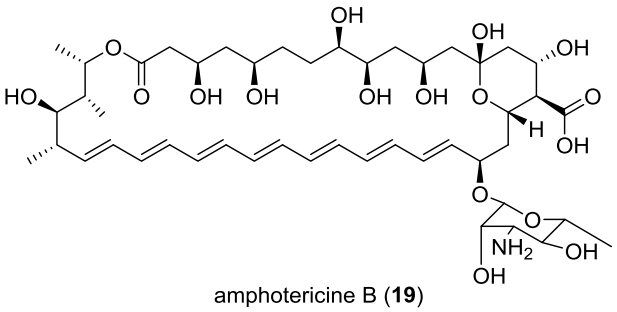
In the 1940s the less toxic pentavalent antimon compounds stibogluconate (**17**) (Pentostam) and meglumine antimoniate (**18**) were introduced (s. Fig. 8). Even though they can save a lot of lifes, these drugs induce severe side effects (nephrotoxicity [46] and cardiotoxicity [47]) and need to be administered intravenously under close medicinal supervision in an hospital environment. However, they are still first-line drugs in regions where resistance against antimonial drugs has not developed yet.



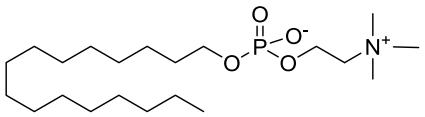
stibogluconate (**17**)



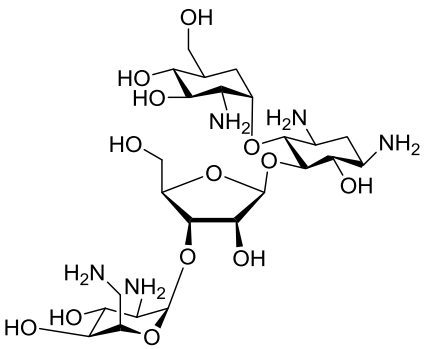
meglumine antimonate (**18**)



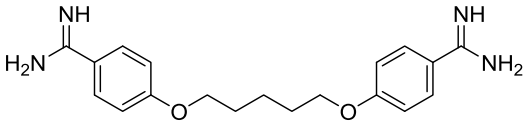
amphotericine B (**19**)



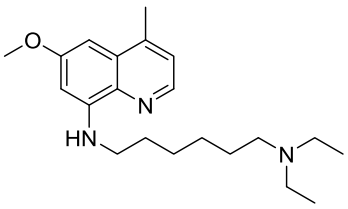
miltefosine (**20**)



paromomycine (**21**)



pentamidine (**22**)



sitamaquine (**23**)

Fig.8. Structures of some important antileishmanial drugs.

In areas with aforementioned resistance amphotericine B (**19**), a macrolide antibiotic, is used as a second-line drug against CL and VL. It needs to be administered intravenously daily up to 20-30 days which means hospitalization for the patient and shows severe side effects like nephrotoxicity [48] as well.
Finally, in the 1980s miltefosine (**20**) was introduced as the first oral antileishmanial agent which had similar healing rates as the effective antimonials, less severe cytotoxic effects, and was active against all forms of Leishmaniasis [49,50]. However, mild hepatotoxicity and nephrotoxicity as well as teratogenicity were observed [51]. Also the sensitivity of some *Leishmania* strains to miltefosine is low.
Another antibiotic in use is paromomycine (**21**) which is also available as injection therapy [52]. The aromatic diamidine pentamidine (**22**) was abandoned due to widespread resistance and severe side effects [53]. Sitamaquine (**23**) is the latest promising antileishmanial drug in the pipeline [54].

1.3. Schistosomiasis

1.3.1. Historical Background

In 1852, during his stay in Kairo, the German physicist and helminthologist Theodor Maximilian Bilharz wrote in his letters to Carl Theodor Ernst von Siebold about his discovery of an unknown species of parasitic leeches (called by him *Distomum haematobium*) in the bladder of a deceased during an autopsy [55,56]. During a later internationalization of technical terms by the international commission of zoological nomenclature in 1949 the term ‘Schistosomiasis’ (coined by Weiland 1858) was recommended and the parasite was named *Schistosoma (S.) haematobium*. The disease has been present for millennia. Eggs of *S. haematobium* were detected in mummies from the old egypt and antigens against schistosoma have been verified with newer methods.
At present around 250 million people are infected with *Schistosoma spp*. which makes this disease one of the most widespread neglected tropical diseases after Malaria. It is common in 79 countries (WHO) of mostly tropical and subtropical climates, especially in Africa, South America, the Far East, the Carribean, China and Japan. Isolated cases in the Mediterrenean were reported recently [57]. Strongly affected is mostly the poor

population in the developing countries of Africa without access to sanitation and clean drinking water, because the infection takes place by contact with contaminated water sources. The distribution of the disease is dependent on the occurrence of some species of water snails (*Biomphalaria*, *Bulinus* or *Oncomelania*) which serve as mediate hosts.

1.3.2. Course of Disease and Pathology

S. haematobium, *S. mansoni* and *S. japonicum* are the most important human pathogenic representatives of the species Schistosoma, next to some local forms found in the Mekong delta (*S. mekongi*) and in West and Central Africa (*S. guineensis* and *S. intercalatum*). *S. mansoni* occurs exclusively in the Americas, while *S. mansoni* and *S. haematobium* are distributed in the Middle East and Africa. In Asia mainly *S. japonicum* is found. They all cause different manifestations of the disease in humans depending on the species. Primarily responsible for the symptoms are not the adult worms, but their released eggs, which distribute themselves through the veins into the liver and other internal organs (*S. mansoni*, *S. japonicum*) thereby evoking an immune response. In contrast to the other two species, *S. haematobium* mainly affects the bladder which may lead to hematuria, chronical fibrosis of the urinary tract and bladder cancer. The chronic inflammation of the genital tract can increase the risk of HIV infection in women. The acute form of Schistosomiasis manifests itself weeks or months after the primary infection, evoked by the first egg deposition of the adult worms living in the body. Typical symptoms are fever, malaise, head and growing pains, and fatigue in a time frame of two to ten weeks. After some time without medical treatment the acute infection transforms into the intestinal (*S. japonicum*, *S. mansoni*) chronical form. In course of time over decades this infection can lead to cirrhosis of the liver and to death.
Because of the permanent inflammation in the body, all species can cause anemia and malnutrition in affected children [58].

1.3.3. Life Cycle

The life cycle of the schistosomes is complex, however it is the same in all species (s. Fig. 9). Sporocysts in infected water snails develop into *cercariae*, multiply and are excreted into the water (7). When humans come in contact with contaminated water the *cercariae* penetrate the skin into the blood stream (1). They lose their tail (2) and are transported by the blood through the lungs into the liver, start to feast on erythrocytes and mature into adult worms (3). The grown leeches can last up to 20 years in the human body and can reach a length of 10 mm. The female usually lies in the gynaecophoric channel of the male and immediately starts producing eggs. The adult blood flukes move into the rectal veins or into the bladder in case of *S. haematobium*. The emerging eggs migrate through the walls of the blood vessels and are excreted by feces or urine, respectively (4). The majority of the eggs remains in the tissue. When the eggs are released into a water source (5) they transform into *miracidiae*, which infect water snails (6), reinitiating the life cycle.

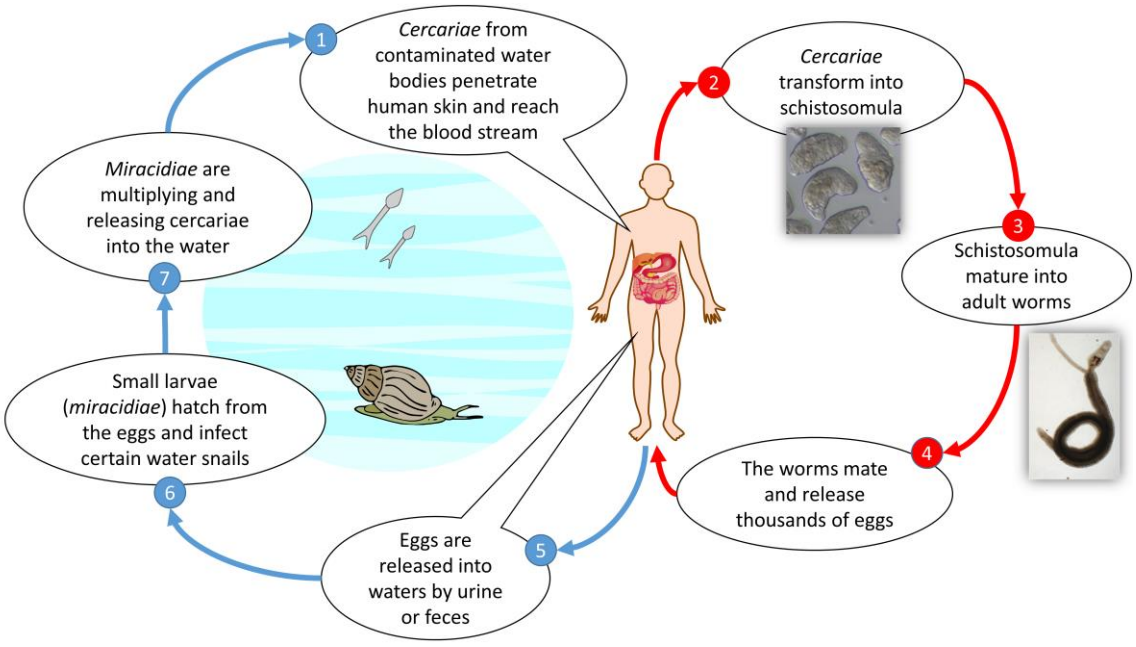
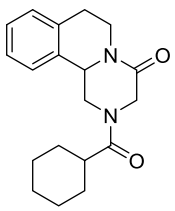


Fig.9. Life cycle of schistosomes.

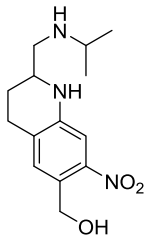
1.3.4. Therapy and Pipeline

The only effective drug against all species of adult worms is praziquantel (**24**), an antihelmintic, which has been used in its racemic form since the 1970s. It is active against trematodes as well as flatworms and tapeworms. Praziqantel can be administered orally in a single dose, is cheap, safe and effective against all species of schistosomes [59]. The goal of the WHO is the control and even the elimination of the disease by a preventive treatment of people living in endangered regions. The alternative to praziquantel, oxamniquine (**25**) is more expensive and only active against *S. mansoni* [60]. A development of general resistance against praziquantel was not observed until today, despite its widespread and intensive use. However, there are signs of a reduced overall susceptibility to the drug [61]. Therefore, new active compounds as potential back-up drugs are needed.

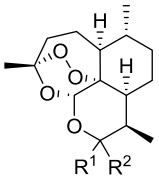
In the last decades, the antiplasmodial artemether (**26**) and artesunate (**27**), semi-synthecial derivatives of antimalaria drug artemisinin (**28**), were investigated for their antischistosomal activity [62,63]. However, the activity is limited to schistosomula, the juvenile form of the worm [64]. Therefore, the drug is only usable as a preventive agent. The combined use with praziquantel is disputable as the danger of resistances against malaria (*Plasmodium falciparum*) may arise due to wide-spread use. Other promising structures are the 1,2,4-trioxolanes [65,66] e.g. arterolan (**29**) (s. Fig. 10).



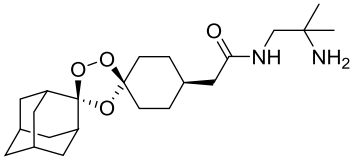
praziquantel (**24**)



oxamniquin (**25**)



26 artemether: R^1 = H, R^2 = β-OCH_3
27 artesunate: R^1 = H; R^2 = α-$OCO(CH_2)_2$-COOH
28 artemisinin: R^1, R^2 = O



arterolan (**29**)

Fig.10. Antischistosomal drugs and drug candidates.

1.4. References

1. Wichtl, M., *Teedrogen. Ein Handbuch fur die Praxis auf wissenschaftlicher Grundlage.* 2. Auflage, Wissenschaftliche Verlagsgesellschaft mbH: Stuttgart, **1989**.
2. Murti, K.; Kaushik M.; Sangwan, Y.; Kaushik, A., Pharmacological properties of *Valeriana officinalis* – A review. *Pharmacologyonline* **2011**, *3*, 641-464.
3. Rücker, G., Über die „Wirkstoffe" der Valerianaceen. *PharmuZ* **1979**, *8*, 78-86.
4. von Bingen, H.; Schulz, H. (Translator), *Ursachen und Behandlung der Krankheiten. (Causae et curae)*. Haug: Heidelberg, **1955**.
5. Marzell, H., *Geschichte und Volkskunde der deutschen Heilpflanzen*. Reichl Verlag: St. Goar, **1938**.
6. Paracelsus; Aschner, B., *Sämtliche Werke,* Gustav Fischer Verlag: Jena **1932**.
7. Brunfels, O., Contrafayt Kreüterbuch, Schott: Straßburg, **1532**.
8. Hufeland, C.W, *Enchiridion Medicum: Manual of the practice of medicine*, William Radde: New York, **1843**.
9. Devi, V. S.; Rao, M. G., *Valeriana wallichii* - A rich aroma root plant. *World J. Pharm. Pharm. Sci.* **2014**, *3*, 1516-1525.
10. Khare, C. P., *Indian herbal remedies: rational Western therapy, ayurvedic, and other traditional usage,* Springer: Berlin, Heidelberg, **2003**.
11. Sah, S. P.; Mathela, C.; Chopra, K., *Valeriana wallichii*: A phyto-pharmacological review. *J. Pharm. Res.* **2010**, *3*, 2337-2339.
12. Houghton, P. J., The scientific basis for the reputed activity of Valerian. *J. Pharm. Pharmacol.* **1999**, *51*, 505-512.
13. Thies, P. W., Die Konstitution der Valepotriate: Mitteilung über die Wirkstoffe des Baldrians. *Tetrahedron* **1968**, *24*, 313-347.
14. Bos, R.; Woerdenbag, H. J.; Pras, N., Determination of valepotriates. *J. Chromatogr. A* **2002**, *967*, 131-146.
15. Lin, S.; Shen, Y.-H.; Zhang, Z.-X.; Li, H.-L.; Shan, L.; Liu, R.-H.; Xu, X.-K.; Zhang, W.-D., Revision of the structures of 1,5-dihydroxy-3,8-epoxyvalechlorine, volvaltrate B, and valeriotetrate C from *Valeriana jatamansi* and *V. officinalis*. *J Nat. Prod.* **2010**, *73*, 1723-1726.

16. Lin, S.; Chen, T.; Liu, X.-H.; Shen, Y.-H.; Li, H.-L.; Shan, L.; Liu, R.-H.; Xu, X.-K.; Zhang, W.-D.; Wang, H., Iridoids and lignans from *Valeriana jatamansi*. *J. Nat. Prod.* **2010**, *73*, 632-638.
17. Fuzzati, N.; Wolfender, J. L.; Hostettmann, K.; Msonthi, J. D.; Mavi, S.; Molleyres, L. P., Isolation of antifungal valepotriates from *Valeriana capense* and the search for valepotriates in crude Valerianaceae extracts. *Phytochem. Anal.* **1996**, *7*, 76-85.
18. Popov, S.; Handjieva, N.; Marekov, N., A new valepotriate: 7-*epi*-deacetylisovaltrate from *Valeriana officinalis*. *Phytochemistry* **1974**, *13*, 2815-2818.
19. Lin, S.; Zhang, Z.-X.; Chen, T.; Ye, J.; Dai, W.-X.; Shan, L.; Su, J.; Shen, Y.-H.; Li, H.-L.; Liu, R.-H., Characterization of chlorinated valepotriates from *Valeriana jatamansi*. *Phytochemistry* **2013**, *85*, 185-193.
20. Thies, P., Über die Wirkstoffe des Baldrians: 2. Mitteilung Zur Konstitution der Isovaleriansäureester Valepotriat, Acetoxyvalepotriat und Dihydrovalepotriat. *Tetrahedron Lett.* **1966**, *7*, 1163-1170.
21. Lin, S.; Shen, Y.-H.; Li, H.-L.; Yang, X.-W.; Chen, T.; Lu, L.-H.; Huang, Z.-S.; Liu, R.-H.; Xu, X.-K.; Zhang, W.-D., Acylated iridoids with cytotoxicity from *Valeriana jatamansi*. *J. Nat. Prod.* **2009**, *72*, 650-655.
22. Bounthanh, C.; Bergmann, C.; Beck, J.; Haag-Berrurier, M.; Anton, R., Valepotriates, a new class of cytotoxic and antitumor agents. *Planta Med.* **1981**, *41*, 21-28.
23. Verma, R. S.; Verma, R. K.; Padalia, R. C.; Chauhan, A.; Singh, A.; Singh, H. P., Chemical diversity in the essential oil of Indian Valerian (*Valeriana jatamansi* Jones). *Chem. Biodivers.* **2011**, *8*, 1921-1929.
24. Bos, R.; Woerdenbag, H. J.; Hendriks, H.; Smit, H. F.; Wikström, H. V.; Scheffer, J. J., Composition of the essential oil from roots and rhizomes of *Valeriana wallichii* DC. *Flavour Frag. J.* **1997**, *12*, 123-131.
25. Houghton, P. J., *2. The chemistry of valeriana. Valerian: the genus valeriana* Harwood Academic Publishers: Amsterdam, **1997**.
26. Marder, M.; Viola, H.; Wasowski, C.; Fernández, S.; Medina, J. H.; Paladini, A. C., 6-Methylapigenin and hesperidin: new valeriana flavonoids with activity on the CNS. *Pharmacol. Biochem. Behav.* **2003**, *75*, 537-545.

27. Wasowski, C.; Marder, M.; Viola, H.; Medina, J. H.; Paladini, A. C., Isolation and identification of 6-methylapigenin, a competitive ligand for the brain GABA(A) receptors, from *Valeriana wallichii*. *Planta Med.* **2002**, *68*, 934-936.
28. Fernández, S.; Wasowski, C.; Paladini, A. C.; Marder, M., Sedative and sleep-enhancing properties of linarin, a flavonoid-isolated from *Valeriana officinalis*. *Pharmacol. Biochem. Behav.* **2004**, *77*, 399-404.
29. Torssell, K.; Wahlberg, K., Isolation, structure and synthesis of alkaloids from *Valeriana officinalis*. *L. Acta Chem. Scand.* **1967**, *21*, 53-62.
30. Torssell, K.; Wahlberg, K., The structure of the principal alkaloid from *Valeriana officinalis* (L.). *Tetrahedron Lett.* **1966**, *7*, 445-448.
31. Janot, M.; Guilhem, J.; Contz, O.; Venera, G.; Cionga, E. In contribution to the study of valerian alcaloids (*Valeriana officinalis*, L.): actinidine and naphthyridylmethylketone, a new alkaloid, *Ann. Pharm. Fr.* **1979**, *37*, 413-420.
32. Schumacher, B.; Scholle, S.; Hölzl, J.; Khudeir, N.; Hess, S.; Müller, C. E., Lignans isolated from Valerian: Identification and characterization of a new olivil derivative with partial agonistic activity at A1 adenosine receptors. *J. Nat. Prod*. **2002**, *65*, 1479-1485.
33. Steinegger, E.; Hänsel, R., *Lehrbuch der Pharmakognosie und Phytopharmazie*. 4. Auflage, Springer: Berlin, Heidelberg, **2013**.
34. Hänsel, R.; Hölzl, J., *Lehrbuch der pharmazeutischen Biologie: Ein Lehrbuch für Studenten der Pharmazie im zweiten Ausbildungsabschnitt*. Springer: Berlin **1996**.
35. Leishman, W. B., On the possibility of the occurrence of trypanosomiasis in India. *Brit. Med. J.* **1903**, *1*, 1252-1254.
36. Ross, R., A new parasite of man. *Thompson Yates and Johnston Laboratories Report* **1903**, *5*, 79-82.
37. Reithinger, R.; Dujardin, J.-C.; Louzir, H.; Pirmez, C.; Alexander, B.; Brooker, S., Cutaneous leishmaniasis. *Lancet Infect. Dis.* **2007**, *7*, 581-596.
38. Sangueza, O. P.; Sangueza, J. M.; Stiller, M. J.; Sangueza, P., Mucocutaneous leishmaniasis: A clinicopathologic classification. *J. Am. Acad. Derm.* **1993**, *28*, 927-932.
39. Davies, C. R.; Reithinger, R.; Campbell-Lendrum, D.; Feliciangeli, D.; Borges, R.; Rodriguez, N., The epidemiology and control of leishmaniasis in Andean countries. *Cad. Saude Publica* **2000**, *16*, 925-950.

40. García, A. L.; Parrado, R.; Rojas, E.; Delgado, R.; Dujardin, J.-C.; Reithinger, R., Leishmaniases in Bolivia: comprehensive review and current status. *Am. J. Trop. Med. Hyg.* **2009**, *80*, 704-711.
41. Calvopina, M.; Armijos, R. X.; Hashiguchi, Y., Epidemiology of leishmaniasis in Ecuador: current status of knowledge-a review. *Mem. Inst. Oswaldo Cruz* **2004**, *99*, 663-672.
42. David, C. V.; Craft, N., Cutaneous and mucocutaneous leishmaniasis. *Dermatol. Ther.* **2009**, *22*, 491-502.
43. Guerin, P. J.; Olliaro, P.; Sundar, S.; Boelaert, M.; Croft, S. L.; Desjeux, P.; Wasunna, M. K.; Bryceson, A. D. M., Visceral leishmaniasis: current status of control, diagnosis, and treatment, and a proposed research and development agenda. *Lancet Inf. Dis.* **2002**, *2*, 494-501.
44. Vianna, G., Tratamento da leishmaniose tegumentar por injeções intravenosas de tártaro emético. *Arq. Bras. Med.* **1912**, *4*, 426-8.
45. Di Cristina, G.; Caronia, G., Sulla terapia della leishmaniosi interna. *Pathologica* **1915**, *7*, 82-83.
46. Jolliffe, D., Nephrotoxicity of pentavalent antimonials. *Lancet* **1985**, *325*, 584.
47. Rijal, S.; Chappuis, F.; Singh, R.; Boelaert, M.; Loutan, L.; Koirala, S., Sodium stibogluconate cardiotoxicity and safety of generics. *Trans. Roy. Soc. Trop. Med. Hyg.* **2003**, *97*, 597-598.
48. Deray, G., Amphotericin B nephrotoxicity. *J. Antimicrob. Chemother.* **2002**, *49*, (suppl. S1), 37-41.
49. Sundar, S.; Jha, T.; Thakur, C.; Engel, J.; Sindermann, H.; Fischer, C.; Junge, K.; Bryceson, A.; Berman, J., Oral miltefosine for Indian visceral leishmaniasis. *N. Engl. J. Med.* **2002**, *347*, 1739-1746.
50. Soto, J.; Arana, B.; Toledo, J.; Rizzo, N.; Vega, J.; Diaz, A.; Luz, M.; Gutierrez, P.; Arboleda, M.; Berman, J., Miltefosine for new world cutaneous leishmaniasis. *Clin. Infect. Dis.* **2004**, *38*, 1266-1272.
51. Arana, B.; Rizzo, N.; Diaz, A., Chemotherapy of cutaneous leishmaniasis: a review. *Med. Microbiol. Immunol.* **2001**, *190*, 93-95.
52. Sundar, S.; Chakravarty, J., Paromomycin in the treatment of leishmaniasis. *Expert Opin. Investig. Drugs* **2008**, *17*, 787-794.
53. Kumar, A., *Leishmania and Leishmaniasis*. Springer: New York, Heidelberg, Dordrecht, London, **2013**.

54. Loiseau, P.; Cojean, S.; Schrével, J., Sitamaquine as a putative antileishmanial drug candidate: from the mechanism of action to the risk of drug resistance. *Parasite* **2011**, *18*, 115-119.
55. Bilharz, T.; Siebold, C. T. E., *Ein Beitrag zur Helminthographia humana*. W. Engelmann: Leipzig, **1852**.
56. Bilharz, T., A contribution to human helminthology. *Rev. Infect. Dis.* **1982**, *4*, 727-732.
57. Holtfreter, M.; Moné, H.; Müller-Stöver, I.; Mouahid, G.; Richter, J., *Schistosoma haematobium* infections acquired in Corsica, France, August 2013. *Euro Surveill.* **2014**, *19*, pii=20821. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20821.
58. Gryseels, B.; Polman, K.; Clerinx, J.; Kestens, L., Human schistosomiasis. *Lancet* **2006***, 368*, 1106-1118.
59. Caffrey, C. R., Chemotherapy of schistosomiasis: present and future. *Curr. Opin. Chem. Biol.* **2007**, *11*, 433-439.
60. Foster, R., A review of clinical experience with oxamniquine. *Trans. Roy. Soc. Trop. Med. Hyg.* **1987**, *81*, 55-59.
61. Wang, W.; Wang, L.; Liang, Y.-S., Susceptibility or resistance of praziquantel in human schistosomiasis: a review. *Parasitol. Res.* **2012**, *111*, 1871-1877.
62. Le, W.; You, J.; Yang, Y.; Mei, J.; Guo, H.; Yang, H.; Zhang, C., Studies on the efficacy of artemether in experimental schistosomiasis. *Acta Pharm. Sin.* **1982**, *17*, 187-193.
63. Utzinger, J.; Chollet, J.; Tu, Z.; Shuhua, X.; Tanner, M., Comparative study of the effects of artemether and artesunate on juvenile and adult *Schistosoma mansoni* in experimentally infected mice. *Trans. Roy. Soc. Trop. Med. Hyg.* **2002**, *96*, 318-323.
64. Xiao, S.; Catto, B. A., *In vitro* and *in vivo* studies of the effect of artemether on S*chistosoma mansoni*. *Antimicrob. Agents Chemother.* **1989**, *33*, 1557-1562.
65. Keiser, J.; Utzinger, J., Artemisinins and synthetic trioxolanes in the treatment of helminth infections. *Curr. Opin. Infect. Dis.* **2007**, *20*, 605-612.
66. Xiao, S.-H.; Keiser, J.; Chollet, J.; Utzinger, J.; Dong, Y.; Endriss, Y.; Vennerstrom, J. L.; Tanner, M., *In vitro* and *in vivo* activities of synthetic trioxolanes against major human schistosome species. *Antimicrob. Agents. Chemother.* **2007**, *51*, 1440-1445.

2. AIM OF THE WORK

Neglected tropical diseases like malaria, leishmaniasis oder sleeping sickness pose a major problem for the affected population of poor countries in the tropical regions. Only a few efficient drugs are available against these severe diseases. Additionally, these drugs are afflicted with strong side-effects, are expensive and difficult to administer in regions with insufficient health-care infrastructure. Furthermore, emerging resistance against the drugs in use is observed worldwide. Hence, the search for new and potent substances is pressing. Nature, with her plethora of possible chemical compounds, can provide new lead structures.

The chloroform extract of pulverized rhizomes of *Valeriana wallichii* showed activity during an *in vitro* screening against *Leishmania major* promastigotes, the pathogen for cutaneous leishmaniasis. The aim of the thesis was to investigate the antileishmanial principles of the extract. Thus, the development of suitable bioactivity-guided fractionation methods was necessary to isolate the active compounds and elucidate their structure by various spectroscopic methods. The compounds should be synthesized to confirm their activity *in vitro*. Additionally, a library of structurally related derivatives should be synthesized and the structure was to be improved in relation to activity and cytotoxicity.

The main goals of the work can be summarized as follows:

a) Development of suitable fractionation methods for a chloroform extract of *Valeriana wallichii* rhizomes.

b) Isolation and structure elucidation of substances with activity against *L. major* promastigotes *in vitro* from the extract.

c) Synthesis of active compounds and a library of derivatives thereof. Improvement of antileishmanial activity and cytotoxicity by structural variation.

d) Screening of the compound library against *Leishmania spp.* and other pathogens.

3. PUBLICATIONS

3.1. Antileishmanial and Cytotoxic Compounds from *Valeriana wallichii* and Identification of a Novel Nepetolactone Derivative

Jan Glaser, Martina Schultheis, Heidrun Moll, Banasri Hazra and Ulrike Holzgrabe

Reprinted from *Molecules* **2015**, *20*, 5740-5753;

Abstract

The chloroform extract of *Valeriana wallichii* (*V. wallichii*) rhizomes was investigated to elucidate the structures responsible for reported antileishmanial activity. Besides bornyl caffeate (**1**, already been reported by us previously), bioassay-guided fractionation resulted in two additional cinnamic acid derivatives (**2**-**3**) with moderate leishmanicidal activity. The structure of a novel nepetolactone derivative (**4**) having a cinnamic acid moiety was elucidated by means of spectral analysis. To the best of our knowledge villoside aglycone (**5**) was isolated from this plant for the first time. The bioassay-guided fractionation yielded two new (compounds **6**–**7**) and two known valtrates (compounds **8**–**9**) with leishmanicidal potential against *Leishmania major* (*L. major*) promastigotes. In addition, β-bisabolol (**10**), α-kessyl alcohol (**11**), valeranone (**12**), bornyl isovalerate (**13**) and linarin-2-*O*-methylbutyrate (**14**) were identified. This is the first report on the isolation of 4'-demethylpodophyllotoxin (**15**), podophyllotoxin (**16**) and pinoresinol (**17**) in *V. wallichii*. In total thirteen known and four new compounds were identified from the extract and their cytotoxic and antileishmanial properties were evaluated.

Introduction

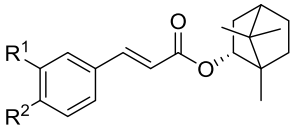
Valeriana wallichii DC (syn. *V. jatamansi* Jones) is endemic to the Himalayan regions of India, Nepal and China and is used in folk and ayurvedic medicine, for instance, against sleep disorders, skin disorders or pain [1]. Antimicrobial [2], anti-inflammatory [3], insecticidal [4], antiviral [5] and antioxidant [6,7] properties of the extract are known. Additionally, the antileishmanial activity of the chloroform extract of the rhizomes was reported previously [8] and studies to isolate and identify the responsible compounds by bioassay-guided fractionation were undertaken and produced bornyl caffeate (**1**) [9]. In continuation of our search for antileishmanial compounds in *V. wallichii* we herein reveal the antileishmanial activity of valtrates and we isolated additional cytotoxic compounds which are to date unheard in *Valeriana*. We successfully isolated and characterized two more cinnamic acid derivatives (**2**, **3)** showing leishmanicidal properties. Additionally, we discovered a nepetolactone derivative (**4**) and villoside aglycone (**5**), both being novel natural products. Two new (compounds **6**–**7**) and two known valtrates (compounds **8**–**9**) were found. For all valtrates we could show strong antileishmanial activity. Furthermore, β-bisabolol (**10**), α-kessyl alcohol (**11**), valeranone (**12**), bornyval (**13**), linarin-*O*-2-methylbutyrate (**14**), 4'-demethylpodophyllotoxin (**15**), podophyllotoxin (**16**) and pinoresinol (**17**) were isolated from the extract (*cf.* Fig. 1).

Results and Discussion

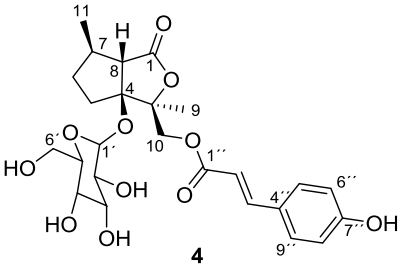
Bioassay-Guided Fractionation of the Extract

The dried chloroform extract of *V. wallichii* rhizomes was slurried in MeOH, filtered and fractionated into 12 fractions F1–F12 by preparative HPLC. After screening for antileishmanial activity on *L. major* promastigotes *in vitro* the most promising fractions F4–F8 were further investigated by subjecting them to preparative HPLC and multiple column chromatographic separations (*cf.* Fig. 2) which led to the isolation and identification of highly active valtrates **6**–**9** and cinnamic acid derivatives **1**–**3** with moderate antileishmanial activity. β-Bisabolol (**10**) and valeranone (**12**) were found in a subfraction of F7 and bornyl isovalerate (**13**) and α-kessyl alcohol (**11**) were isolated from fraction F8. The non-active fractions F1–F3 gave the highest amount of substances. To avoid missing small amounts of potentially active compounds we have

Cinnamic acid derivatives:

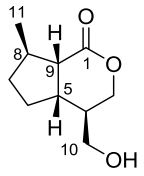


1 $R^1 = R^2 = OH$
2 $R^1 = OH$, $R^2 = OCH_3$
3 $R^1 = H$, $R^2 = OH$



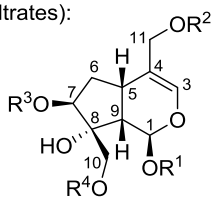
4

Iridoid (Villoside aglycone):

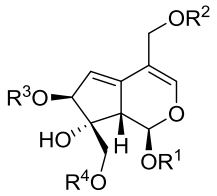


5

Iridoids (Valtrates):



6-7



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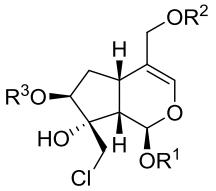
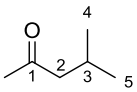


9

	R^1	R^2	R^3	R^4
6	Miv	Iv	Ac	Ivalva
7	Iv	Iv	Ac	Iv
8	Iv	Ac	Iv	Iv
9	Iv	Iv	Ac	-

Iv=



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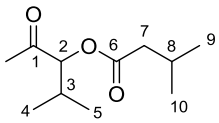
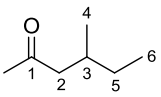
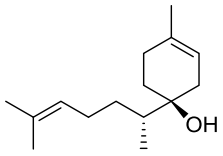


Miv =

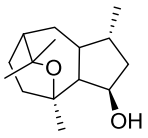


Ac =

Terpenes:



10



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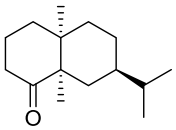
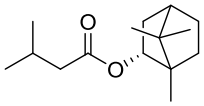
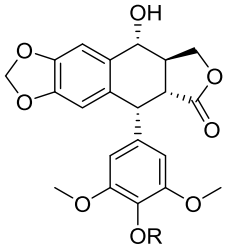


12



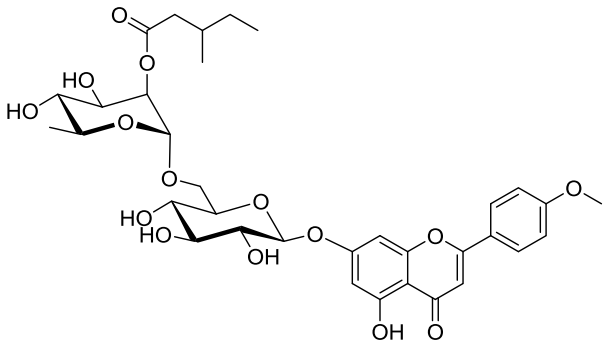
13

Iridoids (Podophyllotoxins):



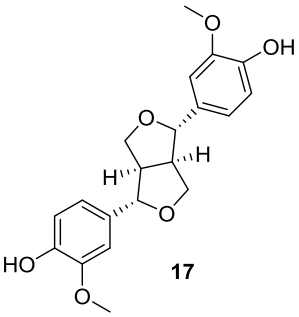
15 R = H
16 R = CH_3

Flavonoid gylcoside:



14

Iridoid (Lignan):



17

Fig.1. Isolated and identified compounds from *V. wallichii* extract. Abbr.: Iv = isovaleryl; Miv = β-methylisovaleryl; Ivalva = α-isovaleroyloxyisovaleryl; Ac = acetyl.

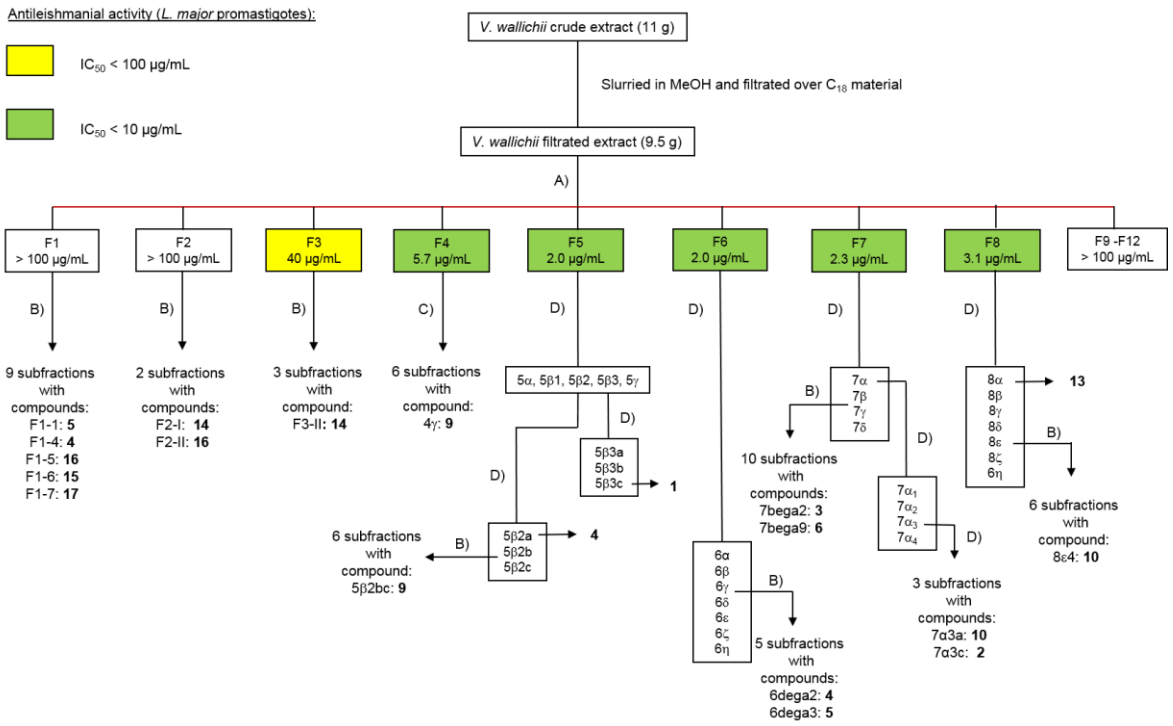


Fig.2. Overview of the bioassay-guided fractionation of *V. wallichii* extract. *Method A*: preparative HPLC (250 mm x 10 mm, 5 µm, Nucleosil 100-5); H_2O (A): MeOH (B), gradient: 70% b (0 min), 75% B (7 min), 100% B (25 min), 70% B (30 min); flow rate: 3.3 mL/min. *Method B*: preparative HPLC (125 mm x 10 mm, 5 µm, Nucleodur Sphinx RP); H_2O (A): CH_3CN (B), gradient: 10% B (0-2 min), 30% B (2-15 min), 65% B (15-22 min), 10% B (22-24 min); flow rate: 4.4 mL/min. *Method C*: flash chromatography (silica gel; MeOH/$CHCl_3$, 4.8:0.2, *v/v*). *Method D*: column chromatography (silica gel; MeOH/$CHCl_3$, 4.8:02, *v/v*).

carefully investigated these fractions. This resulted in the discovery of a novel cinnamic acid derivative **4** with a nepetolactone skeleton and villoside aglycone **5**. From these fractions 4'-demethylpodophyllotoxin (**15**), podophyllotoxin (**16**), pinoresinol (**17**) and linarin-*O*-2-methylbutyrate (**14**) were isolated as well (*cf.* Fig. 1).

*Structure Elucidation of Novel Nepetolactone Derivative **4***

Compound **4** was isolated as a yellowish syrup after multiple fractionation of F1. The IR spectrum showed absorption bands for hydroxyl groups (3382 cm^{-1}), carbonyl functions (1749 and 1697 cm^{-1}) and a double bond (1603 cm^{-1}). The ESI-MS investigation yielded mass signals at *m/z* 506.9 $[M-H]^-$, 621.4 $[M-H+TFA]^-$ and 326.8 $[M-Glc]^-$ in the negative mode, respectively, supporting the proposed structure. In the positive mode multiple adducts (526.2 $[M+H_3O]^+$, 531.1 $[M+Na]^+$) were detected besides the molecular ion signal at *m/z* 509.0 $[M+H]^+$. Fragmentation of *m/z* 526.2 and 531.1 gave a fragment

of *m/z* 347.1 $[M-Glc+Na]^+$. In the NMR spectra the characteristic ^1H and ^{13}C signals for a trans-cinnamic acid with a para-substituted phenyl ring were present. ^{13}C signals inthe range of 70–80 ppm represented a sugar moiety. was supported by comparison of the coupling constants of the protons to data from the literature [10] while the connectivity of the sugar and the cinnamic acid derivative to the nepetolactone skeleton [11] was established by HMBC cross peaks. Interpretation of coupling constants and NOESY experiments revealed the stereochemistry (*cf.* Fig. 3): NOESY correlations of H-8 with other protons were the most prominent ones. H-8 gave NOESY cross peaks with H-1' of the sugar moiety, H-10, H-2" and H-3" of the cinnamic acid rest and the H-11 protons of the methyl group which led to the conclusion that all these groups are in close spatial proximity. The cinnamic acid seems to be located in the space above H-8. Therefore the proposed configuration is consistent with the configuration of the villoside aglycone **5** and other similar compounds from *Valeriana* [12]. Additional evidence came from a weak NOESY correlation between the protons of the C-9 methyl group and H-5b. Thus, compound **4** was assigned to be [(3*S*,4*S*,7*R*,8*S*)-1-oxo-3,7-dimethyl-4-(*O*-β-glucopyranosyl)-oxy]hexahydrocyclopenta-[*c*]-furan-3-yl}-methyl-*trans*-4-hydroxy-cinnamate.

Structure Elucidation of Villoside Aglycone **5**

Villoside is an iridoid glycoside first isolated by Taguchi *et al*. [13] from *Patrinia villosa* Juss., a *Valerianaceae*. However, the villoside aglycone was only obtained semi-

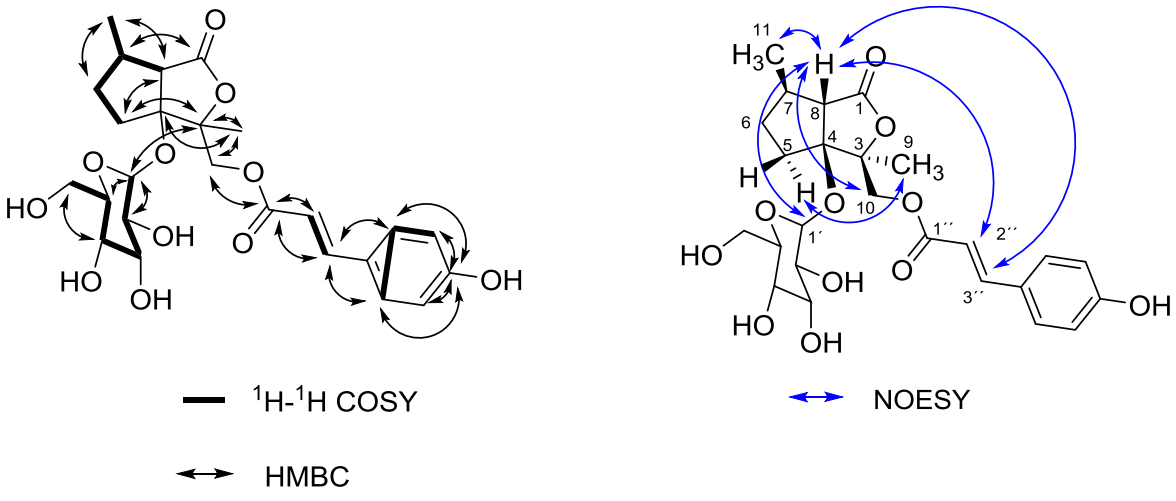


Fig.3. Key ^1H-^1H-COSY, HMBC and NOESY correlations of **4**.

synthetically [13]. This is the first report on the isolation of the aglycone **5** directly from a plant, after chromatographic separation of F1 (*cf.* Fig. 2). Similar compounds (jatamanines) have been isolated from *V. jatamansi* before [12]. Compound **5** was obtained as a yellow syrup. The IR spectrum is characterized by absorption bands at 3387 cm^{-1} indicating a hydroxyl group and at 1705 cm^{-1} caused by a carbonyl function. The ESI-MS showed signals at *m/z* 185.1 $[M+H]^+$ and 207.0 $[M+Na]^+$ which was consistent with the calculated mass of *m/z* 184.1 for the proposed structure. The ^1H spectrum did not show any aromatic proton signals. The ^{13}C spectrum exhibited ten signals including a typical signal for a lactone functionality at 175.7 ppm. A ^{13}C signal at 19.8 ppm with a corresponding ^1H signal at 1.13 ppm for three protons suggested the presence of a methyl group (C-11). The methylene groups next to an oxygen gave characteristic ^{13}C signals at 61.7 and 68.7 ppm (C-3 and C-10, respectively). The remaining signals were found in the aliphatic region. The ^1H-^1H COSY data showed the presence of a spin system involving the hydrogens H-5 to H-9 and, therefore, suggested the presence of a 5-membered ring. All signals have been fully assigned by means of ^1H-^1H COSY, HMBC and HMQC experiments. The configuration was determined by using coupling constants and NOESY data (*cf.* Fig. 4). NOESY correlations between H-11 and H-9, H-5 and H-10, respectively, suggested the methyl group (C-11) and the proton at C-9 as well as the methylene group at C-10 and the proton at C-5 to be located in the same plane of the molecule. The small coupling constant of H-4 and H-3b (3.2 Hz) supports this hypothesis of a quasi-equatorial position of H-4. Additionally, the coupling constant between H-9 and H-8 (J_{aa} = 8.1 Hz) was backing a *trans*-configuration. Furthermore, the coupling constant between H-5 and H-9 (11.0 Hz) was consistent with

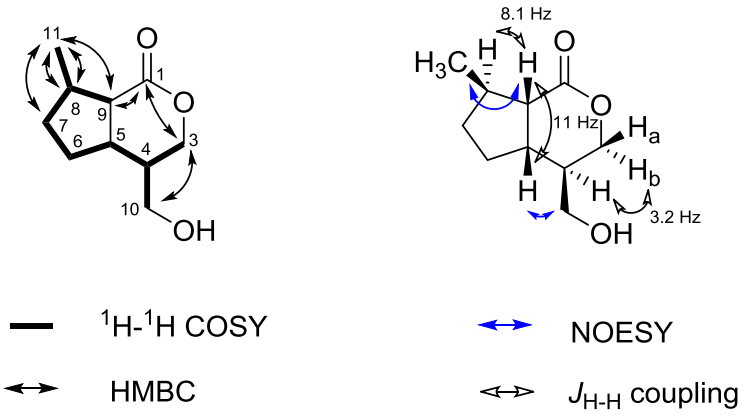


Fig.4. Key ^1H-^1H-COSY, HMBC and NOESY correlations of villoside aglycone **5**.

the reported values of the structurally similar jatamanines having the same configuration [12]. Taking biogenetic reasons into account, compound **5** can be assigned to (4*R*,5*S*,8*R*,9*S*)-4-(hydroxymethyl)-8-methylhexahydro-cyclopenta-[*c*]pyranone (villoside aglycone).

Valtrates with Antileishmanial Activity

Typical and well known constituents of *Valeriana* species are iridoid esters e.g., of the valepotriate and valtrate type, which have been previously investigated in detail because of their *in vitro* antifungal [14] and cytotoxic activity [15–17] and their potential use as antitumor agents [18]. In the active fractions F4–F7 two known valtrates were found, one (compound **8**) of the diene- and one (compound **9**) of the hydrine-type [16], and two iridoid esters **6**, **7** with an as yet unreported substitution pattern. The known valtrates were identified by comparing their spectral data to the literature data (cf. Table 1). Compound **8** has been reported before [14,19,20] and valechlorine (**9**) too [17,21,22]. The new compounds consist of a typical valtrate skeleton of the hydrine-type and are esterified with various acids e.g. isovaleric acid, acetic acid. The connections of the esters were assigned by HMBC experiments (*cf*. Fig. 5). By comparing coupling constants and taking biogenetic considerations into account the configuration was determined to be the same as reported in the literature. Most of the terpenes have been found in subfractions of F7 and F8 (*cf.* Fig. 2). β-Bisabolol (**10**) [23], α-kessyl alcohol (**11**) [24,25], valeranone (**12**) [26] and bornyl isovalerate (**13**) are well known in the Valeriana species [27–30] since a plethora of terpenes has been identified from the essential oil of Valeriana, especially by GC/MS analysis [31,32] in the past. Linarin-*O*-2-methylbutyrate (**14**) has been reported from *V. wallichii* before [33]. Additionally the following known natural products were isolated from *V. wallichii* for the first time: the phenylpropanoids caffeic (**1**), isoferulic (**2**) and *p*-hydroxycinnamic acid bornyl ester (**3**), the lignans 4´-demethylpodophyllotoxin (**15**), podophyllotoxin (**16**) and pinoresinol (**17**)

Table 1. ^{1}H and ^{13}C NMR data of new and known valtrates (**6**-**9**) in $CDCl_3$.

	position	^{13}C of **6**	^{1}H of **6**	^{13}C of **7**	^{1}H of **7**	^{13}C of **8** [20]	^{1}H of **8**	^{13}C of **9** [17]	^{1}H of **9** [17]
	1	89.2	6.22 (d; 4.7; 1H)	89.2	6.27 (d; 4.2; 1H)	92.5	6.25 (d; 10.0; 1H)	89.4	6.19 (d; 5.1; 1H)
	3	141.0	6.44 (br s; 1H)	140.9	6.44 (br s; 1H)	148.0	6.68 (br s; 1H)	141.0	6.45 (br s; 1H)
	4	112.8		112.9		108.7		113.0	
	5	31.8	2.90 (dd; 9.2; 11,0; 1H)	31.4	2.90 (dd; 7.8; 8.0; 1H)	139.1		32.6	2.91 (m; 1H)
	6	34.9	2.1 (m; 2H)	34.7	2.12 (m; 2H)	117.6	5.77 (t; 2.8; 1H)	35.0	2.04–2.13 (m; 2H)
	7	80.1	4.99 (t; 4.5; 1H)	80.4	5.03 (t; 4.7; 1H)	83.1	5.47 (d; 2.8; 1H)	80.0	5.01 (t; 3.9; 1H)
	8	80.9		80.9		80.2		81.8	
	9	44.5	2.42 (dd; 6.1; 15.0; 1H)	44.7	2.44 (dd; 4.1; 9.6; 1H)	48.4	2.94 (dd; 2.5; 10.0; 1H)	45.4	2.45 (dd; 5.9; 8.1; 1H)
	10	66.8	4.29 (d; 11.5; 1H)	66.6	4.22 (s; 2H)	65.5	4.32 (d; 11.6; 1H)	49.1	3.71 (d; 11.4; 1H)
			4.35 (d; 11.5; 1H)				4.39 (d; 11.6; 1H)		3.82 (d; 11.4; 1H)
	11	63.4	4.42 (d; 12.3; 1H)	63.3	4.42 (d; 12.3; 1H)	60.9	4.64 (d; 12.5; 1H)	63.3	4.42 (d; 12.3; 1H)
			4.62 (d; 12.3; 1H)		4.62 (d; 12.3; 1H)		4.71 (d; 12.5; 1H)		4.60 (d; 12.3; 1H)
R^1	1	171.3		171.1		170.8 [d]		171.1	
	2	41.3	2.40 (m; 2H)	43.3 [b]	2.23 (m; 4H)	43.2	2.32 (d; 7,0; 2H)	43.4 [g]	2.09–2.13 (m; 4H)
	3	31.8	1.90 (m; 1H)	25.6 [c]	2.07–2.15 (m; 3H)	25.7	2.05–2.09 (m; 2H)	25.7 [h]	2.00–2.06 (m; 2H)
	4	19.5	0.97 (m; 3H)	22.3 [a]	0.96 (m; 18H)	22.3 [f]	0.93 (d; 6.6; 6H) [g]	22.4 [i]	0.97 (d; 6.7; 6H) [g]
	5	29.3	1.40 (m; 2H)	22.3 [a]	0.96 (m; 18H)	22.3 [f]	0.93 (d; 6.6; 6H) [g]	22.4 [i]	0.97 (d; 6.7; 6H) [g]
	6	11.2	0.90 (m; 3H)						
R^2	1	169.7		173.0		170.8 [d]		172.9	
	2	43.0	2.30–2.35 (m; 4H)	43.5	2.19 (m; 2H)	20.9	2.04 (s; 3H)	43.3 [g]	2.09–2.13 (m; 4H)
	3	25.7	2.11 (m; 2H)	25.7 [c]	2.07–2.15 (m; 3H)			25.6 [h]	2.00–2.06 (m; 2H)
	4 and 5	22.4	0.98 (d; 6.9; 12H)	22.4 [a]	0.96 (m; 18H)			22.4 [i]	0.95 (d; 6.6; 6H) [g]
R^3	1	170.2		170.3		171.8		169.8	
	2	21.0	2.07 (s; 3H)	21.0	2.05 (s; 3H)	43.4	2.14 (m; 2H)	21.0	2.08 (s; 3H)
	3					25.6 [e]	2.05–2.09 (m; 2H)		
	4 and 5					22.3 [f]	0.95 (d; 6.6; 6H) [g]		
R^4	1	173.2		172.9		173.8			
	2	76.9	4.74 (d; 4.8; 1H)	43.1 [b]	2.23 (m; 4H)	43.0	2.20 (m; 2H)		
	3	30.0	2.23 (m; 1H)	25.7 [c]	2.07–2.15 (m; 3H)	25.6 [e]	2.17–2.25 (m; 1H)		
	4	18.8	1.02 (m; 3H)	22.3 [a]	0.96 (m; 18H)	22.3 [f]	0.99 (d; 6.6; 6H) [g]		
	5	17.3	1.00 (m; 3H)	22.3 [a]	0.96 (m; 18H)	22.3 [f]	0.99 (d; 6.6; 6H) [g]		
	6	172.9							
	7	43.0	2.30–2.35 (m; 4H)						
	8	25.7	2.11 (m; 2H)						
	9 and 10	22.4	0.98 (d; 6.9; 12H)						

a-i Assignments may be interchanged.

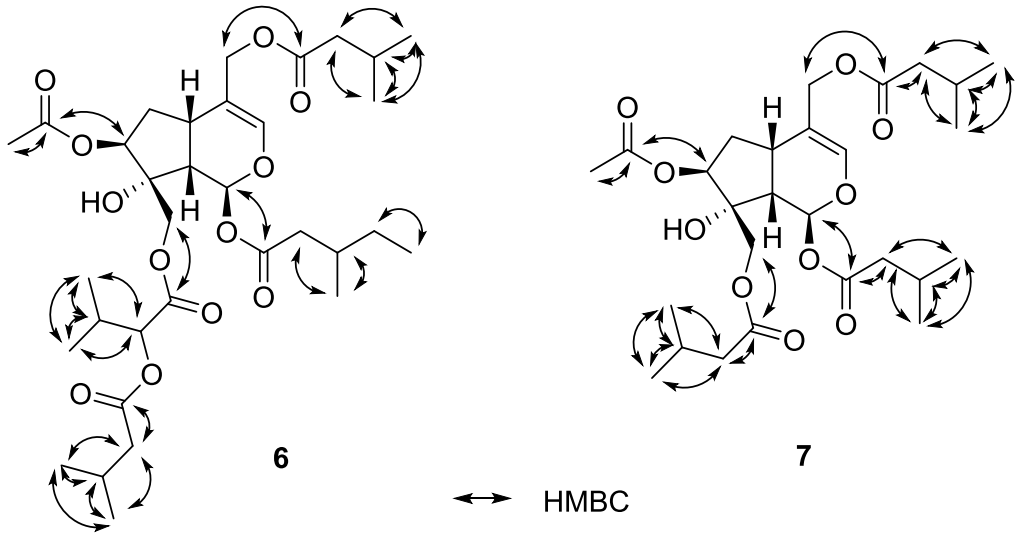


Fig.5. Key HMBC correlations of valtrates **6** and **7**.

Antileishmanial Activity and Cytotoxicity

All compounds isolated were subjected to the evaluation of their antileishmanial activity against *L. major* promastigotes *in vitro* and cytotoxicity against macrophages (J774.1 murine cell line) using the corresponding AlamarBlue© assays [34] (*cf.* Table 2).

The valtrates **6**–**9** showed the highest antileishmanial activity (IC_{50} 0.8–2.3 µg/mL). Their cytotoxicity is in the same range. It is unknown whether the cytotoxicity is directly responsible for the leishmanicidal potential. Nevertheless, the valtrates represent the main active principle of the extract. Fractions with moderate antileishmanial activity in the range of the standard drug miltefosine were phenylpropanoids bornyl caffeate (**1**), bornyl isoferulate (**2**) and bornyl 3-hydroxycinnamate (**3**). This has led to the synthesis of a compound library and QSAR studies recently reported by us [2].

Furthermore, valeranone (**12**) and β-bisabolol (**10**) exhibited moderate antileishmanial and cytotoxic activity. The kessyl alcohol fraction showed rather good antileishmanial activity. However, it was contaminated with an active but unidentified valtrate, thus it remains unclear whether kessyl alcohol (**8**) is really active. Bornyl isovalerate (**13**), linarin-*O*-2-methylbutyrate (**14**), podophyllotoxin (**11**), 4'-demethylpodophyllotoxin (**15**) and pinoresinol (**16**) showed no antileishmanial activity. However, podophyllotoxin and 4'-demethylpodophyllotoxin were highly cytotoxic (IC_{50} < 0.8 µg/mL). This is not surprising as podophyllotoxin and derivatives are widely used as anticancer agents. However, cytotoxicity and antileishmanial activity are not linked to each other in this

Table 2. Antileishmanial activity and cytotoxicity of fractions containing isolated compounds.

Compound	IC_{50} (*L. major*) [µg/mL]	IC_{50} (J774.1) [µg/mL]
Bornyl caffeate (1)	48.8 *	8.3 *
2	16.7	34.7
3	12.2	8.6
4	> 100	> 100
5	> 100	17.5
6	**1.9** [a]	**< 0.8** [a]
7	**0.8**	**< 0.8**
8	**1.7** *	**1.0** *
9	**2.3**	**1.9**
β-Bisabolol (10)	52.2	37.1
α-Kessyl alcohol (11)	**5.8** [b]	**1.7** [b]
Valeranone (12)	60.8	42.6
Bornyl isovalerate (13)	> 100 *	> 100 *
Linarin-*O*-2-methylbutyrate (14)	> 100	10.8
15	> 100	< **0.8**
16	> 100	**< 0.8**
Pinoresinol (17)	> 100	< **0.8**
Miltefosine	36.2 *	56.5 *

*This data was gained from pure or synthesized compounds and is therefore given in µM. [a] There was a small amount of another valtrate present. [b] The fraction of kessyl alcohol was contaminated with an non-identified valtrate (~10 % by NMR integrals).

case. Fractions with novel compounds **4** and **5** exhibited no antileishmanial activity (IC_{50} > 100 µg/mL). Nevertheless, villoside aglycone (**5**) showed moderate cytotoxicity (IC_{50} 17.5 µg/mL). Taken together the antileishmanial activity was found in valtrate derivatives, mainly, and cinnamic acid derivatives with exception of novel compound **4**. However the valtrates were found to be highly cytotoxic in contrast to the cinnamic acid derivatives having moderate cytotoxicity.

Experimental Section

General

^{1}H (400.132 MHz), ^{13}C (100.613 MHz) and 2D NMR spectra were recorded on a Bruker Avance 400 Ultra Shield™ (Bruker Biospin, Ettlingen, Germany) spectrometer. As internal standard the signals of the deuterated solvents were used ($CDCl_3$: ^{1}H 7.26 ppm, ^{13}C 77.0 ppm). IR spectra were recorded on a Jasco FT/IR-6100 spectrometer (Groß-Umstadt, Germany) with an ATR unit at room temperature. LC/MS was conducted on an Agilent 1100 analytical HPLC with DAD detection and an Agilent LC/MSD Trap (Agilent Technologies, Böblingen, Germany). For flash chromatography a Reveleris Flash system (Grace, Columbia, MD, USA) was used. Preparative HPLC was conducted using an Agilent 1100 preparative HPLC with fraction collector and multiple wavelength detector (MWD). TLC plates (60 F254) were purchased from Macherey-Nagel. Column chromatography was carried out on silica gel (Kieselgel 60, 0.063–0.2 mm, 70–320 mesh, Merck, Darmstadt, Germany), filtration on LiChroprep© RP-18 (40–63 µm, Merck).

Plant Material

Dried rhizomes of *V. wallichii* were acquired from a local herb shop in Kolkata, India. The identity as *V. jatamansi* Jones syn. *V. wallichii* DC. was confirmed by comparison with an authentic sample cultivated at the Institute of Himalayan Bioresource Technology in Palampur, Himachal Pradesh, India by R.D. Singh. A voucher specimen of the herbarium was preserved in the laboratory of Banasri Hazra (IHBT Ref. No. 11666).

Extraction and Isolation

The pulverized rhizome (10 g) was refluxed with chloroform (100 mL) for 2 h and filtered. The solvent was removed from the filtrate in a rotary evaporator, followed by complete drying *in vacuo*. Thus, the chloroform extract was obtained (yield = 1.1%; *w/w*) and preserved at 4 °C. The process was repeated to get higher amounts of the extract as

per experimental requirements. Dry extract (11 g) was slurried in MeOH and filtrated using a short column filled with LiChroprep RP-18 silica gel which yielded 9.5 g of extract after evaporation. This material was fractionated into 12 fractions (F1–F12) by *preparative HPLC method A* (250 mm × 10 mm, 5 µm, Macherey-Nagel Nucleosil 100-5; A) H_2O, B) MeOH, gradient: 70% B (0 min), 75% B (7 min), 100% B (25 min), 70% B (30 min); flow rate: 3.3 mL/min).

A portion of 600 mg of fraction F1 was further fractionated by *preparative HPLC method B* (125 mm × 10 mm, 5 µm, Macherey-Nagel Nucleodur Sphinx RP; A) H_2O, B) CH_3CN, gradient: 10% B (0–2 min), 30% B (2–15 min), 65% B (15–22 min), 10% B (22–24 min); flow rate: 4.4 mL/min) resulting in 9 fractions (F1-1 to F1-9). F1-1 (1.5 mg) yielded villosid aglycone **5**, F1-4 (9 mg) the novel nepetolactone derivative **4**. From F1-5 (3.7 mg) and F1-6 (9.1 mg) podophyllotoxin (**16**) and 4'-demethylpodophyllotoxin (**15**) were identified and F1-7 (2.9 mg) contained pinoresinol (**17**).

A 150 mg portion of fraction F2 was partitioned in two fractions (F2-I and F2-II) using the above *HPLC method B*. In F2-I linarin-*O*-2-methylbutyrate (**14**) (6.0 mg) was found and F2-II (12.5 mg) yielded podophyllotoxin (**16**)

Fractionation of F3 into three fractions (F3-I to F3-III) yielded again the linarin derivative **14** in F3-II. Fraction F4 was subjected to flash chromatography (silica gel, MeOH/$CHCl_3$, 4.8:0.2 *v/v*) and gave 6 fractions (4α–4ζ) from which valechlorine (**9**) was identified in fraction 4γ (32.6 mg).

Fraction F5 was separated by column chromatography (CC) on silica gel (MeOH/$CHCl_3$, 4.8:0.2 v/v) and yielded 5 fractions (5α, 5β1, 5β2, 5β3, 5γ). Repeating the chromatography with fraction 5β3 (258 mg) gave three fractions (5β3a–5β3c) of which the last fraction 5β3c yielded 135 mg of bornyl caffeate (**1**). Fractionation of 5β2 (168 mg) by CC gave four fractions (5β2a–5β2c) of which fraction 5β2a gave 14.2 mg of valtrate **7**. Fractions 5β2b and 5β2c were combined and subjected to *preparative HPLC method B*). This process resulted in six fractions (5β2bc1–5β2bc6) from which fraction 5β2bc yielded 6.1 mg of valtrate **9**.

Fraction F6 was again fractionated like F5 by CC which gave seven fractions 6α–6η. 6γ (24.2 mg) and 6δ (25.7 mg) were combined and subjected to *preparative HPLC method B* which gave fractions $6dega_1$-$6dega_5$. $6dega_2$ again yielded valtrate **7** (4.9 mg) and $6dega_3$ valtrate **8** (4.0 mg).

F7 was fractionated by column chromatography as above and gave four fractions (7α–7δ). 7α (57.6 mg) was further partitioned by CC into four fractions ($7\alpha_1$–$7\alpha_4$) of which $7\alpha_2$ yielded 1.5 mg of a mixture of valeranone (**12**) and β-bisabolol (**10**). Further fractionation of $7\alpha_3$ (19.7 mg) by the same CC method gave another 3 fractions ($7\alpha_{3a}$–$7\alpha_{3c}$) of which $7\alpha_{3a}$ consisted of 5.0 mg pure β-bisabolol (**10**). $7\alpha_{3c}$ yielded 2.1 mg of caffeic acid derivative **2**. Fractions 7β and 7γ were combined and fractionated by *preparative HPLC method B* which resulted in 10 fractions ($7bega_1$–$7bega_{10}$) of which fraction $7bega_2$ gave 7.6 mg cinnamic acid derivative **3** and fraction $7bega_9$ 2.5 mg of valtrate **6**.

F8 was fractionated into 6 fractions (8α–8ζ) by CC. Fraction 8β yielded 1.7 mg of bornyl isovalerate (**13**). 8ε was fractionated further by *HPLC method B* and gave six fractions ($8\varepsilon_1$–$8\varepsilon_6$) with fraction $8\varepsilon_4$ yielding 6.8 mg kessyl alcohol (**11**).

Characterization of Novel Nepetolactone Derivative [(3S,4S,7R,8S)-1-Oxo-3,7-dimethyl-4-(O-β-d-glucopyranosyl)oxy]hexahydrocyclopenta[c]furan-3-yl}-methyl-trans-4-hydroxycinnamate (**4**)

Yellowish syrup; IR (ATR): 3382 (OH); 1749, 1697 (C=O), 1603 (C=C) cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ = 1.19 (3H, d, *J* = 6.9 Hz, H-11), 1.73 (3H, s, H-9), 1.41 (1H, m, H-6b), 1.68 (1H, m, H-5b), 1.97 (1H, m, H-6a), 2.20 (1H, m, H-5a), 2.35 (1H, m, H-7), 2.83 (1H, m, H-8), 3.22 (1H, m, H-2'), 3.26 (1H, m, H-5'), 3.41 (1H, t, *J* = 9.1 Hz, H-3'), 3.50 (1H, t, *J* = 9.6 Hz, H-4'), 3.79 (2H, dd, *J* = 3.2, 7.6 Hz, H-6'), 4.37 (1H, d, *J* = 7.5 Hz, H-1'), 4.52 (2H, m, H-10), 6.21 (1H, d, *J* = 15.9 Hz, H-2"), 6.79 (2H, *J* = 8.6 Hz, H-6", H-8"), 7.36 (2H, d, *J* = 8.6 Hz, H-5", H-9"), 7.60 (1H, d, *J* = 15.9 Hz, H-3") ppm; ^{13}C-NMR ($CDCl_3$): δ = 17.1 (C-9), 21.5 (C-11), 30.6 (C-5), 33.0 (C-6), 37.6 (C-7), 59.8 (C-8), 61.3 (C-6'), 67.3 (C-10), 69.4 (C-4'), 73.2 (C-2'), 76.0 (C-5'), 76.3 (C-3'), 86.0 (C-4), 93.4 (C-3), 96.8 (C-1'), 113.5 (C-2"), 115.8 (C-6", C-8"), 125.7 (C-4"), 130.2 (C-5", C-9"), 146.4 (C-3"), 159.5 (C-7"), 167.5 (C-1"), 177.2 (C-1) ppm; ESI-MS: *m/z* 621.4 $[M-H+TFA]^-$, 506.9 $[M-H]^-$, 326.8 $[M-Glc]^-$; HRMS (ESI-TOF$^+$): *m/z* $[M+Na]^+$ calculated for $C_{25}H_{32}O_{11}Na$: 531.1842; found: 531.1836.

Characterization of Villoside Aglycone (4R,5S,8R,9S)-4-(Hydroxymethyl)-8-methylhexahydro-cyclopenta[c]pyranone (**5**)

Yellowish syrup; IR (ATR): 3387 (OH); 1705 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ = 1.11–1.18 (1H, m, H-7a), 1.13 (3H, d, *J* = 6.4 Hz, H-11), 1.21–1.31 (1H, m, H-6a), 1.63–1.72 (1H, m; H-4), 1.82–1.88 (1H, m, H-7b), 1.97–2.05 (1H, m, H-6b), 2.12–2.27 (2H, m, H-8, H-5), 2.32 (1H, dd, *J* = 8.4, 11.0 Hz, H-9), 3.44 (1H, dd, *J* = 8.00, 11.1 Hz, H-10a), 3.64 (1H, dd, *J* = 4.70, 11.1 Hz, H-10b), 4.10 (1H, m, H-3a), 4.35 (1H, dd, *J* = 3.20, 11.1 Hz, H-3b) ppm; ^{13}C-NMR ($CDCl_3$): δ = 19.8 (C-11), 32.3 (C-6), 34.6 (C-7), 38.4 (C-8), 38.9 (C-5), 42.4 (C-4), 48.9 (C-9), 61.7 (C-10), 68.7 (C-3), 175.7 (C-1) ppm; ESI-MS: *m/z* 185.1 $[M+H]^+$, 207.0 $[M+Na]^+$; HRMS (ESI-TOF$^+$): *m/z* $[M+Na]^+$ calculated for $C_{10}H_{16}O_3Na$: 207.0997; found: 207.0993.

Antileishmanial and Cytotoxicity Assays

Antileishmanial activity against *L. major* promastigotes and cytotoxicity against a murine macrophage cell line (J774.1) was determined using an AlamarBlue© assay. Methods for both procedures have been reported previously [34]. IC_{50} values are presented as mean values of two experiments.

Conclusions

The valtrates **6**–**9** represent the main active antileishmanial principles of the *V. wallichii* extract, but they are toxic. Additionally, the cinnamic acid derivatives **1**–**3** showed moderate activity, interestingly except for novel compound **4**. This could be due to the larger size of the molecule or the higher polarity caused by the sugar moiety. However, the previously reported cinnamic acid derivatives exhibited higher activity, even without the double bond [2].

Acknowledgments

Thanks are due to the SFB 630 (DFG) for financial support and to MS and Svetlana Sologub for conducting the antileishmanial and cytotoxicity assays, respectively. BH

wishes to dedicate this work to the memory of R.D. Singh, Chief Scientist, CSIR, at IHBT, Palampur, and acknowledge his immense contribution towards identification of the *V. wallichii* plant sample used in this work. We regret his sad demise in a tragic road accident in October 2014. This publication was funded by the German Research Foundation (DFG) and the University of Wuerzburg in the funding programme Open Access Publishing.

Author Contributions

JG fractionated the extract, isolated the compounds, elucidated structures and prepared the manuscript. MS performed the antileishmanial assays. BH provided the plant material and prepared the extract. UH and BH helped preparing the manuscript and provided scientific input and discussion. HM read the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Sah, S.P.; Mathela, C.; Chopra, K. *Valeriana wallichii*: A phyto-pharmacological review. *J. Pharm. Res.* **2010**, *3*, 2337–2339.
2. Khuda, F.; Iqbal, Z.; Khan, A.; Nasir, F. Antimicrobial and anti-inflammatory activities of leaf extract of *Valeriana wallichii* DC. *Pak. J. Pharm. Sci.* **2012**, *25*, 715–719.
3. Agnihotri, S.; Wakode, S.; Ali, M. Chemical composition, antimicrobial and topical anti-inflammatory activity of *Valeriana jatamansi* Jones. essential oil. *J. Essent. Oil Bear. Pl.* **2011**, *14*, 417–422.
4. Dua, V.; Alam, M.; Pandey, A.; Rai, S.; Chopra, A.; Kaul, V.; Dash, A. Insecticidal activity of *Valeriana jatamansi* (Valerianaceae) against mosquitoes. *J. Am. Mosq. Control Assoc.* **2008**, *24*, 315–318.

5. Ming, D.S.; Yu, D.Q.; Yang, Y.Y.; He, C.H. The structures of three novel sesquiterpenoids from *Valeriana jatamansi* Jones. *Tetrahedron Lett.* **1997**, *38*, 5205–5208.
6. Sudhanshu, N.R.; Sandhya, M.; Ekta, M. Evaluation of antioxidant properties of *Valeriana wallichi* to scavenge free radicals. *Asian J. Pharm. Clin. Res.* **2012**, *5*, 238–240.
7. Thusoo, S.; Gupta, S.; Sudan, R.; Kour, J.; Bhagat, S.; Hussain, R.; Bhagat, M. Antioxidant activity of essential oil and extracts of *Valeriana jatamansi* roots. *BioMed. Res. Int.* **2014**, doi:10.1155/2014/614187.
8. Ghosh, S.; Debnath, S.; Hazra, S.; Hartung, A.; Thomale, K.; Schultheis, M.; Kapkova, P.; Schurigt, U.; Moll, H.; Holzgrabe, U. *Valeriana wallichii* root extracts and fractions with activity against *Leishmania spp*. *Parasitol. Res.* **2011**, *108*, 861–871.
9. Glaser, J.; Schultheis, M.; Hazra, S.; Hazra, B.; Moll, H.; Schurigt, U.; Holzgrabe, U. Antileishmanial lead structures from nature: Analysis of structure-activity relationships of a compound library derived from caffeic acid bornyl ester. *Molecules* **2014**, *19*, 1394–1410.
10. Sobolev, A.P.; Brosio, E.; Gianferri, R.; Segre, A.L. Metabolic profile of lettuce leaves by high-field NMR spectra. *Magn. Reson. Chem.* **2005**, *43*, 625–638.
11. Eisenbraun, E.J.; Browne, C.E.; Irvin-Willis, R.L.; McGurk, D.J.; Eliel, E.L.; Harris, D.L. Structure and stereochemistry of 4aβ,7α,7aβ-nepetalactone from *Nepeta mussini* and its relationship to the 4aα,7α,7aα -and 4aα,7α,7aβ-nepetalactones from *N. cataria*. *J. Org. Chem.* **1980**, *45*, 3811–3814.
12. Lin, S.; Chen, T.; Liu, X.-H.; Shen, Y.-H.; Li, H.-L.; Shan, L.; Liu, R.-H.; Xu, X.-K.; Zhang, W.-D.; Wang, H. Iridoids and lignans from *Valeriana jatamansi*. *J. Nat. Prod.* **2010**, *73*, 632–638.
13. Taguchi, H.; Yokokawa, Y.; Endo, T. Studies on the constituents of *Patrinia villosa Juss*. *Yakugaku Zasshi* **1973**, *93*, 607–611.
14. Fuzzati, N.; Wolfender, J.; Hostettmann, K.; Msonthi, J.; Mavi, S.; Molleyres, L. Isolation of antifungal valepotriates from *Valeriana capense* and the search for valepotriates in crude Valerianaceae extracts. *Phytochem. Anal.* **1996**, *7*, 76–85.
15. Bos, R.; Hendriks, H.; Scheffer, J.J.C.; Woerdenbag, H.J. Cytotoxic potential of valerian constituents and valerian tinctures. *Phytomedicine* **1998**, *5*, 219–225.

16. Lin, S.; Shen, Y.-H.; Li, H.-L.; Yang, X.-W.; Chen, T.; Lu, L.-H.; Huang, Z.-S.; Liu, R.-H.; Xu, X.-K.; Zhang, W.-D*., et al.* Acylated iridoids with cytotoxicity from *Valeriana jatamansi*. *J. Nat. Prod.* **2009**, *72*, 650–655.
17. Lin, S.; Zhang, Z.-X.; Chen, T.; Ye, J.; Dai, W.-X.; Shan, L.; Su, J.; Shen, Y.-H.; Li, H.-L.; Liu, R.-H.; *et al.* Characterization of chlorinated valepotriates from *Valeriana jatamansi*. *Phytochemistry* **2013**, *85*, 185–193.
18. Bounthanh, C.; Bergmann, C.; Beck, J.P.; Haag-Berrurier, M.; Anton, R. Valepotriates, a new class of cytotoxic and antitumor agents. *Planta Med.* **1981**, *41*, 21–28.
19. Finner, E.; David, S.; Thies, P. Über die Wirkstoffe des Baldrians. *Planta Med.* **1984**, *50*, 4–6.
20. Koch, U.; Hölzl, J. The compounds of *Valeriana alliariifolia*. *Planta Med.* **1985**, *51*, 172–173.
21. Xu, J.; Li, Y.; Guo, Y.; Guo, P.; Yamakuni, T.; Ohizumi, Y. Isolation, structural elucidation, and neuroprotective effects of iridoids from *Valeriana jatamansi*. *Biosci. Biotechnol. Biochem.* **2012**, *76*, 1401–1403.
22. Yang, X.P.; Li, E.W.; Zhang, Q.; Yuan, C.S.; Jia, Z.J. Five new iridoids from *Patrinia rupestris*. *Chem. Biodivers.* **2006**, *3*, 762–770.
23. Minyard, J.P.; Thompson, A.C.; Hedin, P.A. Constituents of the cotton bud. VIII. β-Bisabolol, a new sesquiterpene alcohol. *J. Org. Chem.* **1968**, *33*, 909–911.
24. Itô, S.; Kodama, M.; Nozoe, T.; Hikino, H.; Hikino, Y.; Takeshita, Y.; Takemoto, T. Structure and absolute configuration of α-kessyl alcohol and kessyl glycol. *Tetrahedron* **1967**, *23*, 553–563.
25. Nishiya, K.; Kimura, T.; Takeya, K.; Itokawa, H. Sesquiterpenoids and iridoid glycosides from *Valeriana fauriei*. *Phytochemistry* **1992**, *31*, 3511–3514.
26. Vite, G.D.; Spencer, T.A. Synthesis of (+)-7-epivaleranone and (+)-valeranone. *J. Org. Chem.* **1988**, *53*, 2560–2565.
27. Gränicher, F.; Christen, P.; Kapetanidis, I. Essential oils from normal and hairy roots of *Valeriana officinalis* var. *sambucifolia*. *Phytochemistry* **1995**, *40*, 1421–1424.
28. Mathela, C.S.; Chanotiya, C.S.; Sammal, S.S.; Pant, A.K.; Pandey, S. Compositional diversity of terpenoids in the Himalayan *Valeriana* genera. *Chem. Biodivers.* **2005**, *2*, 1174–1182.

29. Wang, J.; Zhao, J.; Liu, H.; Zhou, L.; Liu, Z.; Wang, J.; Han, J.; Yu, Z.; Yang, F. Chemical analysis and biological activity of the essential oils of two valerianaceous species from China: *Nardostachys chinensis* and *Valeriana officinalis*. *Molecules* **2010**, *15*, 6411–6422.
30. Houghton, P.J. The biological activity of valerian and related plants. *J. Ethnopharmacol.* **1988**, *22*, 121–142.
31. Bos, R.; Woerdenbag, H.J.; Hendriks, H.; Smit, H.F.; Wikström, H.V.; Scheffer, J.J. Composition of the essential oil from roots and rhizomes of *Valeriana wallichii DC*. *Flavour Fragr. J.* **1997**, *12*, 123–131.
32. Sati, S.; Mathela, C. Essential oil composition of *Valeriana hardwickii* var. *arnottiana* from the Himalayas. *Flavour Fragr. J.* **2005**, *20*, 299–301.
33. Chari, V.M.; Jordan, M.; Wagner, H.; Thies, P.W. A ^{13}C-NMR study of the structure of an acyl-linarin from *Valeriana wallichii*. *Phytochemistry* **1977**, *16*, 1110–1112.
34. Bringmann, G.; Thomale, K.; Bischof, S.; Schneider, C.; Schultheis, M.; Schwarz, T.; Moll, H.; Schurigt, U. A novel *Leishmania major* amastigote assay in 96-well format for rapid drug screening and its use for discovery and evaluation of a new class of leishmanicidal quinolinium salts. *Antimicrob. Agents Chemother.* **2013**, *57*, 3003–3011.

Sample Availability: Samples of novel compounds **4** and **5** are available from the authors.

3.2. Antileishmanial Lead Structures from Nature: Analysis of Structure-Activity Relationships of a Compound Library Derived from Caffeic Acid Bornyl Ester

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Reprinted from *Molecules*, **2014**, *19*, 1394-1410;

Abstract

Bioassay-guided fractionation of a chloroform extract of *Valeriana wallichii* (*V. wallichii*) rhizomes lead to the isolation and identification of caffeic acid bornyl ester (**1**) as the active component against *Leishmania major* (*L. major*) promastigotes (IC_{50} = 11.8 µg/mL). To investigate the structure-activity relationship (SAR), a library of compounds based on **1** was synthesized and tested *in vitro* against *L. major* and *L. donovani* promastigotes, and *L. major* amastigotes. Cytotoxicity was determined using a murine J774.1 cell line and bone marrow derived macrophages (BMDM). Some compounds showed antileishmanial activity in the range of pentamidine and miltefosine which are the standard drugs in use. In the *L. major* amastigote assay compounds **15**, **19** and **20** showed good activity with relatively low cytotoxicity against BMDM resulting in acceptable selectivity indices. Molecules with adjacent phenolic hydroxyl groups exhibited elevated cytotoxicity against murine cell lines J774.1 and BMDM. The Michael system seems not to be essential for antileishmanial activity. Based on the results compound **27** proves to be the new lead structure for further structure optimization.

Introduction

Leishmaniasis is one of the most dreaded infectious diseases worldwide. It is endemic in 98 countries and almost 350 million people on five continents are at risk of infection [1,2]. The disease has an especially high impact on the population of the disadvantaged regions of South America, Africa and Asia (especially India), all of them suffering from poverty and low health care standards. Leishmaniasis is caused by protozoan parasites, which are transmitted by the bite of the sand fly. It comprises a variety of diseases. Cutaneous leishmaniasis mostly caused by *L. major* produces skin sores, ulcers and eventually heals by leaving defacing scars. The most severe form is the visceral leishmaniasis also known as *kala-azar* or black fever. Here the parasites invade internal organs, especially the liver and the spleen. This is fatal if left untreated. The species known to cause visceral leishmaniasis is *L. donovani* which is prevalent in India and East Africa. 70 % of the global burden of *kala-azar* is concentrated in India [1,3]. The estimated incidence of visceral leishmaniasis in India is 146,700 to 282,800 cases per year [1].

The infection can be treated but the drugs in use like miltefosine or amphotericin B show severe side effects. Additionally these drugs are expensive and require adequate medicinal care which is not readily available in the most affected regions of India or Africa. Therefore, the need for new antileishmanial drugs is evident. As reported previously the chloroform extract of *V. wallichii* roots showed antileishmanial activity [4]. In this paper we describe the isolation and structure elucidation of one active ingredient, which was assigned to be caffeic acid bornyl ester (**1**). In order to analyse structure-activity relationship for activity optimization a library of derivatives has been synthesized. The respective cytotoxicity against diverse species of *Leishmania* promastigotes, amastigotes and macrophages was determined.

Results and Discussion

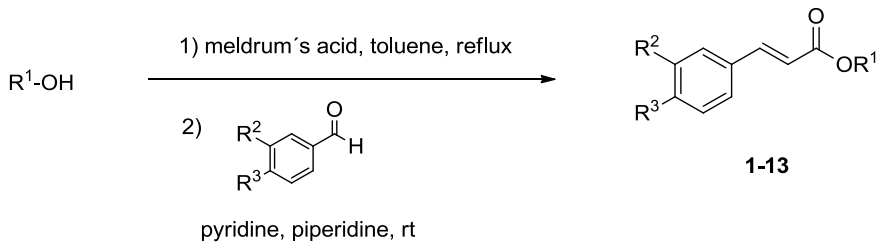
Isolation

The crude chloroform extract obtained from the pulverized rhizomes of *V. wallichii* was subjected to bioactivity-guided fractionation. The extract was roughly fractionated into

twelve fractions by means of semi-preparative HPLC using reverse phase material and a gradient of H_2O/MeOH as the eluent. The most active fraction was repeatedly partitioned by normal phase silica gel column chromatography with $CHCl_3$/MeOH (4.8:0.2 *v/v*) as mobile phase. From a relatively polar sub-fraction a pure compound with an IC_{50} of 11.8 µM against *L. major* promastigotes could be isolated. ^1H NMR data showed two doublets for vinyl protons at 6.3 and 7.6 ppm, respectively, with the typical coupling constants of 15.9 Hz indicating a double bond with *trans* configuration. Signals for three aromatic protons with a characteristic coupling pattern suggested *meta* and *para* substitution in relation to the side chain. Characteristic of the borneol moiety were the three -CH_3 singuletts at 0.90, 0.95 and 0.99 ppm. ESIMS data confirmed the assumed ester structure with two hydroxyl groups as substituents on the aromatic ring by giving a mass of *m/z* 315 $[M-H]^-$. The obtained spectroscopic data are in accordance to literature data [5-7].

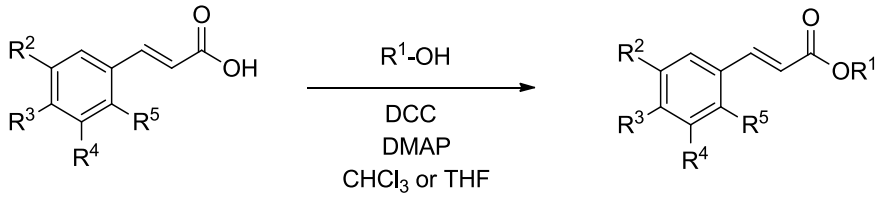
Chemistry

Since the caffeoyl-skeleton has been described to be a valuable pharmacophore for antiviral [5,8], antibacterial [9] and antiprotozoal activity [10] we tried to enhance the antileishmanial activity and to decrease the cytotoxicity by the synthesis of a library of derivatives of **1** with systematic structure variations with regard to the terpenoid part on the one hand and the caffeic acid part on the other hand. To investigate structure-activity relationship borneol was replaced by moieties varying in size and bulkiness, and substituents with different properties such as hydroxyl, methoxy and nitro groups as well as halogen atoms were attached to the aromatic ring in varying positions. The general synthesis of phenolic compound **1** and its derivatives **2**-**13** via Knoevenagel-Doebner condensation is outlined in Scheme 1.



Scheme 1. Preparation of compounds **1**-**13** (substituents are listed in Table 1).

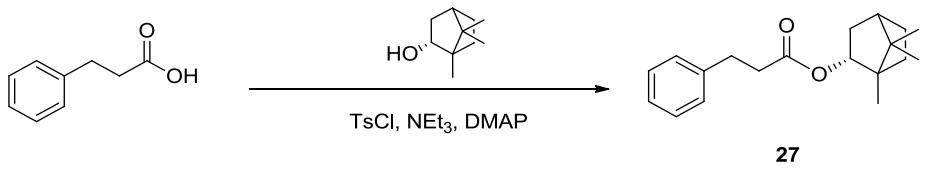
Activation of the corresponding alcohol with Meldrum´s acid without isolation of the activated product and subsequent aldol condensation using the substituted aldehyde lead to the cinnamic acid derivatives **1-13** in acceptable yields [5]. Compounds **14**-**26** were accessible by facile esterification using the Steglich concept composed of *N*,*N*'-dicyclohexylcarbodiimid (DCC) and 4-dimethylaminopyridine (DMAP) in CH_3Cl or THF (Scheme 2).



14-26

Scheme 2. Preparation of compounds **14**-**26** (substituents are listed in Table 2).

The α,β-unsaturated carbonyl moiety of the caffeic acid is a Michael acceptor which is highly reactive to nucleophilic attacks e.g. by amino or thiol groups. To investigate whether the double bond is essential for antileishmanial activity compound **27** has been synthesized via a different synthesis route as described before [11]. By esterification of 3-phenyl propanoic acid with borneol using tosyl chloride, Et_3N and DMAP as a catalyst (Scheme 3) product **27** was obtained.



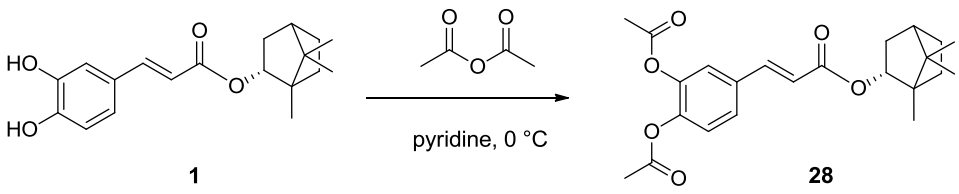
27

Scheme 3. Preparation of compound **27**.

It might be possible that the catechol moiety of compound **1** contributes to cytotoxicity or antileishmanial activity. Hence both phenolic hydroxyl groups were acetylated by adding acetic acid anhydride to caffeic acid bornyl ester **1** (isolated from the plant) in pyridine at 0 °C and stirring for 24 h at room temperature (Scheme 4) to give compound **28**.

Structure-Activity Relationship Investigation

Antileishmanial activities of the synthesized compounds were evaluated in *L. major* promastigotes as described before [12] and in *L. donovani* promastigotes according to

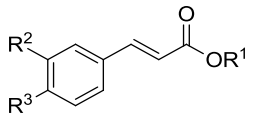


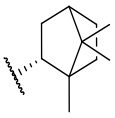
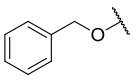
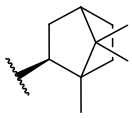
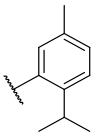
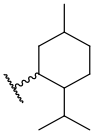
1 **28**

Scheme 4. Preparation of compound **28**.

Hazra et al. [13]. Cytotoxicity was determined on a J774.1 murine cell line [12]. The antiprotozoal activities are presented in Tables 1-3 as inhibitory concentrations (IC_{50}). The most active compound is the cinnamic acid bornyl ester **15** having an IC_{50} value of 39.6 µM against *L. major* and 15.6 µM against *L. donovani* promastigotes, which is in the concentration range of pentamidine and miltefosine, both drugs currently in use.

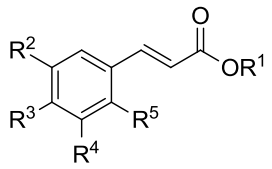
Table 1. Antileishmanial activity and cytotoxicity (compounds **1**-**13**).

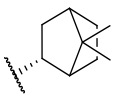
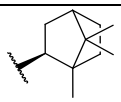
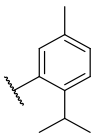
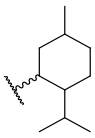
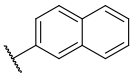
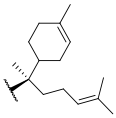
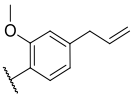
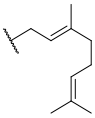
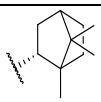


Compd.	R^1	R^2	R^3	IC_{50} (µM) * *L. major* promastigotes	IC_{50} (µM) * *L. donovani* promastigotes	IC_{50} (µM) * J774.1
1		-OH	-OH	48.8	27.3	8.3
2		-OH	-OCH_3	64.4	41.3	48.7
3		-H	-Cl	71.2	> 100	49.5
4		-H	-Br	> 100	> 100	54.6
5		-H	-$N(CH_3)_2$	> 100	> 100	> 100
6		-H		> 100	> 100	> 100
7		-OH	-OH	45.8	34.8	8.8
8		-OH	-OCH_3	60.6	74.5	44.3
9		-H	-NO_2	> 100	> 100	> 100
10		-OH	-OH	57.6	42.1	9.5
11		-OH	-OCH_3	59.8	79.6	45.6
12		-OH	-OH	59.5	79.4	1.95
13		-OH	-OCH_3	54.2	> 100	44.6

* Positive control: pentamidine 82 µM (*L. major*), 38.6 µM (J774.1); miltefosine: 36.2 µM (*L. major*), 56.5 µM (J774.1); amphotericin B 0.4 µM (*L. donovani*).

Table 2. Antileishmanial activity and cytotoxicity (compounds **14**-**26**).



Compd.	R^1	R^2	R^3	R^4	R^5	IC_{50} (*μM*)* *L. major* promastigotes	IC_{50} (*μM*)* *L. donovani* promastigotes	IC_{50} (*μM*)* J774.1
14		-H	-H	-Cl	-Cl	65.6	> 100	58.9
15		-H	-H	-H	-H	39.6	15.6	45.2
16		-H	$-OCH_3$	-H	-H	60.9	> 100	42.6
17		-H	-Cl	-H	-Cl	80.5	> 100	60.6
18		-H	-H	-H	-H	64.3	51.2	46.0
19						55	> 100	44.8
20						53.1	79.7	44.8
21						60	> 100	46.7
22						67	28.7	44.5
23						> 100	23.4	> 100
24						> 100	> 100	> 100
25						> 100	80.3	32.2
26						> 100	41.9	> 100
28		-OAc	-OAc	-H	-H	30.7	-	2.1

*Positive control: pentamidine 82 μM (*L. major*), 38.6 μM (J774.1); miltefosine: 36.2 μM (*L. major*), 56.5 μM (J774.1); amphotericin B 0.4 μM (*L. donovani*).

Influence of the Bornyl Moiety

The bornyl moiety is of particular importance of preserving antileishmanial activity. The most active compounds (**1**, **7**, **15** and **27**) are esters of borneol. There is no significant change in activity using isoborneol (**1**) instead of borneol (**7**). Substituting borneol with a less sterically demanding alcohol e.g. cyclohexanol (**23**) or eugenol (**24**) gives IC_{50} values higher than 100 µM (*L. major*). Using thymol (**10**, **11**, **19**), menthol (**12**, **13**, **20**) or other bulky substituents like naphthol (**22**) or adamantol (**21**) preserves the activity. A similar pattern is found with *L. donovani* promastigotes. Again the bornyl esters show the best IC_{50} values and activity stays the same using thymol, menthol or naphthol. In contrast to *L. major* some of the sterically less demanding compounds exhibit activity against *L. donovani* promastigotes (e.g. 23.4 µM (**23**), 41.9 µM (**26**)). This might be due to biological differences between the two species. However borneol seems to be the most advantageous substituent fulfilling the requirement of bulkiness in this position.

The Catechol Moiety

The caffeic acid derivative (**1**) isolated from *V. wallichii* having two hydroxyl groups in position 3 and 4 of the aromatic ring shows an IC_{50} value of 48.8 µM against *L. major* promastigotes and relatively high cytotoxicity (8.3 µM) against a J774.1 cell line. The toxicity is an attribute of all *meta* and *para* dihydroxylated caffeic acid derivatives (**7**, **10**, **12**) and has been reported in the literature for similar compounds [10,14]. The catechol moiety is prone to oxidation resulting in an *o*-quinone which can easily react with amino groups of proteins [15]. Hence, omitting the hydroxyl groups lead to **15** with antileishmanial activity in the same range as **1** but with a fivefold lower cytotoxicity. The same effect is observed by replacement of the hydroxyl group in 4-position with a methoxy group (**2**, **8**, **11**, **13**). However, the cytotoxicity is not correlating with antileishmanial activity, the latter stays in the same concentration range for compounds whether with or without hydroxyl groups. Acetylation to “mask” the catechol structure (**28**) slightly increased antileishmanial activity but cytotoxicity persisted in the single-digit micromolar range.

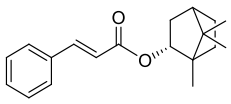
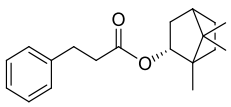
Substitution on the Aromatic Ring

In order to analyse the influence of the substituents of the aromatic ring on antileishmanial activity and cytotoxicity, bornyl esters are compared in the following. Compound **15** with an unsubstituted benzene ring is the most promising compound with an antileishmanial activity in a low micromolar concentration and low cytotoxicity. The comparison of all derivatives with substituents in *para* position reveals compounds **3** (-Cl) and **16** (-OMe) to have minor antileishmanial activity against *L. major* (71.2 µM and 60.9 µM). Bigger substituents are not tolerated in this place (**4**-**6**, **9**) whereas chlorination in position 2 and 3 (**14**), and 2 and 4 (**17**), respectively, is acceptable. None of these compounds exhibited any activity against *L. donovani* promastigotes. Thus, a non-substituted aromatic ring is advantageous for a good antiprotozoal activity against multiple species.

Michael System

All compounds are characterized by the highly reactive enone Michael system, which is prone to unspecific covalent reactions with proteins of both parasite and host. In order to check whether the Michael moiety is necessary for antileishmanial activity, compound **27** characterized by a simple carbonyl group was synthesized. This compound is as active as the corresponding compound **15** and did not show cytotoxicity (Table 3). This is in contrast to the results observed for caffeic acid alkyl ester derivatives which were found to be inactive without the double bond [10].

Table 3. Antileishmanial activity of compounds **15** and **27**.

Compound	*L. major* promastigotes IC_{50} (µM)	Cytotoxicity J774.1 IC_{50} (µM)	Selectivity index SI [a]
 **15**	39.6	45.2	1.1
 **27**	50.2	> 100	> 2.0

[a] SI = IC_{50} for J774.1/IC_{50} for *L. major*.

Amastigote Results

The amastigote is the intracellular pathogenic form of the parasite in the vertebrate host and therefore the important target. Compounds showing high activity against *L. major* promastigotes (**1**, **2**, **7**, **10**, **15**, **19**, **20**) were selected for screening against *L. major* amastigotes and cytotoxicity against BMDM. The amastigote assay was conducted as reported by Bringmann et al. [12] and the results are presented in Table 4. The cytotoxicity against BMDM mirrors those against the macrophages J774.1. With regard to antileishmanial activity cinnamic acid menthyl (**20**) and thymyl ester (**19**) show good activities combined with low cytotoxicity. Again the cinnamic acid bornyl ester (**15**) is the most active compound. In general, the activity lies in the range of miltefosine with an IC_{50} value of 33.0 µM. These results corroborate the theory that the compounds could be active as antileishmanial agents against amastigotes *in vivo*.

Table 4. Antileishmanial activity and cytotoxicity of selected compounds.

Compound	*L.major* amastigotes IC_{50} (µM)	Cytotoxicity BMDM IC_{50} (µM)	Selectivity index SI [a]
1	47.6	**5.3**	0.1
7	47.0	**10.9**	0.2
15	**10.9**	54.3	***5.0***
2	39.2	49.0	1.2
19	49.1	***> 148***	***> 3.0***
20	**19.5**	***> 126***	***> 6.4***
10	54.1	**9.8**	0.2
Miltefosine	33.0	65.5	2.0

[a]SI = IC_{50} for BMDM/IC_{50} for *L. major*.

Experimental Section

General Experimental Procedures

Starting materials and reagents were purchased from Sigma-Aldrich and VWR. Solvents were of synthetic or analytical grade. Melting points were determined on a Stuart melting point apparatus SMP10 (Bibby Scientific) and are uncorrected. Optical rotations were measured on a CHIRALYSER 6.3 (IBZ Messtechnik). IR spectra were acquired on a JASCO FT/IR-6100 Fourier Transformation Infrared Spectrometer equipped with an ATR unit. ^{1}H (400.132 MHz) and ^{13}C (100.613 MHz) NMR spectra were recorded on a Bruker Avance 400 Ultra Shield™ (Bruker Biospin, Ettlingen,

Germany) spectrometer. The signals of the deuterated solvents were used as internal standards ($CDCl_3$: ^1H 7.26 ppm, ^{13}C 77.0 ppm; MeOD: ^1H 4.84 ppm, ^{13}C 49.05 ppm). LC/MS was conducted on an Agilent 1100 analytical HPLC with DAD detection and an Agilent LC/MSD Trap. ESIMS data was conducted in positive and negative mode. For LC/MS, following conditions were used: Nucleodur Sphinx RP18 (Macherey-Nagel) 150 mm x 4.6 mm, 5 µm, A) CH_3CN + 0.1 % FA, B) H_2O + 0.1 % FA, gradient: 10 % B (0-2 min), 30 % B (2-3 min), 100 % B (3-25 min), 80 % B (25-33 min), 40 % B (33-37 min), 10 % B (38-40 min), 0.8 mL/min, MS-detection: ESI, nebulizer pressure: 50 psi, drying gas: 10 L/min, drying gas temperature: 350 °C, capillary voltage: 3500 V. An Agilent 1100 preparative HPLC with fraction collector and multiple wavelength detector (MWD) was used for fractionation of the extract.

Plant Material and Preparation of Extract

The plant material was obtained as described previously [4]. The rhizomes were pulverized and a portion of 10 g was refluxed for 2 h with 100 mL chloroform. Evaporation of the solvent yielded a black syrup with a characteristic smell.

*Bioactivity-Guided Fractionation and Isolation of **1***

10.9 g of the crude chloroform extract were subjected to a bioactivity-guided fractionation. The extract was slurried in MeOH and the insoluble parts were removed by filtration over LiChroprep© RP 18 material (Yield: 9.5 g). The extract was subjected to semi-preparative HPLC (250 mm x 10 mm, 5 µm, Macherey-Nagel Nucleosil 100-5; A) H_2O, B) MeOH, gradient: 70 % B (0 min), 75 % B (7 min), 100 % B (25 min), 70 % B (30 min); flow rate: 3.3 mL/min) and partitioned into 12 fractions. Fraction 4-8 exhibited significant antileishmanial activity against *L. major* promastigotes. Fraction 5 (559 mg) was further fractionated by column chromatography using silica gel and MeOH/$CHCl_3$ (4.8:0.2 *v/v*) as mobile phase. Fraction $5\beta_3$ (258 mg) was partitioned a second time by column chromatography using silica gel and MeOH/$CHCl_3$ (4.8:0.2 *v/v*) to yield three fractions ($5\beta_{3a}$, $5\beta_{3b}$, $5\beta_{3c}$) from which the last fraction afforded 135 mg of caffeic acid (-)-bornyl ester (IC_{50} 42.4 µM /11.8 µg/mL). The structure was identified by means of NMR and LC/MS data.

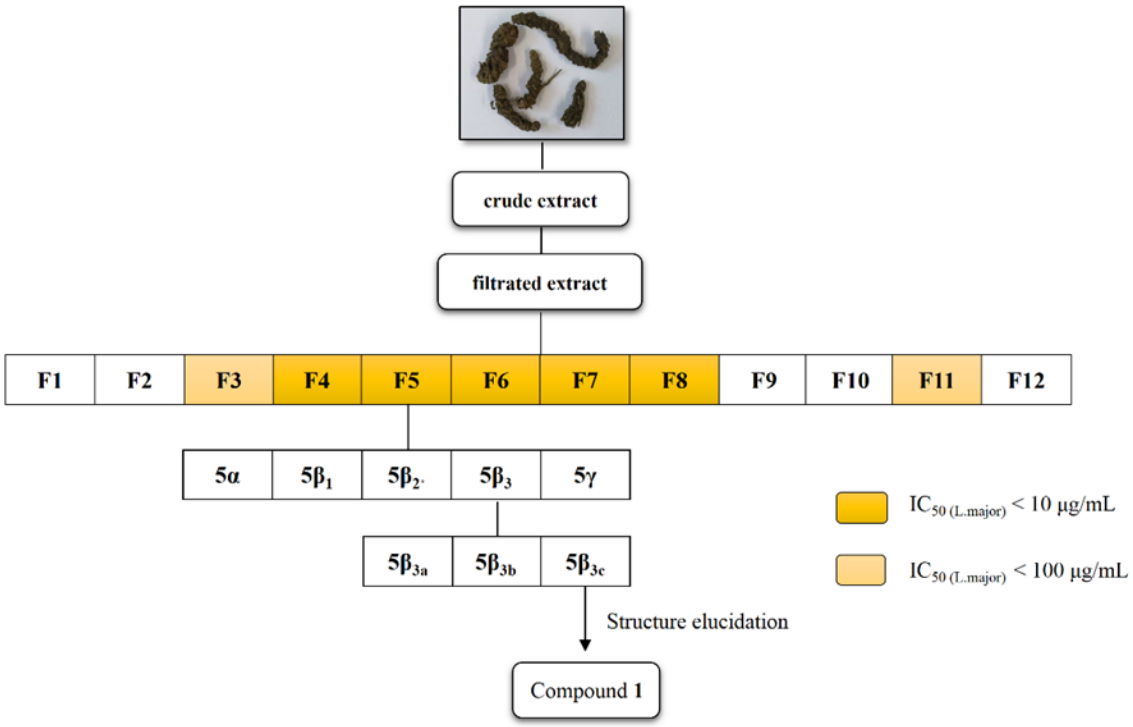


Fig.1. Overview of bioactivity-guided fractionation.

Caffeic acid (-)-bornyl ester (**1**). Brownish solid (MeOH); m.p. 150 °C (lit.[6] 148-151 °C); $[\alpha]_{426}$ -5.1 ° (c 0.1, MeOH); IR: 3444, 3165, 1661, 1604, 1273, 1186 cm^{-1}; ^{1}H NMR (MeOD) *δ* (ppm) *J* (Hz): 0.90 (s;3H); 0.94 (s;3H); 0.98 (s;3H); 1.06 (dd;*3.7*;*13.7*;1H); 1.26-1.35 (m;2H); 1.70 (t;*4.5*;1H); 1.79-1.88 (m;1H); 2.07-2.13 (m;1H); 2.38-2.46 (m;1H); 5.00 (ddd;*2.2*;*3.4*;*9.9*;1H); 6.30 (d;*15.9*;1H); 6.80 (d;*8.2*;1H); 7.00 (dd;*2.1*;*8.2*;1H); 7.05 (d;*2.1*;1H); 7.54 (d;*15.9*;1H); ^{13}C NMR (MeOD) *δ* (ppm) 13.9; 19.2; 20.1; 28.2; 29.0; 37.9; 46.4; 48.9; 49.5; 81.2; 115.1; 115.5; 116.5; 122.9; 127.8; 146.7; 146.8; 149.6; 169.7; ESIMS: *m/z* 315 $[M-H]^-$, 339 $[M+Na]^+$.

Synthesis

General Procedure for the Synthesis of Compounds **1**-**13**

Synthesis was conducted according to the method of Xia et al. [5]. The alcohol (2.94 mmol) and Meldrum´s acid (2.94 mmol) were dissolved in 20 mL toluene and refluxed for 4 hours. To the resulting activated alcohol the corresponding aldehyde (2.94 mmol), pyridine (2.5 mL) and piperidine (250 µL) were added. This mixture was stirred at room temperature for 24-48 hours. The solvent was evaporated, the residue

dissolved in 20 mL of diethyl ether and washed three times with 10 mL of an aqueous saturated $NaHCO_3$ solution, 10 mL 20 % HCl, and 10 mL H_2O, respectively. After drying over $MgSO_4$ the solvent was evaporated and the residue was chromatographed using silica gel (petroleum ether/ethyl acetate 60:40) to yield the desired product. (Yields overall of two steps)

Synthesized Caffeic acid (-)-bornyl ester (**1**). Yield 19%; off-white solid; m.p. 150 °C; $[\alpha]_{426}$ - 4.5 ° (c 0.1, MeOH); IR and NMR data in accordance with above and literature [6].

Isoferulic acid (-)-bornyl ester (**2**). Yield 9%; colorless syrup; IR: 3384, 2952, 2877, 1698, 1631, 1263, 1172, 1155 cm^{-1}; ^1H NMR ($CDCl_3$) *δ* (ppm) *J* (Hz): 0.88 (s;3H); 0.90 (s;3H); 0.95 (s;3H); 1.04-1.08 (m;1H); 1.25-1.32 (m;2H); 1.71 (t;*4.5*;1H); 1.75-1.85 (m;1H); 2.02-2.09 (m;1H); 2.38-2.46 (m;1H); 3.94 (s;3H); 5.02 (ddd;*2.1*;*3.4*;*9.9*;1H); 6.30 (d;*16.0*;1H); 6.92 (d;*8.2*;1H); 7.05 (d;*1.9*;1H); 7.09 (dd;*8.2*;*1.9*;1H); 7.60 (d;*16.0*;1H); ^{13}C NMR ($CDCl_3$) *δ* (ppm): 13.6; 18.9; 19.7; 27.3; 28.1; 36.9; 45.0; 47.9; 48.9; 56.0; 79.8; 109.3; 114.7; 116.2; 123.0; 127.1; 144.3; 146.7; 147.8; 167.6.

4-Chloro cinnamic acid (-)-bornyl ester (**3**). Yield 14%; slightly yellow solid; m.p. 80-83 °C; IR: 2952, 2876, 1704, 1636, 1490, 1308, 1184, 820 cm^{-1}; ^1H NMR ($CDCl_3$) *δ* (ppm) *J* (Hz): 0.88 (s;3H); 0.90 (s;3H); 0.94 (s;3H); 1.03-1.08 (m;1H); 1.25-1.32 (m;2H); 1.71 (t;*4.6*;1H); 1.74-1.84 (m;1H); 2.01-2.06 (m;1H); 2,38-2.46 (m;1H); 5.02 (ddd;*2.2*;*3.4*;*10.0*;1H); 6.44 (d;*16.0*;1H); 7.36 (d;*8.5*;2H); 7.47 (d;*8.5*;2H); 7.61 (d;*16.0*;1H); ^{13}C NMR ($CDCl_3$) *δ* (ppm): 13.5; 18.9; 19.7; 27.2; 28.1; 36.9; 45.0; 47.9; 49.0; 80.2; 119.4; 129.1 (2C); 129.2 (2C); 133.1; 136.0; 142.7; 167.1.

4-Bromo cinnamic acid (-)-bornyl ester (**4**)**.** Yield 9%; yellow solid; m.p. 71-74 °C; IR: 2953, 2875, 1705, 1635, 1203, 1158, 816 cm^{-1}; ^1H NMR ($CDCl_3$) *δ* (ppm) *J* (Hz): 0.88 (s;3H); 0.90 (s;3H); 0.94 (s;3H); 1.03-1.08 (m;1H); 1.25-1.32 (m;2H); 1.71 (t;*4.6*;1H); 1.74-1.84 (m;1H); 2.01-2.06 (m;1H); 2.38-2.46 (m;1H); 5.03 (ddd;*2.2*;*3.4*;*9.9*;1H); 6.44 (d;*16.0*;1H); 7.36 (d;*8.5*;2H); 7.47 (d;*8.5*;2H); 7.61 (d;*16.0*;1H); ^{13}C NMR ($CDCl_3$) *δ* (ppm): 13.5; 18.9; 19.7; 27.2; 28.1; 36.9; 45.0; 47.9; 48.9; 80.2; 119.4; 124.4; 129.4 (2C); 132.1 (2C); 133.4; 142.7; 167.1.

4-N-Dimethylamino cinnamic acid (-)-bornyl ester (**5**). Yield 25%**;** yellowish solid; m.p. 114-116 °C; IR: 2950, 2877, 1696, 1603, 1524, 1151, 810 cm^{-1}; ^{1}H NMR ($CDCl_3$) *δ* (ppm) *J* (Hz): 0.88 (s;3H); 0.90 (s;3H); 0.94 (s;3H); 1.04-1.08 (m;1H); 1.25-1.37 (m;2H); 1.70 (t;*4.5*;1H); 1.74-1.84 (m;1H); 2.03-2.10 (m;1H); 2.37-2.45 (m;1H); 3.02 (s;6H); 5.01 (ddd;*2.0*;*3.3*;*9.9*;1H); 6.25 (d;*15.9*;1H); 6.67 (d;*8.8*;2H); 7.43 (d;*8.8*;2H); 7.61 (d;*15.9;*1H); ^{13}C NMR ($CDCl_3$) *δ* (ppm): 13.6; 18.9; 19.8; 27.3; 28.1; 36.9; 40.1 (2C); 45.0; 47.8; 48.9; 79.4; 111.8 (2C); 113.2; 123.0; 129.7 (2C); 144.7; 151.7; 168.2.

4-O-Benzoyl cinnamic acid (-)-bornyl ester (**6**). Yield 13%**;** lightly yellow solid; m.p. 93-95 °C; IR: 2951, 2878, 1695, 1627, 1600, 1510, 1255, 1171, 998 cm^{-1}; ^{1}H NMR ($CDCl_3$) *δ* (ppm) *J* (Hz): 0.88 (s;3H); 0.90 (s;3H); 0.95 (s;3H); 1.04-1.08 (m;1H); 1.25-1.37 (m;2H); 1.70 (t;*4.5*;1H); 1.73-1.84 (m;1H); 2.02-2.08 (m;1H); 2.37-2.45 (m;1H); 5.01 (ddd;*2.0*;*3.4*;*9.9*;1H); 5,10 (s;2H); 6.34 (d;*16.0*;1H); 7.00 (d;*8.7*;2H); 7.34-7.44 (m;5H); 7.49 (d;*8.7*;2H); 7.60 (d;*16.0*;1H); ^{13}C NMR ($CDCl_3$) *δ* (ppm): 13.5; 18.9; 19.7; 27.2; 28.1; 36.9; 45.0; 47.8; 48.9; 70.1; 79.8; 115.2 (2C); 116.5; 127.4 (2C); 127.5; 128.0; 128.7 (2C); 129.7 (2C); 136.5; 143.8; 160.4; 167.6.

Caffeic acid isobornyl ester (**7**). Yield 12%; brownish solid; m.p. 160-161 °C; IR: 3444, 3168, 2956, 1666, 1604, 1439, 1277, 1182 cm^{-1}; ^{1}H NMR ($CDCl_3$) *δ* (ppm) *J* (Hz): 0.86 (s;3H); 0.89 (s;3H); 1.05 (s;3H); 1.09-1.25 (m;2H); 1.55-1.62 (m;1H); 1.68-1.88 (m;4H); 4.80 (dd;*4.6*;*6.9*;1H); 6.23 (d;*15.9*;1H); 6.87 (d;*8.2*;1H); 6.99 (dd;1.7;*8.2*;1H); 7.12 (d;*1.7*;1H); 7.53 (d;*15.9*;1H); ^{13}C NMR ($CDCl_3$) *δ* (ppm) 11.2; 19.7; 19.8; 26.7; 33.4; 38.5; 44.8; 46.7; 48.6; 81.2; 114.1; 115.2; 115.7; 122.1; 127.1; 143.6; 144.5; 146.3; 167.4.

Isoferulic acid isobornyl ester (**8**). Yield 11%; colourless syrup; IR: 3387, 2952, 2876, 1696, 1631, 1591, 1512, 1263, 1154 cm^{-1}; ^{1}H NMR ($CDCl_3$) *δ* (ppm) *J* (Hz): 0.87 (s;3H); 0.90 (s;3H); 1.07 (s;3H); 1.09-1.26 (m;2H); 1.54-1.61 (m;1H); 1.68-1.90 (m;4H); 3.94 (s;3H); 4.80 (dd;*4.3*;*7.4*;1H); 6.25 (d;*15.9*;1H); 6.91 (d;*8.2*;1H); 7.02 (d;*1.9*;1H); 7.07 (dd;*1.9*;*8.2*;1H); 7.55 (d;*15.9*;1H); ^{13}C NMR ($CDCl_3$) *δ* (ppm) 11.5; 20.0; 20.1; 27.0; 33.8; 38.9; 45.1; 47.0; 48.9; 56.0; 81.0; 109.3; 114.7; 116.3; 123.0; 127.1; 144.3; 146.8; 147.8; 167.0.

4-Nitro cinnamic acid isobornyl ester (**9**)**.** Yield 16%; yellowish solid; m.p. 121-123 °C; IR: 2950, 2876, 1700, 1516, 1340, 1301, 1163, 842 cm^{-1}; ^{1}H NMR ($CDCl_3$) *δ* (ppm) *J* (Hz): 0.87 (s;3H); 0.90 (s;3H); 1.06 (s;3H); 1.07-1.25 (m;2H); 1.54-1.61 (m;1H); 1.70-1.92 (m;4H); 4.83 (dd;*4.1*;*7.5*;1H); 6.53 (d;*16.0*;1H); 7.65 (d;*16.0*;1H); 7.67 (d;*8,7*;2H); 8.24 (d;*8.7*;2H); ^{13}C NMR ($CDCl_3$) *δ* (ppm) 11.5; 20.0; 20.1; 27.0; 33.8; 38.8; 45.1; 47.0; 49.0; 81.8; 123.2; 124.1 (2C); 128.6 (2C); 140.7; 141.3; 165.6.

Caffeic acid thymyl ester (**10**). Yield 14%; brownish solid; m.p. 115-117 °C; IR: 3339, 2960, 1726, 1695, 1616, 1514, 1235, 1136, 1114 cm^{-1}; ^{1}H NMR ($CDCl_3$) *δ* (ppm) *J* (Hz): 1.20 (s;3H); 1.21 (s;3H); 2.32 (s;3H); 3.03 (sept;*6.9*;1H); 5.84 (br s;-OH); 5.89 (br s;-OH); 6.48 (d;*15.9*;1H); 6.87 (d;*8.2*;1H); 6.87 (d;*0.9*;1H); 7.04 (dd;*0.9*;*7.9*;1H); 7.07 (dd;*2.0*;*8.2*;1H); 7.22 (d;*7.9*;1H); 7.77 (d;*15.9*;1H); ^{13}C NMR ($CDCl_3$) *δ* (ppm) 20.5; 22.7 (2C); 26.9; 114.2; 114.4; 115.2; 122.4; 122.5; 126.2; 126.9; 127.0; 136.3; 136.9; 143.5; 146.3; 146.4; 147.6; 166.2.

Isoferulic acid thymyl ester (**11**). Yield 15%; colourless syrup; IR according to [16]; ^{1}H NMR ($CDCl_3$) *δ* (ppm) *J* (Hz): 1.21 (s;3H); 1.23 (s;3H); 2.32 (s;3H); 2.34 (s;3H); 3.05 (sept;*6.9*;1H); 3.96 (s;3H); 6.52 (d;*15.9*;1H); 6.89 (d;*0.9*;1H); 6.96 (d;*8.2*;1H); 7.05 (dd;*0.9*;*7.9*;1H); 7.11 (d;*1.9*;1H); 7.12 (dd;*1.9*;*8.2*;1H); 7.23 (d;*7.9*;1H); 7.81 (d;*15.9*;1H); ^{13}C NMR ($CDCl_3$) *δ* (ppm) 21.2; 23.4 (2C); 27.5; 56.3; 109.8; 114.9; 115.1; 123.2; 123.7; 126.7; 127.1; 127.4; 136.8; 137.5; 146.8; 147.1; 148.3; 148.6; 166.3.

Caffeic acid menthyl ester (**12**). Yield 25%; colourless syrup; IR: 3384, 2953, 2868, 1694, 1632, 1591, 1511, 1264, 1170 cm^{-1}; ^{1}H NMR ($CDCl_3$) *δ* (ppm) *J* (Hz): 0.78 (d;*6.9*;3H); 0.90 (d;*2.7*;3H); 0.90 (d;*2.7*;3H); 1.00-1.15 (m;2H); 1.42-1.55 (m;2H); 1.69-1.72 (m;2H); 1.87-1.95 (m;1H); 2.03-2.06 (m;1H); 4.8 (dt;*4.4*;*10.9*;1H); 6.25 (d;*15.9*;1H); 6.87 (d;*8.2*;1H); 6.99 (dd;*1.9*;*8.2*;1H); 7.11 (d;*1.9*;1H); 7.57 (d;*15.9*;1H); ^{13}C NMR ($CDCl_3$) *δ* (ppm) 16.8; 21.1; 22.4; 24.0; 26.8; 31.8; 34.7; 41.4; 47.6; 75.1; 114.8; 115.8; 116.2; 122.7; 127.8; 144.4; 145.4; 147.0; 168.2.

Isoferulic acid menthyl ester (**13**). Yield 24%; colourless syrup; IR: 3398, 2953, 2926, 2868, 1695, 1513, 1263, 1158 cm^{-1}; ^{1}H NMR ($CDCl_3$) *δ* (ppm) *J* (Hz) 0.79 (d;*6.9*;3H); 0.91 (d;*2.9*;3H); 0.92 (d;*2.6*;3H); 1.00-1.16 (m;2H); 1.40-1.55 (m;2H); 1.67-1.73 (m;2H);

1.90-1.99 (m;1H); 2.03-2.09 (m;1H); 3.93 (s;3H); 4.82 (dt;*4.4*;*10.9*;1H); 6.28 (d;*15.8*;1H); 6.91 (d;*8.2*;1H); 7.04 (d;*1.8*;1H); 7.07 (dd;*1.8*;*8.2*;1H); 7.60 (d;*15.8*;1H); ^{13}C NMR ($CDCl_3$) *δ* (ppm) 16.4; 20.8; 22.0; 23.6; 26.3; 31.4; 34.3; 41.1; 47.3; 55.9; 74.1; 109.2; 114.7; 116.1; 123.0; 127.1; 144.4; 146.7; 147.8; 166.8.

General Procedure for the Synthesis of Compounds **14**-**27**.

Cinnamic acid (500 mg, 3.37 mmol) and the respective alcohol (3.37 mmol) were dissolved in THF or $CHCl_3$ (20 mL). After addition of DCC and DMAP the resulting mixture was stirred for 16 h at room temperature. After evaporation and column chromatography on silica gel ($CHCl_3$/MeOH 4.8:0.2) the desired product was obtained.

2,3-Dichloro cinnamic acid (-)-bornyl ester (**14**). Yield 18%; colourless crystals; m.p. 86-87 °C; IR: 2930, 2875, 2118, 1711, 1635, 1315, 1178 cm^{-1}; ^{1}H NMR ($CDCl_3$) *δ* (ppm) *J* (Hz) 0.89 (s;3H); 0.90 (s;3H); 0.95 (s;3H); 1.05-1.09 (m;1H); 1.25-1.38 (m;2H); 1.72 (t;*4.5*;1H); 1.75-1.83 (m;1H); 2.00-2.06 (m;1H); 2.39-2.47 (m;1H); 5.03 (ddd;*2.2;3.4;9.9*;1H); 6.44 (d;*16.0*;1H); 7.22 (t;*7.9*;1H); 7.48 (dd;*1.5*;*7.9*;1H); 7.54 (dd;*1.5*;*7.9*;1H); 8.09 (d;*16.0*;1H); ^{13}C NMR ($CDCl_3$) *δ* (ppm) 13.2; 18.6; 19.4; 26.9; 27.7; 36.5; 44.6; 47.6; 48.6; 80.1; 122.3; 125.5; 127.0; 131.1; 132.6; 133.7; 134.9; 139.7; 166.2.

Cinnamic acid (-)-bornyl ester (**15**). Yield 7%; colourless syrup; Spectral data are in accordance with literature data [17,18].

4-Methoxy cinnamic acid (-)-bornyl ester (**16**). Yield 25%; crystalline solid; m.p. 87-90 °C; IR: 2952, 2929, 2117, 1701, 1627, 1602, 1513, 1152 cm^{-1}; ^{1}H NMR ($CDCl_3$) *δ* (ppm) *J* (Hz) 0.87 (s;3H); 0.88 (s;3H); 0.94 (s;3H); 1.03-1.08 (m;1H); 1.24-1.39 (m;2H); 1.70 (t;*4.5*;1H); 1.73-1.80 (m;1H); 2.02-2.08 (m;1H); 2.37-2.45 (m;1H); 3.84 (s;3H); 5.02 (ddd;*2.2;3.4;9.9*;1H); 6.34 (d;*15.9*;1H); 6.90 (d;*8.7*;2H); 7.49 (d;*8.7*;2H); 7.63 (d;*15.9*;1H); ^{13}C NMR ($CDCl_3$) *δ* (ppm) 13.2; 18.6; 19.4; 26.9; 27.7; 36.6; 44.7; 47.5; 48.6; 55.0; 79.4; 114.0 (2C); 116.0; 127.0; 129.3 (2C); 143.5; 160.9; 167.3.

2,4-Dichloro cinnamic acid (-)-bornyl ester (**17**). Yield 85%; colourless crystalline solid; m.p. 90-92 °C; IR: 2930, 2882, 2118, 1712, 1638, 1469, 1312, 1177 cm^{-1}; ^{1}H NMR

($CDCl_3$) *δ* (ppm) *J* (Hz): 0.90 (s;3H); 0.92 (s;3H); 0.97 (s;3H); 1.07-1.11 (m;1H); 1.27-1.40 (m;2H); 1.74 (t;*4.5*;1H); 1.76-1.85 (m;1H); 2.02-2.09 (m;1H); 2.41-2.49 (m;1H); 5.03 (ddd;*2.2;3.4;9.9*;1H); 6.44 (d;*16.0*;1H); 7.22 (t;*7.9*;1H); 7.48 (dd;*1.5*;*7.9*;1H); 7.54 (dd;*1.5*;*7.9*;1H); 8.09 (d;*16.0*;1H); ^{13}C NMR ($CDCl_3$) *δ* (ppm) 13.5; 18.9; 19.7; 27.2; 28.0; 36.8; 44.9; 47.9; 49.0; 80.4; 121.8; 127.5; 128.3; 130.0; 131.4; 135.5; 136.2; 138.8; 166.5.

Cinnamic acid isobornyl ester (**18**). Yield 14%; colourless syrup at room temperature; m.p. 42-43 °C; IR: 2952, 2877, 1708, 1637, 1309, 1160 cm^{-1}; ^1H NMR ($CDCl_3$) *δ* (ppm) *J* (Hz) 0.88 (s;3H); 0.90 (s;3H); 1.07 (s;3H); 1.09-1.24 (m;2H); 1.55-1.62 (m;1H); 1.68-1.91 (m;4H); 4.81 (dd;*4.3*;*7.3*;1H); 6.41 (d;*16.0*;1H); 7.36-7.40 (m;3H); 7.00-7.54 (m;2H); 7.63 (d;*16.0*;1H); ^{13}C NMR ($CDCl_3$) *δ* (ppm) 11.5; 20.0; 20.1; 27.0; 33.8; 38.9; 45.1; 47.0; 48.9; 81.1; 118.9; 128.0 (2C); 128.8 (2C); 130.1; 134.5; 144.2; 167.0.

Cinnamic acid thymyl ester (**19**). Yield 24%; colourless solid; m.p. 64 °C; spectral data are in accordance with literature [19].

Cinnamic acid menthyl ester (**20**). Yield 25%; colourless crystals; m.p. 51-53 °C; spectral data are in accordance with literature [20-22].

Cinnamic acid adamantyl ester (**21**). Yield 10%; colourless powder; m.p. 66 °C [lit.[23]: 63-65 °C]; IR: 2896, 2866, 2848, 1703, 1687, 1641, 1170 cm^{-1}; ^1H NMR ($CDCl_3$) *δ* (ppm) *J* (Hz) 1.70 (m;6H); 2.20 (s;9H); 6.36 (d;*15.9*;1H); 7.36-7.38 (m;3H); 7.49 (m;2H); 7.57 (d;*15.9*;1H); ^{13}C NMR ($CDCl_3$) *δ* (ppm) 30.9 (3C); 36.2 (3C); 41.4 (3C); 80.6; 102.4; 128.0; 128.8; 129.9; 134.7; 143.4; 166.0.

Cinnamic acid naphthyl ester (**22**). Yield 15%; colourless crystalline solid; m.p. 109-110 °C [lit.[19] 106-107 °C]; spectral data are in accordance with literature [19].

Cinnamic acid cyclohexyl ester (**23**). Yield 33%; colourless liquid; spectral data are in accordance with literature [24-26].

Cinnamic acid bisabolyl ester (**24**). Yield 11%; yellowish syrup; IR: 2962, 2924, 1703, 1636, 1160 cm^{-1}; ^{1}H NMR ($CDCl_3$) *δ* (ppm) *J* (Hz) 1.28-1.33 (m;1H); 1.41 (s;3H); 1.53 (s;3H); 1.58 (s;3H); 1.59 (s;3H); 1.74-2.00 (m;9H); 5.04 (br m;1H); 5.31 (br m;1H); 6.32 (d;*16.0*;1H); 7.29-7.32 (m;3H); 7.43-7.46 (m;2H); 7.52 (d;*16.0*;1H); ^{13}C NMR ($CDCl_3$) *δ* (ppm) 17.6; 20.6; 22.1; 23.3; 23.7; 25.7; 26.4; 30.9; 35.7; 40.5; 87.3; 120.1; 120.3; 124.1; 128.0 (2C); 128.8 (2C); 130.0; 131.6; 134.1; 134.7; 143.5; 166.1.

Cinnamic acid eugenyl ester (**25**). Yield 50%; colourless plates; all data are in accordance with literature [16,27].

Cinnamic acid geranyl ester (**26**). Yield 28%; colourless liquid; spectral data are in accordance with literature [28].

*Synthesis of Phenyl Propanoic Acid (-)-Bornyl Ester (**27**).*

Phenyl propanoic acid (700 mg, 4.66 mmol) was dissolved in 3.2 mL NEt_3 (5 eq.) and cooled in an ice bath. 114 mg (0.2 eq.) 4-dimethylaminopyridine in 2 mL CH_3CN and 1.07 g tosyl chloride (1.2 eq.) in 3 mL CH_3CN were added and the solution stirred for 30 min until a red colour developed. Then 719 mg (4.66 mmol) of (-)-borneol were dissolved in 2 mL CH_3CN and added to the solution. The mixture was allowed to warm up to room temperature and stirred for two hours. After evaporation of the solvent the reaction mixture was suspended in 10 mL of water and extracted three times with 10 mL of diethyl ether. The organic phase was washed three times each with water, brine and Na_2SO_4. After drying the organic phase over $MgSO_4$ the product was purified twice by column chromatography on silica gel using ethyl acetate/petroleum ether (60:40) as eluent to yield 278 mg of **27**. Yield 21%; colourless liquid; IR: 2952, 2877, 1730 cm^{-1}; ^{1}H NMR (MeOD) *δ* (ppm) *J* (Hz) 0.78 (s;3H); 0.89 (s;3H); 0.91 (s;3H); 0.84-0.88 (m;2H); 1.14-1.20 (m;1H); 1.26-1.34 (m;1H); 1.63 (t;*4.5*;1H); 1.72-1.80 (m;1H); 1.87-1.94 (m;1H); 2.26-2.34 (m;1H); 2.67 (t;*7.4*;2H); 2.95 (t;*7.4*;2H); 4.84 (m;1H); 7.11-7.29 (m;5H); ^{13}C NMR (MeOD) *δ* (ppm) 13.8; 19.2; 20.1; 28.1; 28.9; 32.2; 37.0; 37.7; 46.3; 48.8; 49,7; 81.4; 127.3; 129.4 (2C); 129.5 (2C); 141.8; 175.0.

*Synthesis of 3-(3,4-Bis(acetyloxy))phenyl Propenoic Acid (-)-Bornyl Ester (**28**).*

Compound **1** (20 mg) was suspended in 2 mL pyridine at 0 °C. One equivalent of acetic acid anhydride was added and the solution stirred for 2 h at room temperature. After evaporation the residue was subjected to column chromatography on silica gel using $CHCl_3$/MeOH (4.8:0.2 *v/v*) as eluent to yield the desired product. Yield 51%; orange syrup; IR: 2953, 2877, 1771, 1707 cm^{-1}; ^1H NMR ($CDCl_3$) *δ* (ppm) *J* (Hz) 0.87 (s;3H); 0.90 (s;3H); 0.94 (s;3H);); 1.03-1.07 (m;1H); 1.25-1.35 (m;2H); 1.71 (t;*4.5*;1H); 1.75-1.81 (m;1H); 1.99-2.05 (m;1H); 2.30 (s;3H); 2.31 (s;3H); 2.37-2.47 (m;1H); 5.01 (ddd;*2.1;3.4;9.9*;1H); 6,41 (d;*16.0*;1H); 7.22 (d;*8.4*;1H); 7.38 (d;*2.0*;1H); 7,42 (dd;*2.0*;*8.4*;1H); 7.60 (d;*16.0*;1H); ^{13}C NMR ($CDCl_3$) *δ* (ppm) 13.5; 18.9; 19.7; 20.6; 20.7; 27.2; 28.0; 36.8; 45.0; 47.9; 49.0; 80.2; 120.0; 122.7; 123.9; 126.3; 133.5; 142.2; 142.4; 143.4; 166.9; 168.0; 168.1.

Biological Assays

Materials and methods for the biological assays have been described before by the authors. AlamarBlue assays for investigation of antileishmanial activities against *L. major* promastigotes, amastigotes and J774.1 and BMDM cytotoxicity were conducted as previously reported [12]. The protocol for the *L. donovani* quantitative colorimetric assay is outlined by Hazra et al. [13]. The IC_{50} values are presented as mean values of two independent experiments against the parasite and macrophages.

Conclusions

In summary, caffeic acid bornyl ester (**1**) was isolated as the antileishmanial component of the chloroform extract of *V. wallichii* rhizome. Structure-activity relationships of the compound library of 27 derivatives were analysed and revealed the size of the bornyl moiety to govern the antileishmanial activity. The hydroxyl groups in 3 and 4 position of the aromatic ring increase cytotoxicity and the Michael system in the side chain is not essential for antiprotozoal activity (Table 2). The cinnamic acid bornyl ester **15** showed the best activity with regard to *L. major* and *L. donovani* promastigotes with acceptable cytotoxicity. Compound **27** with no Michael acceptor moiety was almost as active as

compound **15.** Since **27** is less toxic it presents an attractive new lead structure derived from nature for further optimization.

Additionally esters of caffeic and ferulic acid under certain conditions may face the problem of limited bioavailability because of the possible formation of a polar phenolate ion which prevents the compound from penetrating the cell membrane. By omitting the hydroxyl groups and thereby increasing the lipophilicity this problem should be avoided. Since bornyl hydroxycinnamic esters have been shown to inhibit the trypanosomal rhodesain [29] it is tempting to speculate whether they are able to inhibit the corresponding leishmanial proteases. Preliminary experiments using *L. major* promastigote full-lysate in a cysteine-cathepsin fluorescence activity assay [12,30] showed protease inhibitory activity of compound **1** and **15** and, thus, point to this target.

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Author Contributions

The listed authors contributed to this work as described in the following. Jan Glaser fractionated the plant extract and isolated and identified the antileishmanial compound. He also carried out the synthesis and prepared the manuscript. As corresponding author for infection biology Uta Schurigt together with Martina Schultheis conducted assays against *L. major* amastigotes and promastigotes and helped interpreting the results. Sudipta Hazra and Banasri Hazra were in charge of the *L. donovani* promastigote assays. As corresponding author for medicinal chemistry Ulrike Holzgrabe proposed the subject and monitored the progress of the ongoing research. Banasri Hazra, Heidrun Moll and Ulrike Holzgrabe contributed with valuable discussions and scientific input. All authors helped preparing the manuscript and approved the final version.

Conflicts of Interest

The authors declare no conflict of interest.

References and Notes

1. Alvar, J.; Vélez, I.D.; Bern, C.; Herrero, M.; Desjeux, P.; Cano, J.; Jannin, J.; den Boer, M., Leishmaniasis worldwide and global estimates of its incidence. *PLOS ONE* **2012**, *7*, e35671.
2. Desjeux, P., Leishmaniasis: current situation and new perspectives. *Comp. Immun. Microbiol. Infect. Dis.* **2004**, *27*, 305-318.
3. Hotez, P.; Singh, S.K.; Zhou, X.-N., Advancing Sino-Indian cooperation to combat tropical diseases. *PLoS Neglected Tropical Diseases* **2013**, *7*, e2204.
4. Ghosh, S.; Debnath, S.; Hazra, S.; Hartung, A.; Thomale, K.; Schultheis, M.; Kapkova, P.; Schurigt, U.; Moll, H.; Holzgrabe, U., *Valeriana wallichii* root extracts and fractions with activity against *Leishmania* spp. *Parasitol. Res.* **2011**, *108*, 861-871.
5. Xia, C.-N.; Li, H.-B.; Hu, W.-X., Synthesis of *trans*-caffeate analogues and their bioactivities against HIV-1 integrase and cancer cell lines. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 6553-6557.
6. Xia, C.-N.; Hu, W.-X., Synthesis of caffeic acid esters. *J. Chem. Res.* **2005**, 332-334.
7. Bohlmann, F.; Zdero, C., Neue Terpen-Inhaltsstoffe aus *Verbesina*-Arten. *Phytochemistry* **1976**, *15*, 1310-1311.
8. Toyota, M.; Saito, T.; Matsunami, J.; Asakawa, Y., A comparative study on three chemo-types of the liverwort *Conocephalum Conicum* using volatile constituents. *Phytochemistry* **1997**, *44*, 1265-1270.
9. Suire, C.; Asakawa, Y.; Toyota, M.; Takemoto, T., Chirality of terpenoids isolated from the liverwort *Conocephalum Conicum*. *Phytochemistry* **1982**, *21*, 349-352.
10. Ogungbe, I.V.; Crouch, R.A.; Haber, W.A.; Setzer, W.N., Phytochemical investigation of *Verbesina turbacensis* Kunth: trypanosome cysteine protease inhibition by (-)-bornyl esters. *Nat. Prod. Commun.* **2010**, *5*, 1161-1166.

11. Bohlmann, F.; Lonitz, M., Natürlich vorkommende Terpen-Derivate, 109: Neue Eudesman-Derivate und andere Sesquiterpene aus Verbesina-Arten. *Chemische Berichte* **1978**, *111*, 254-263.
12. König, B.; Dustmann, J., The caffeoylics as a new family of natural antiviral compounds. *Naturwissenschaften* **1985**, *72*, 659-661.
13. Michalet, S.; Cartier, G.; David, B.; Mariotte, A.-M.; Dijoux-franca, M.-G.; Kaatz, G.W.; Stavri, M.; Gibbons, S., *N*-Caffeoylphenalkylamide derivatives as bacterial efflux pump inhibitors. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 1755-1758.
14. Otero, E.; Robledo, S.M.; Díaz, S.; Carda, M.; Muñoz, D.; Paños, J.; Vélez, I.D.; Cardona, W., Synthesis and leishmanicidal activity of cinnamic acid esters: structure–activity relationship. *Med. Chem. Res.*, in press, DOI: 10.1007/s00044-00013-00741-y.
15. Wakasugi, K.; Iida, A.; Misaki, T.; Nishii, Y.; Tanabe, Y., Simple, mild, and practical esterification, thioesterification, and amide formation utilizing *p*-toluenesulfonyl chloride and *N*-methylimidazole. *Adv. Synth. Catal.* **2003**, *345*, 1209-1214.
16. Bringmann, G.; Thomale, K.; Bischof, S.; Schneider, C.; Schultheis, M.; Schwarz, T.; Moll, H.; Schurigt, U., A novel *Leishmania major* amastigote assay in 96-well format for rapid drug screening and its use for discovery and evaluation of a new class of leishmanicidal quinolinium salts. *Antimicrob. Agents Chemother.* **2013**, *57*, 3003-3011.
17. Hazra, S.; Ghosh, S.; Debnath, S.; Seville, S.; Prajapati, V.K.; Wright, C.W.; Sundar, S.; Hazra, B., Antileishmanial activity of cryptolepine analogues and apoptotic effects of 2,7-dibromocryptolepine against *Leishmania donovani* promastigotes. *Parasitol. Res.* **2012**, *111*, 195-203.
18. Aponte, J.C.; Castillo, D.; Estevez, Y.; Gonzalez, G.; Arevalo, J.; Hammond, G.B.; Sauvain, M., *In vitro* and *in vivo* anti-leishmania activity of polysubstituted synthetic chalcones. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 100-103.
19. Jensen, S.; Omarsdottir, S.; Bwalya, A.G.; Nielsen, M.A.; Tasdemir, D.; Olafsdottir, E.S., Marchantin A, a macrocyclic bisbibenzyl ether, lisolated from the liverwort *Marchantia polymorpha*, inhibits protozoal growth *in vitro*. *Phytomedicine* **2012**, *19*, 1191-1195.
20. Rawel, H.M.; Rohn, S., Nature of hydroxycinnamate-protein interactions. *Phytochem. Rev.* **2010**, *9*, 93-109.

21. Tawata, S.; Taira, S.; Kobamoto, N.; Zhu, J.; Ishihara, M.; Toyama, S., Synthesis and antifungal activity of cinnamic acid esters. *Biosci. Biotech. Biochem.* **1996**, *60*, 909.
22. Wu, D.; Nair, M.G.; DeWitt, D.L., Novel compounds from *Piper Methysticum* Forst (Kava-kava) roots and their effect on cyclooxygenase enzyme. *J. Agric. Food Chem.* **2002**, *50*, 701-705.
23. Monteiro, A.L.; Lando, V.R.; Gasparini, V., Synthesis of chiral 2-arylpropenoic esters by the palladium catalyzed carbonylation of arylacetylenes. *Synth. Commun.* **1997**, *27*, 3605-3611.
24. Palermo, V.; Ruiz, D.M.; Autino, J.C.; Vázquez, P.G.; Romanelli, G.P., Simple halogen-free synthesis of aryl cinnamates using Mo-Keggin heteropoly acids as catalyst. *Pure Appl. Chem.* **2012**, *84*, 529.
25. Meth-Cohn, O., Transesterification of methyl esters of aromatic and α,β-unsaturated acids with bulky alcohols: (-)-menthyl cinnamate and (-)-menthyl nicotinate. *Org. Synth.* **1990**, *68*, 155-159.
26. Ye, S.; Tang, Y.; Dai, L.-X., Highly diastereoselective synthesis of vinylcyclopropane derivatives with (-)-8-phenylmenthol as chiral auxiliary. *J. Org. Chem.* **2001**, *66*, 5717-5722.
27. Sakakura, A.; Kawajiri, K.; Ohkubo, T.; Kosugi, Y.; Ishihara, K., Widely useful DMAP-catalyzed esterification under suxiliary base- and solvent-free conditions. *J. Am. Chem. Soc.* **2007**, *129*, 14775-14779.
28. Mikhajlovich, G.B.; Pastukhova, N.P.; Kamneva, E.A., Method of producing adamantyl esters of unsaturated acids. *RU 2448950 C1* **2012**.
29. Shi, Z.H.; Li, N.G.; Shi, Q.P.; Tang, Y.P., Design, synthesis, and preliminary evaluation of substituted cinnamic acid esters as selective matrix metalloproteinase inhibitors. *Drug Dev. Res.* **2012**, *73*, 317-324.
30. Gupta, A.K.; Reddy, S.A.D.; Boomishankar, R., Facile formation of stable tris(imido)phosphate trianions as their tri-and hexanuclear Pd(II) complexes in protic solvents. *Inorg. Chem.* **2013**.
31. Sova, M.; Perdih, A.; Kotnik, M.; Kristan, K.; Rižner, T.L.; Solmajer, T.; Gobec, S., Flavonoids and cinnamic acid esters as inhibitors of fungal 17β-hydroxysteroid dehydrogenase: A synthesis, QSAR and modelling study. *Bioorg. Med. Chem.* **2006**, *14*, 7404-7418.

32. Mali, R.S.; Papalkar, A.S., Useful synthesis of aryl cinnamates: synthesis of difengpin. *J. Chem. Res.* **2003**, *2003*, 603-604.
33. Jakupovic, J.; Pathak, V.P.; Bohlmann, F.; King, R.M.; Robinson, H., Obliquin derivatives and other constituents from Australian *Helichrysum* species. *Phytochemistry* **1987**, *26*, 803-807.
34. Schurigt, U.; Schad, C.; Glowa, C.; Baum, U.; Thomale, K.; Schnitzer, J.K.; Schultheis, M.; Schaschke, N.; Schirmeister, T.; Moll, H., Aziridine-2,3-dicarboxylate-based cysteine cathepsin inhibitors induce cell death in *Leishmania major* associated with accumulation of debris in autophagy-related lysosome-like vacuoles. *Antimicrob. Agents Chemother.* **2010**, *54*, 5028-5041.

Sample Availability: Samples of the compounds **1**-**28** are available from the authors.

3.3. Cinnamic Acid Bornyl Ester Derivatives from *Valeriana wallichii* Exhibit Antileishmanial *In Vivo* Activity in *Leishmania major*-Infected BALB/c Mice

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Abstract

Human leishmaniasis covers a broad spectrum of clinical manifestations ranging from self-healing cutaneous leishmaniasis to severe and lethal visceral leishmaniasis caused among other species by *Leishmania major* or *Leishmania donovani*, respectively. Some drug candidates are in clinical trials to substitute current therapies, which are facing emerging drug-resistance accompanied with serious side effects. Here, two cinnamic acid bornyl ester derivatives (**1** and **2**) were assessed for their antileishmanial activity. Good selectivity and antileishmanial activity of bornyl 3-phenylpropanoate (**2**) *in vitro* prompted the antileishmanial assessment *in vivo*. For this purpose, BALB/c mice were infected with *Leishmania major* promastigotes and treated with three doses of 50 mg/kg/day of compound **2**. The treatment prevented the characteristic swelling at the site of infection and correlated with reduced parasite burden. Transmitted light microscopy and transmission electron microscopy of *Leishmania major* promastigotes revealed that compounds **1** and **2** induce mitochondrial swelling. Subsequent studies on *Leishmania major* promastigotes showed the loss of mitochondrial transmembrane potential ($\Delta\Psi m$) as a putative mode of action. As the cinnamic acid bornyl ester derivatives **1** and **2** had exhibited antileishmanial activity *in vitro*, and compound **2** in *Leishmania major*-infected BALB/c mice *in vivo*, they can be regarded as possible lead structures for the development of new antileishmanial therapeutic approaches.

Introduction

Human leishmaniasis is a vector-borne parasitic disease that is caused by more than 20 species of the protozoan genus *Leishmania* [1]. The term “leishmaniasis” covers a variety of clinical manifestations ranging from self-healing to lethal infections. Cutaneous leishmaniasis (CL) causes substantial morbidity with an estimated annual incidence of 800.000 cases, whereas the visceral form of leishmaniasis (VL) is responsible for about 300.000 new infections per year with a total mortality rate of 30.000 [2]. Currently, the few clinically approved drugs against human leishmaniasis are becoming more unreliable due to severe side effects [3] and the appearance of drug resistance problems [4,5]. Therefore, there is an urgent need for new therapeutic approaches against the most common forms of leishmaniasis.

Following the identification of antileishmanial lead compounds by means of *in vitro* assays, well established animal models are usually chosen to investigate the potential of drug candidates *in vivo*. BALB/c mice have been extensively used, mainly due to their susceptibility to a variety of *Leishmania* species, and particularly for *Leishmania* (*L.) major* infection studies [6,7].

For the last few years, many naturally occurring compound classes such as chalcones and quinolines were found to have antileishmanial activity *in vitro* and in experimental mouse models [8,9]. Recently, a semi-pure fraction of a chloroform extract of *Valeriana* (*V.*) *wallichii* with significant antileishmanial activities and with IC_{50} values lower than 10 µg/mL in addition to an apparent apoptosis-like cell death induction were identified [10]. Further fractionation resulted in caffeic acid bornyl ester exhibiting good antileishmanial activity against *L. major* promastigotes [11]. This finding prompted the synthesis of a library of related compounds in order to establish structure-activity relationships (SAR). In general, compounds having a brenzcatechin moiety were found to be active, but also cytotoxic against host cells due to oxidation and formation of a highly reactive Michael system, whereas analogue compounds without the 3,4-dihydroxy substitution at the phenyl ring (e.g. compound **1**) were still active but less toxic [12]. Furthermore, the typical Michael system of the caffeic acid moiety could be omitted to obtain an active but non-toxic phenylpropanoic acid ester **2** [12].

The present study aims to determine the effect of compound **2** treatment on CL *in vivo* and to elucidate the cell death mechanism induced by both the compounds (**1** and **2**) in

L. major parasites. Our results suggest that the approach of using bornyl caffeate isolated from *V. wallichii* and related derivatives is efficient to identify and optimize antileishmanial lead structures showing *in vitro* and *in vivo* activity towards *L. major* in BALB/c mice.

Materials and Methods

*Synthesis of Compounds **1** and **2***

(-)-Bornyl cinnamate (**1**) and (-)-bornyl 3-phenylpropionate (**2**) were synthesized as described previously [12].

Leishmania Strains and Cultivation Methods

The cloned virulent *L. major* parasite (strain: MHOM/IL/81/FE/BNI) was maintained by continuous passage in female BALB/c mice (Government of Lower Franconia, Germany, permission number: 55.2-2531.01-26/12). *L. major* amastigotes were isolated from lesions as described previously [13] and promastigotes were grown *in vitro* in blood-agar cultures at 27 °C, 5% CO_2, and 95% humidity.

Luciferase-transgenic (Luc-tg) *L. major* has been generated as described previously [14]. The virulence of Luc-tg *L. major* was maintained by passage in female BALB/c mice and promastigotes were grown *in vitro* in blood agar cultures with addition of 50 µg/mL hygromycin B using the same conditions as described above.

L. donovani promastigotes (strain: MHOM/IN/1983/AG83) were obtained from the Indian Institute of Chemical Biology, Kolkata, India. Promastigotes were cultured in Schneider's insect medium (Sigma-Aldrich Co., St. Louis, USA) as described elsewhere [15].

In Vitro Antileishmanial Activity Against Leishmania Parasites and Cytotoxicity Studies

Antiparasitic activities of compounds **1** and **2** dissolved in dimethyl sulfoxide (DMSO, E. Merck, India) against *L. donovani* (AG83) promastigotes were determined by a quantitative colorimetric assay using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sisco Research Laboratory, Mumbai, India) test as described elsewhere [16]. The compounds were tested at concentrations of 10 µM,

50 µM, and 100 µM and IC_{50} values (concentration of compound, which inhibited at least 50% cell metabolic activity) for each compound were determined from respective dose–response curves using Origin 5.0 software (Microcal Software, Inc., Northampton, MA, USA).

The activity of the two tested compounds **1** and **2** against *L. major* parasites was evaluated using the colorimetric AlamarBlue® assay as described previously [17]. The compounds were tested at increasing concentrations ranging from 0.1 µM - 100 µM for 24 h. AlamarBlue® was added for additional 48 h and the optical density was measured with Multiskan Ascent enzyme-linked immunosorbent assay (ELISA) reader (Thermo Electron Corporation, Dreieich, Germany) using a test wavelength of 540 nm and a reference wavelength of 630 nm. Absorbance in the absence of compounds was set as 100% parasite growth.

A detailed protocol of antileishmanial investigations against intracellular *L. major* amastigotes residing within bone marrow-derived macrophages (BMDM) is described elsewhere [14]. Briefly, 2×10^5/ml BMDM were infected with Luc-tg *L. major* promastigotes at a ratio of 1:15 for 24 h. These infected BMDM were cultured in the absence or presence of increasing concentrations of the tested compounds **1** and **2** for 24 h prior to the supplement of Britelite™ plus (PerkinElmer, Waltham, MA, USA) for 5 min. Britelite™ plus is a very sensitive assay for the quantification of firefly luciferase in *Leishmania* parasites [14]. The luminescence was measured using a Victor X Light 2030 luminometer (PerkinElmer, Waltham, MA, USA).

BMDM were generated in complete RPMI medium as described previously [14]. On day six, the cytotoxicity against 2×10^5 cells/ml was tested at increasing concentrations of compounds **1** and **2** for 48 h. Human embryonic kidney HEK 293 T cells (ATCC®, Wesel, Germany) were cultured in Dulbecco´s Modified Eagle Medium (DMEM) (Life Technologies, Darmstadt, Germany), with 10% FCS (PAA Laboratories, Germany). According to the standard operating procedures (SOP) within the Collaborative Research Center 630 (SFB 630) 2×10^4 HEK 293 T cells/ml were tested at increasing concentrations of compounds **1** and **2** for 48 h. Human liver carcinoma HepG2 cells (ATCC®) were cultured in RPMI medium (Gibco) with 10% FCS. 1×10^5 cells/ml HepG2 were tested at increasing concentrations of **1** and **2** for 72 h according to the SOPs. The AlamarBlue® assay was performed as described above for *L. major* parasites.

2.4 Ethics Statement

All *L. major* animal experiments were designed and performed in strict accordance with the German Animal Welfare Act (TierSchG) according to the experimental guidelines and procedures approved by the government of Lower Franconia, Germany (permission number: 55.2-2531.01-25/13). The *L. major* parasite (strain: MHOM/IL/81/FE/BNI) was originally isolated from an Israeli patient with oriental sore in 1981 and was received from the Bernhard-Nocht-Institute, Hamburg (Germany) [18].
The *L. donovani* parasite (strain: BHU 1216) was originally isolated from a North Indian VL patient unresponsive to treatment with sodium stibogluconate and was received as a generous gift from Prof. Shyam Sundar, Institute of Medical Sciences, Banaras Hindu University, India [19].

Mice

Naïve female inbred BALB/c mice weighing 16-18 g were purchased (Charles River Breeding Laboratory, Sulzfeld, Germany), housed in groups of four and were given five days to acclimate prior to treatment. At the onset of the *L. major* infection studies the mice were 6-8 weeks of age. All mice were kept under specific pathogen-free conditions in individually ventilated cages (IVC). Environmental conditions were a temperature of 21 °C ±2 °, humidity of 50% ±10%, lighting of 60 lux and a 12:12 light: dark cycle with lights on at 7 a.m. and off at 7 p.m. Animals were housed in 391×199×160 mm cages (Techniplast GmbH, Hohenpeißenberg, Germany; Seal Safe PLUS cages X-TEMP PPSU) and given access to mouse maintenance food (ssniff® Spezialdiaeten GmbH, Soest, Germany; R/M-H maintenance food) and water *ad libitum*. Environmental enrichment included bedding (Abedd LAB and VET Service GmbH, Vienna, Austria; Espe-classic – H0234-40), and one handful of paper tissue nesting material. During housing, animals were monitored daily for health status. No adverse events were observed.

In Vivo Antileishmanial Activity Against L. major

Female BALB/c mice (11 per group; three groups in total) were infected with 2×10^5 *L. major* promastigotes in 30 µL phosphate-buffered saline (PBS) into the right hind footpad under isoflurane inhalation anaesthesia using the UniVet Porta system

(Groppler, Deggendorf, Germany) [7]. The clinical manifestation of *L. major* infection was monitored twice a week by determination of the body weight (g) and footpad size (mm) of the infected (iFP) and the non-infected footpad (niFP) serving as negative control. The footpad swelling describes the difference in mm between the iFP and niFP of individual mice. Three weeks post infection (p.i.) the mice were randomly divided into three groups of 11 for different treatment regimens. On day 21, 25, and 28 p.i. the respective groups were either left untreated, treated intraperitoneally (i.p.) with 100 µl of solvent (infected, 50% DMSO/PBS) or 100 µl 50 mg/kg/day of compound **2** in 50% DMSO/PBS. This high dose of compound **2** was chosen to identify its antileishmanial *in vivo* efficacy in *L. major*-infected BALB/c mice. The close monitoring of body weight and footpad swelling was continued until the end of experiment. On day 35 p.i. mice were sacrificed by CO_2 inhalation and single cell suspensions from the iFP, draining popliteal lymph nodes (pLN) and spleens were obtained. The parasite burden was determined by limiting dilution assays as described previously [20]. It was not necessary to apply any analgesics or anaesthetics during the animal trials. Compound **2** did not have any strong side effects.

*Cultivation Conditions for Compound **1**- and Compound **2**-Induced Phenotypic Changes in L. major Promastigotes*

L. major parasites harvested from blood agar cultures were washed twice with Dulbecco's phosphate-buffered saline (DPBS; Invitrogen, Darmstadt, Germany) and centrifuged at 3.000 × g for 10 min. The pellet was suspended in RPMI 1640 medium at a final concentration of 1×10^8 cells/ml. Cells were treated with 1% DMSO, 122.7 µM miltefosine (1-hexadecylphorylcholine; apoptosis inducer), or 100 µM of the tested compounds **1** and **2** in a final volume of 200 µl in 96 well plates. Samples were incubated at 27 °C in 5% CO_2 for several time points between 30 min and 24 h. Transmitted light microscopic, transmission electron microscopic (TEM), and flow cytometric techniques were used to find phenotypic patterns caused by the treatment with the tested compounds.

Diff-Quik Staining for Transmitted Light Microscopy

After incubation for 30 min, 1 h, 2 h, 4 h, and 24 h in the presence of the tested compounds **1** and **2,** promastigotes were harvested and centrifuged in a Cytospin 3 centrifuge (Shandon, Frankfurt, Germany) on microscope slides at 253 × g for 5 min at room temperature (RT). Cytospin preparations of cells were stained using the Differential Quick Stain (Diff-Quik) dye (Medion Diagnostics AG, Duedingen, Switzerland), according to the manufacturers' protocol with some modifications. Diff-Quik stains the leishmanial nuclei and the kinetoplasts dark purple and the cytoplasm light purple allowing the observation of phenotypic changes within the parasite. Briefly, air-dried cells were fixed by dipping the slides 5 times for 3 seconds in fixative solution. Subsequently the slides were stained with Solution I following Solution II (from Diff-Quik kit) and air-dried between each staining steps. Finally, the samples were rapidly dipped once in ultra-pure water (produced by a TKA purification system apparatus, Niederelbert, Germany) to remove excessive staining solutions. The air-dried cells were analyzed by transmitted light microscopy under a 50× objective on a Nikon ECLIPSE 50*i* microscope equipped with a digital camera (Nikon, Tokyo, Japan). The images were processed using NIS Elements D software (Nikon).

Transmission Electron Microscopy (TEM)

L. major promastigotes were incubated for 2 h in the presence of DMSO, 100 µM of compound **1** and compound **2**, respectively. After the incubation period, the parasites were harvested, contrasted and embedded following the procedure described previously [21]. Ultrathin sections were mounted on 300-mesh grids, stained with uranyl acetate and lead citrate, and analyzed with an EM 10 transmission electron microscope (Carl Zeiss AG, Oberkochen, Germany). To describe morphological changes in a statistical manner, six photographs with approximately 50 cells were taken and the percentage of cells with swollen mitochondria per treatment was determined.

MitoTracker® Staining of L. major Promastigotes

L. major promastigotes were harvested from blood-agar cultures and washed twice with DPBS (900 × g for 5 min at RT). 2 × 10^6 promastigotes were dissolved in 180 µl RPMI complete medium and seeded in a 96 well plate. Parasites were incubated with 20 µL

of compound **1** (final concentration: 100 µM), compound **2** (final concentration: 100 µM) and with 20 µL of DMSO, respectively, for 30 min, 45 min, 60 min, or 120 min at 27 °C in a 5% CO_2. After incubation 2 µl of MitoTracker® Red CMXRos (final concentration: 100 mM) (Life Technologies, Darmstadt, Germany) was added for 15 min at 27 °C. Samples were directly acquired using a MACSQuant Analyzer (Miltenyi Biotech, Bergisch Gladbach, Germany) and MitoTracker®-positive parasites were analyzed using MACS Quant Analyzing software.

Determination of the Cell Death Phenotype by Flow Cytometric Analysis

The loss of membrane integrity and translocation of phosphatidylserine (PS) to the outside of the cellular membrane can be determined by annexin V (AV) and propidium iodide (PI) staining. Double staining with AV-fluorescein isothiocyanate (FITC) and PI during flow cytometry analysis leads to discrimination between four populations: double negatives or alive cells (AV^-/PI^-), double positive or late necrotic/late apoptotic cells (AV^+/PI^+), PI-positive and AV-negative or early necrotic cells (AV^-/PI^+), and PI-negative and AV-positive or early apoptotic cells (AV^+/PI^-) [22,23].

Parasites were harvested after incubation for 6 h, 10 h, and 24 h in the presence of compounds **1**, **2** and miltefosine (Cayman Chemical Company, MI, USA), respectively, the latter serving as positive control for the induction of apoptotic cell death. DMSO was used as solvent control. Cell staining was performed using an AV-FITC Apoptosis detection kit (Sigma-Aldrich, Saint Louis, MO, USA). Cells were harvested, washed and suspended in 500 µL of binding buffer, 5 µL of AV-FITC and 10 µl of PI. The cells were incubated for 10 min in the dark at RT. Finally, stained samples were immediately analyzed by flow cytometry using MACSQuant analyzer or a flow cytometer (Becton Dickinson, San José, CA, USA).

Statistical Analyses

Data were analyzed using the GraphPad Prism 5 (San Diego, USA) software and values are given as mean ± standard deviation (SD). Footpad swelling of *Leishmania*-infected mice as well as the parasite burden of infected tissues is displayed per individual mouse with 11 mice per group. The unpaired *t*-test was used to compare two independent groups. Differences were considered significant at **, $p<0.005$.

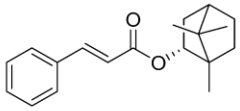
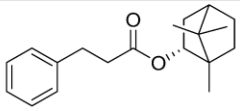
Results

*Compounds **1** and **2** Revealed Antileishmanial Activity against L. major and L. donovani*

For the initial evaluation of the antileishmanial activity of compounds **1** and **2**, a MTT reduction and an AlamarBlue® assay with *L. donovani* and *L. major* parasites were employed, respectively. The cytotoxicity effects of the compounds were investigated in BMDM, HEP G2, and HEK 293T cells (Fig. 1). Compound **1** showed the best activity against *L. donovani* promastigotes as well as *L. major* amastigotes and promastigotes (Fig. 1). The half maximal inhibitory concentrations (IC_{50}) for compound **1** were between 39.6 µM against *L. major* promastigotes and 10.9 µM against *L. major* amastigotes, being in the range of miltefosine used as a positive control with an IC_{50} value of 36.2 µM against promastigotes and 33 µM against amastigotes [12]. The cytotoxicity was variable for the three tested cells up to the highest dose of 100 µM.

50% of growth inhibition was not evident after treatment of *L. donovani* promastigotes with the bornyl 3-phenylpropanoate (compound **2**) with a dose up to 100 µM, but after treatment of *L. major* promastigotes and amastigotes at 50.2 µM and 89.1 µM, respectively.

Interestingly, no cytotoxicity effects against host cell lines were found for the three cell types up to 100 µM, whereas miltefosine displayed an IC_{50} value of 65.5 µM against BMDM.

	Cytotoxicity (µM)					
	L. donovani promastigotes	*L. major* promastigotes	*L. major* amastigotes	BMDM	HEP G2	HEK 293T
 (-)-bornyl cinnamate (1)	15.6	39.6	10.9	54.3	> 100	83.1
 (-)-bornyl 3-phenylpropanoate (2)	> 100	50.2	89.1	> 100	> 100	> 100

IC_{50}: Concentration with 50% growth inhibition. IC_{50} values for positive control: Miltefosine 36.2 µM (*L. major* promastigotes), 33.0 µM (*L. major* amastigotes), 65.5 µM (BMDM).

Fig.1: Determination of cytotoxicity and IC_{50} values against *Leishmania* parasites.

The determination of IC_{50} values showed strain-specific differences. The cytotoxicity against host cells in compound **1**-treated cells was reduced after omitting the Michael system from this compound resulting in compound **2**, with a moderate activity against *L. major* promastigotes and amastigotes.

*Compound **2** Exhibited Antileishmanial Activity against L. major Infection in BALB/c Mice*

Since compound **2** exhibited no detectable cytotoxicity against the tested mammalian cell types but showed antileishmanial activity against *L. major* parasites only (Fig. 1), the effect of this compound on *L. major*-infected BALB/c mice was investigated. As the translation from *in vitro* findings to *in vivo* efficacy is the bottleneck in successful drug research, an experimental *Leishmania*-infection model was chosen to provide evidence for their antileishmanial activity *in vivo* [24]. Severe swelling and lesion development at the site of infection with ascending parasite dissemination into organs during the onset of infection characterizes this CL infection model [25]. An established CL infection in non-healing BALB/c mice can be observed from week three post infection and is characterized by significant footpad swelling and elevated parasite burden [7]. To investigate the antileishmanial effect in an already established *L. major* infection, compound **2** was assessed for its activity in *L. major*-infected female BALB/c mice (Fig. 2). Consequently, the treatment regimen was started on day 21 with repetitions on days 25 and 28 p.i. to evaluate the efficacy of a short-term treatment regimen [26]. *L. major*-infected BALB/c mice treated i.p. with 50 mg/kg/day of compound **2** on days 21, 25, and 28 showed reduced footpad swelling compared to untreated or solvent control-treated mice (Fig. 2A). Significant reduction in footpad swelling was already achieved after the second administration of compound **2** on day 25 compared with the footpad swelling of untreated or solvent-treated mice. The triple administration of compound **2** resulted in a reduction of the foot pad swelling, indicating a recovering phenotype upon 28 days post infection. In contrast, an ongoing infection as observed in untreated or solvent-treated mice was accompanied with an increasing footpad swelling. Infected but untreated BALB/c mice reflected the manifestations of experimental CL, characterized by footpad swelling of ~3 mm difference between infected and non-infected footpad (Fig. 2A) and significant loss of body weight (Fig. 2B) after five weeks post infection.

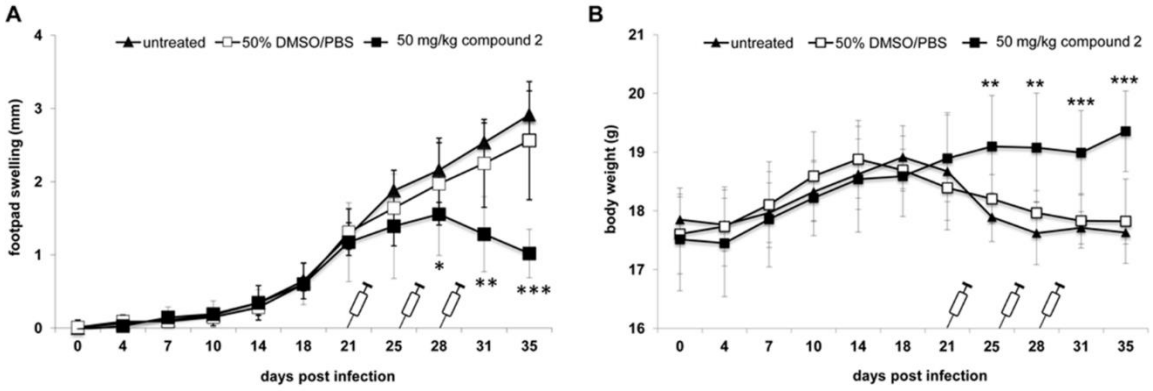


Fig.2. Compound 2 treatment mediates recovery from *L. major*-induced disease progression in BALB/c mice. Female BALB/c mice (total: n = 33) were infected with 5 × 10^6 *L. major* promastigotes into the right hind footpad. On d 21 post infection BALB/c mice were either treated intraperitoneally with 50 mg/kg compound **2** (■) (n = 11), 50 % DMSO/PBS (□) (n = 11) or left untreated (▲) (n = 11). Individual treatment was repeated on d 25 and d 28 post infection. **A.** Footpad swelling (difference between infected and non-infected footpad in mm) and **B.** body weight (g) was determined twice a week to monitor clinical manifestation of CL. n = number of animals.

Mice treated with the solvent control showed also clinical manifestations with footpad swelling and loss of body weight, similar to untreated mice. The clinical manifestation and progression of leishmaniasis in the two control groups was also associated with loss of body weight, whereas the body weight of infected mice treated with compound **2** was not affected during the treatment regimen (Fig. 2B).

Progressing *Leishmania* infection in BALB/c mice is associated with elevated parasite burdens within infected tissues and organs, whereas a recovering phenotype is associated with reduced parasite levels. Limiting dilutions assays (LDA) confirmed high parasite burden in infected footpads (Fig. 3A), at the site of infection-draining popliteal lymph nodes (Fig. 3C), and spleens (Fig. 3D) of diseased mice. The recovering phenotype in compound **2**-treated BALB/c mice correlated with a ~100 fold reduced parasite burden within the infected footpads, a ~10.000 fold reduced parasite burden within the popliteal lymph nodes (Fig. 3C). The parasite burden in the spleen was only numerically reduced, due to a low sample number (Fig. 3D). The photographic documentation of three exemplary infected footpads per group demonstrated the clear difference between a compound **2**-treated recovering phenotype and the diseased footpads of control mice at the end of the experiment five weeks post infection (Fig. 3B).Taken together, the short-term treatment of *L. major*-infected BALB/c mice with compound **2** was successful in terms of the observed recovery of footpad swelling and parasite burdens compared to untreated mice (Fig. 2 and 3).

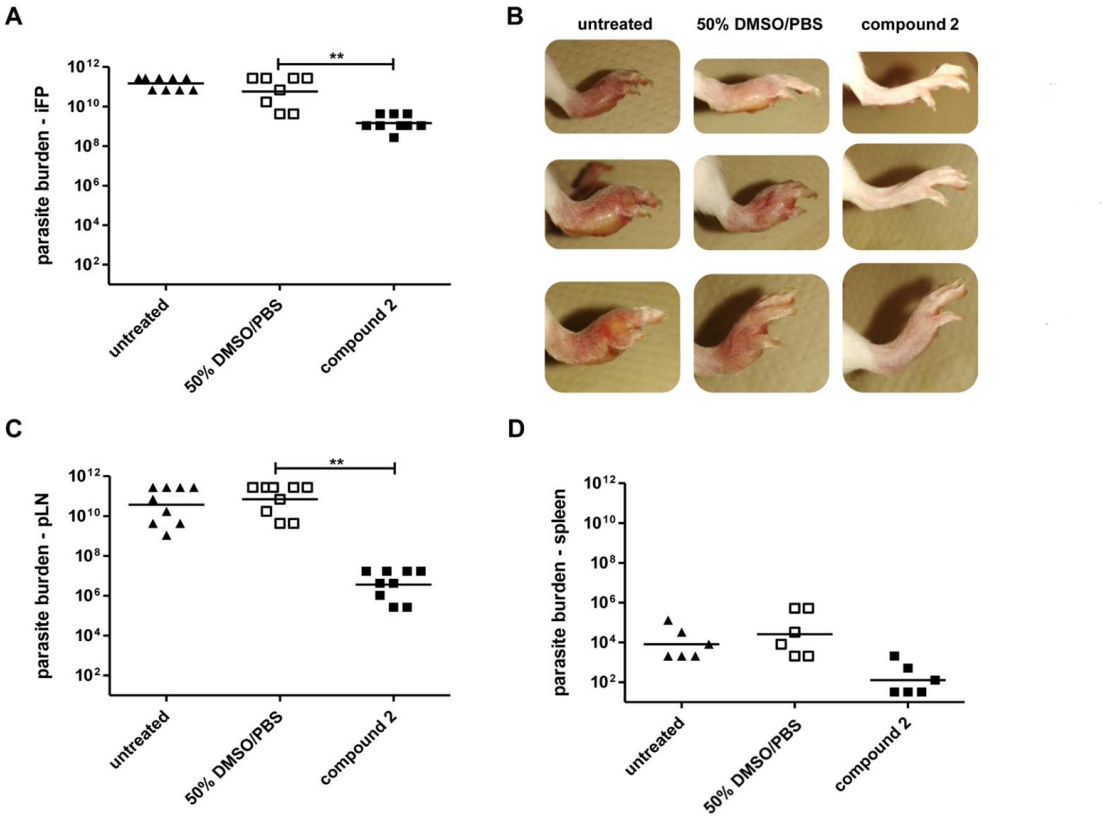


Fig.3. Compound 2 treatment of *L. major*-infected BALB/c mice is associated with reduced parasite burden in infected tissues and organs. Female BALB/c mice (n = 33) were infected with 5 × 10^6 *L. major* promastigotes into the right hind footpad. Treatment regimen started on d 21 post infection either using 50% DMSO/PBS (□) (n = 11), 50 mg/kg compound **2** (■) (n = 11) or mice were left untreated (▲) (n = 11). **A.** At the end of experiment on d 35 all mice (n = 33) were sacrificed and the parasite burden of the infected footpads (iFP) (n = 9) was determined using Limited Dilution Assays (LDA). **B.** Photographic documentation of the infected footpads of three exemplary mice per group shall reveal the severity of disease progression in non-healing mice and the control of clinical manifestation in compound **2**-treated BALB/c mice. **C.** The parasite burden of the infection site-draining popliteal lymph nodes (pLN) of individual mice (n = 9) or **D.** spleens of individual mice (n = 6) was determined using LDA. n = number of animals; ** p<0.005; differences in parasite load between 50% DMSO/PBS-treated and compound **2**-treated animals did not reach statistical significance for **D**.

*Rapid Changes in Cell Morphology and Induction of Mitochondrial Swelling were Mediated by Compounds **1** and **2** in L. major Promastigotes*

Since IC_{50} values and cytotoxicity assays for compounds **1** and additionally *in vivo* efficiency for compound **2** revealed antileishmanial activity and relatively good selectivity, the effect of these two compounds against *L. major* promastigotes was further investigated. First, potential changes of the cell morphology induced by the compounds **1** and **2** were studied by means of transmitted light microscopy. The visual examination of promastigotes incubated with compounds **1** and **2** revealed that the early

rounding of cell shape during the first hour of incubation became more prominent over time when compared to control cells (Fig. 4). Beside changes in the cell shape of compound-treated promastigotes, the formation of intracellular vacuole-like structures has also been observed within the first hour leading to larger aggregates of cells in vacuolization processes compared to control cells (intracellular vacuoles are better shown in insets of Fig. 4). After treatment with miltefosine, compound **1** and **2** for 24 h rounding of shape and shrinkage of almost all cells was observed. No morphological changes were

evident in the control cells along the 24 h incubation period (Fig. 4). Whereas the apoptosis inducer miltefosine promoted morphological changes and cell disruption after 2 h of treatment, compounds **1** and **2** induced vacuolization and changes in cell morphology. This phenotype was observable in compound **1**-treated promastigotes just after 30 min and in compound **2**-treated promastigotes after 1 h. The antileishmanial effect of compound **1** and **2** as seen by *in vitro* and *in vivo* studies together with the transmission light microscopic investigations identified the potential mode of action against *L. major* parasites as a result of changes in the cell morphology and formation of vacuole-like structures within the parasite. The observation of prominent vacuoles and swelling of parasites prompted the elucidation of the origin of those vacuoles by investigating the ultrastructure using TEM. Compared to DMSO-treated promastigotes showing typical morphological characteristics (Fig. 5A), parasites treated with compound **1** or **2** displayed large intracellular vacuoles (Fig. 5B and 5C) as seen in the light microscopic pictures above (Fig. 4). *Leishmania* belongs to the class of *Kinetoplastida*, which are characterized by the presence of a DNA-containing kinetoplast within the giant single mitochondrion being usually localized near the flagellar pocket of the parasite [27]. Considering this, it can be postulated that compound **1** and **2** induced alterations in the ultrastructure of the mitochondria in *L. major* promastigotes, as the kinetoplast DNA was clearly visible within the observed vacuoles (Fig. 5B and 5C). A difference in percentage of swollen mitochondria in compound- or DMSO-treated parasites was noted. None of the DMSO-treated parasites displayed swollen mitochondria, whereas compound **1** and **2** induced mitochondrial swelling in 39% or 36% of counted parasites. after 2 h, respectively. Additionally, compound **1**- and **2**-treated promastigotes (Fig. 5B and 5C) displayed a slimmer cell body compared to the DMSO-treated control parasites (Fig. 5A).

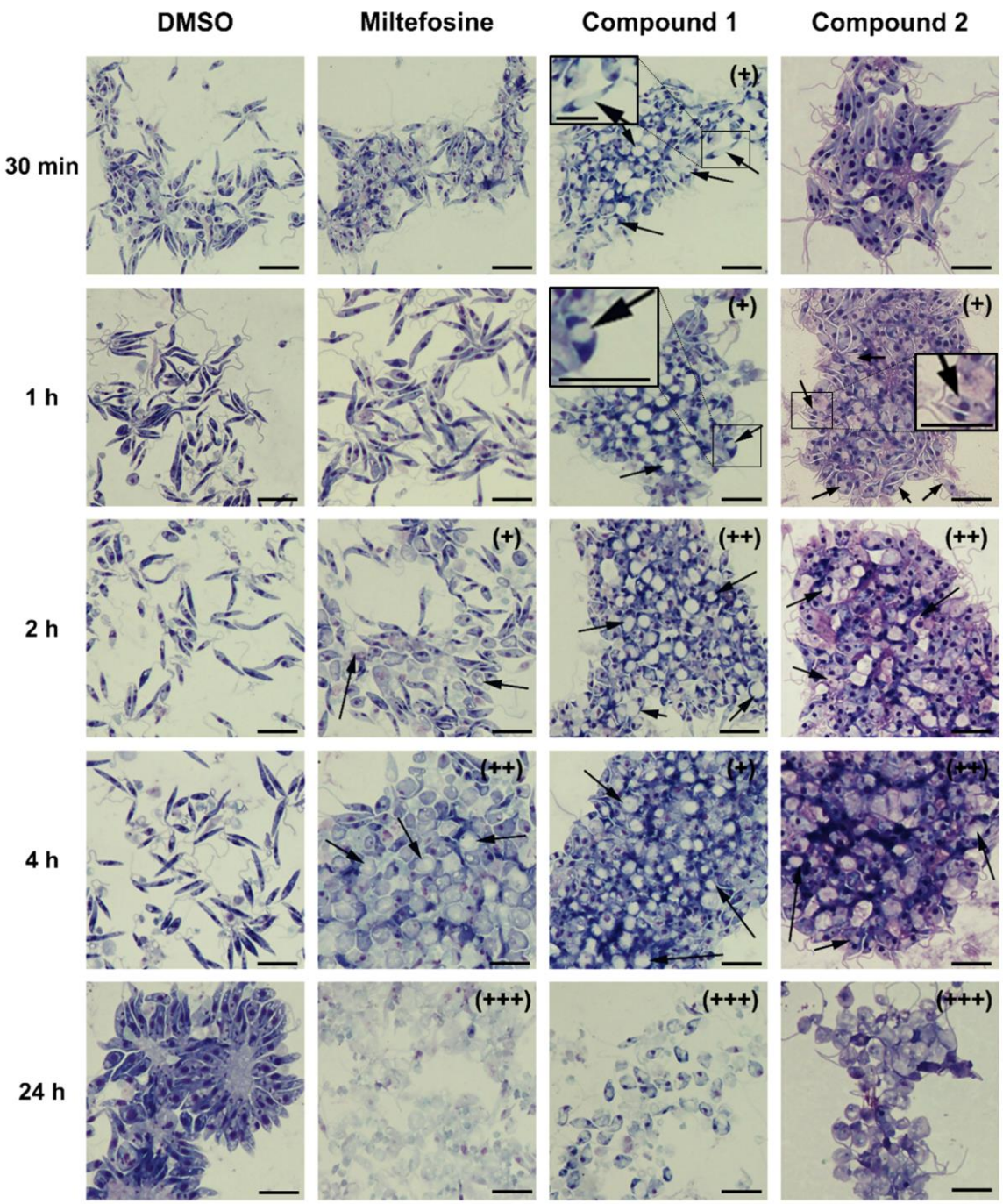


Fig.4. **Changes in cell morphology in *L. major* promastigotes upon treatment.** Compound **1** and **2** induce parasite swelling and vacuolization of *L. major* promastigotes at very early time points. *L. major* promastigotes were treated with solvent control (1% DMSO), miltefosine (122.7 μM), compound **1** (100 μM), and **2** (100 μM) for 30 min, 1 h, 2 h, 4 h, and 24 h. Cytospin preparations of the cells were stained with Diff-Quik dye and analyzed by transmitted light microscopy under 50× objective. "Plus" symbol (+) was used to represent changes in morphology; two (++) and three (+++) symbols were used as a reference for the severity of the phenotype induced by the different tested compounds and the black arrows to indicate the presence of vacuole-like structures. Black bar indicates 20 μm.

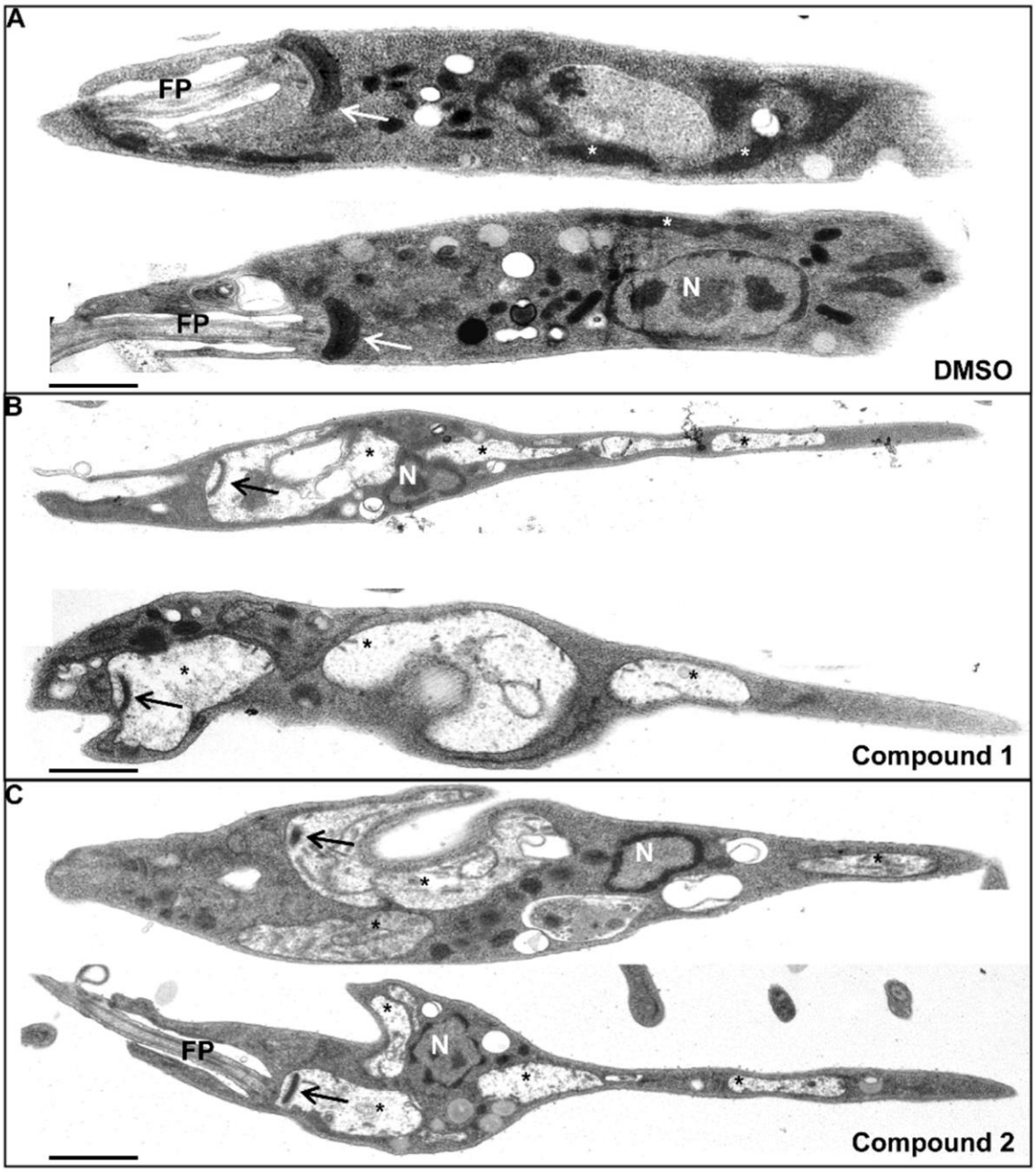


Fig.5. Treatment with compound 1 or 2 induces mitochondrial swelling in *L. major* promastigotes. Ultrastructural alterations of mitochondria in compound **1**- and compound **2**-treated *L. major* promastigotes were detected by TEM. **A.** *L. major* promastigotes were incubated for 2 h with DMSO-containing RPMI medium and served as solvent control. **B** and **C** show TEM pictures of *L. major* promastigotes treated for 2 h with 100 µM compound **1** or 100 µM compound **2**, respectively. Two TEM pictures of characteristic parasites are shown per sample. FP, flagellar pocket; N, nucleus; arrow, kinetoplast; white asterisk, mitochondrion; black asterisk, swollen mitochondria. Black bar indicates 1 µm.

The origin of this phenotype is unclear and must be investigated in further studies. So far, the leishmanicidal mode of action of the compounds **1** and **2** is most probably due to alterations of the mitochondrial ultrastructure through marked swelling and loss

of matrix content, resulting in cell death of the *L. major* parasite. Other organelles within the parasite seemed not to be affected by these two compounds, as i.e. the nucleus and other contrasted organelles showed typical morphology.

*Antileishmanial Activity of Compounds **1** and **2** was Attributed to Changes in the Mitochondrial Transmembrane Potential of L. major*

The mitochondrial transmembrane potential was investigated as compound-induced mitochondrial dysfunction would lead to cell death of *Leishmania* parasites and could explain the antileishmanial activity of the compounds **1** and **2** tested against *L. major*. For this reason, *L. major* promastigotes were treated either with DMSO or with compound **1** or **2** for 30 min, 45 min, 60 min, and 120 min prior to MitoTracker® Red CMXRos fluorometric staining (Fig. 6). As the accumulation of MitoTracker® Red CMXRos indicates functional mitochondria, alterations of its function were detected over time in compound-treated *L. major* promastigotes. A significant drop in MitoTracker®-positive cells has been observed in compound **1**-treated parasites compared to parasites treated with DMSO that were set as 100% functional mitochondria (Fig. 5). The reduction of MitoTracker®-positive cells after 60 min to 75% indicated alterations of mitochondrial functions that are becoming more prominent after 120 min of compound **1** treatment (63%).
Similar to these observations, also compound **2**-treatment altered the mitochondrial function of the parasite, as after 60 min and 120 min 75.2% and 60.2% MitoTracker®-positive cells were observed, respectively (Fig. 6). Differences between DMSO- and compound-treated promastigotes have been observed as early as 60 min. As MitoTracker® Red CMXRos stains the mitochondria of live cells and its accumulation depends on the mitochondrial transmembrane potential it can be concluded that treatment with these compounds mediated a membrane depolarization in *L. major* promastigotes as a decreased accumulation of MitoTracker® was observed. These results support the hypothesis that the antileishmanial activities of the compounds tested in the present study are mitochondria-associated as a significant depolarization of the mitochondrial transmembrane potential was observed in compound-treated *L. major* promastigotes.

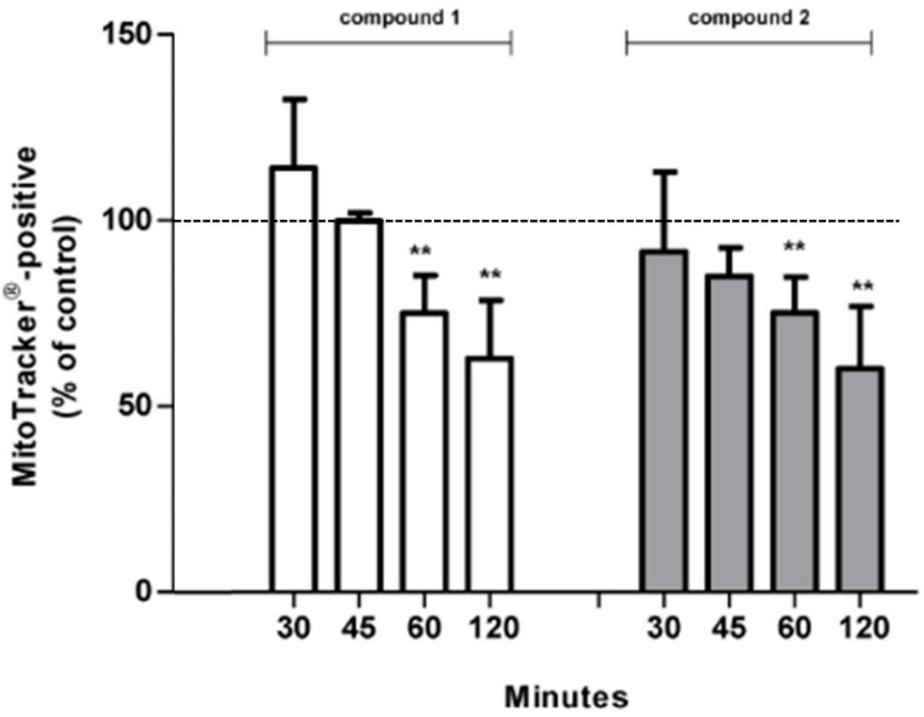


Fig.6. The tested compounds 1 and 2 affect mitochondrial integrity and membrane potential. Mitochondrial integrity of compound-treated *L. major* promastigotes was investigated using flowcytometric determination of MitoTracker® Red CMXRos accumulation within active organelles. *L. major* promastigotes were incubated in 1% DMSO or treated with 100 µM compound **1** or **2** for indicated time points prior to MitoTracker® Red CMXRos staining. MitoTracker-positive cell populations were determined by histograms analysis and were presented as percentage of control cells (set as 100%, inserted line).

*Loss of Cell Membrane Integrity in L. major Promastigotes Because of Compound **1**- and **2**-Treatment*

The type of cell death induced by the compounds **1** and **2** was investigated using flow cytometric approaches. Since the translocation of PS to the outside of the cellular membrane without simultaneous loss of membrane integrity represents one of the features of early apoptosis in mammalian cells [28], staining with AV (binds to PS) and PI (DNA-binding) was applied to discriminate between four *Leishmania* populations as defined elsewhere [29]: live (AV^-/PI^-), necrotic/late apoptotic (AV^+/PI^+), necrotic (AV^-/PI^+) and early apoptotic cells (AV^+/PI^-) using per definition miltefosine-treated parasites as positive control for apoptosis induction in *Leishmania* [30].

After exposure of *L. major* promastigotes to bornyl cinnamate (**1**) and bornyl 3-phenylpropanoate (**2**) for 24 h, a percentage of only 33% and 21% cells were alive (AV^-/PI^-), respectively, whereas at the same time under normal growth conditions 71%

and 79% of cells were unaffected und alive (Fig. 7). Compound-mediated cell death was found to increase over time. Compound **1** treatment for 24 h resulted in increased cell permeability in 30% of all cells being characterized as necrotic (AV^-/PI^+) whereas additional binding of AV to the parasites surface was observed in 33% of all treated cells, leading to necrotic/late apoptotic (AV^+/PI^+) cells (Fig. 7). These phenotypes increased over time, especially between the late time points from 10 h to 24 h of incubation. Interestingly, compound **2** induced a broad spectrum of phenotypes; after 24 h of treatment 24% of all cells showed per definition a necrotic (AV^-/PI^+), 19% per definition an early apoptotic (AV^+/PI^-), and 37% per definition a necrotic/late apoptotic cell death phenotype (AV^+/PI^+) (Fig. 7). These phenotypic effects increased over time and were predominant after 24 h of incubation.

The presence of miltefosine induced a strong affinity of the parasitic surface towards AV in 88% of the *L. major* promastigotes within 24 h. Between 6 h and 24 h a substantial shift from 8% to 13% of early apoptotic cells (AV^+/PI^-) and a shift from 45% to 75% of necrotic/late apoptotic cells (AV^+/PI^+), respectively, was observed (Fig. 7). Miltefosine is described as an inducer of early apoptosis in *L. major* using AV as read out [30]. Here, miltefosine induced a broad spectrum of cell deaths with a predominant late necrotic/late apoptotic phenotype (AV^+/PI^+).

In summary, investigations in *L. major* promastigotes treated with bornyl cinnamate (**1**) and bornyl 3-phenylpropanoate (**2**) for 24 h revealed their antileishmanial *in vitro* activity. Per definition, compound **1** induced a necrotic cell death phenotype and compound **2** a miltefosine-like cell death being per definition associated with elevated levels of early apoptotic cells. The mechanism underlying the antileishmanial activity of compound **1** and **2** is associated with mitochondria deformation and inhibition of its transmembrane potential with the consequence of cell death as defined by the loss of membrane integrity in *L. major* promastigotes.

Discussion

Although leishmaniasis is one of the infectious diseases causing 20.000 to 30.000 deaths each year, it still belongs to the tropical neglected diseases [1]. Indeed, there is a huge clinical need, but no adequate treatment is available against leishmaniasis,

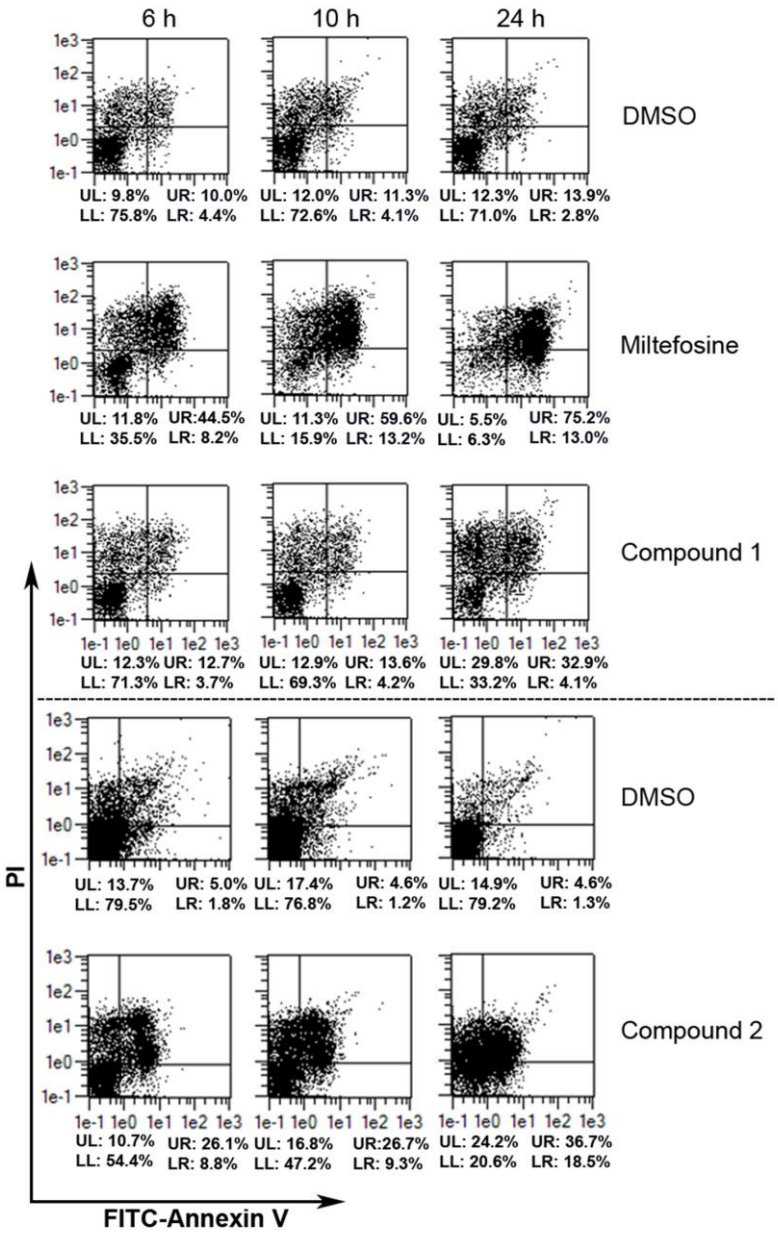


Fig. 7. Compound-associated cell death is time-dependent. *L. major* promastigotes were treated with solvent control (1% DMSO), apoptosis inducer miltefosine (122.7 µM), compound **1** (100 µM), and **2** (100 µM) in a time course experiment for 6 h, 10 h, and 24 h. Cells were harvested, stained with AV and PI and subsequently analyzed by flow cytometry. Analysis for compound **2** was performed in an independent experiment. Percentage for each quadrant is written below the corresponding dot blot figure. Lower-left (LL): live cells (AV^-/PI^-); upper-left (UL): early necrotic cells (AV^-/PI^+); upper-right (UR): necrotic/late apoptotic cells (AV^+/PI^+); and lower-right (LR): early apoptotic cells (AV^+/PI^-).

mainly because the efficacy of the chemotherapy is limited by the continuous development of drug resistance to the first-line drugs [4]. Traditionally, different cultures around the world have been using native plants for treatment of systemic forms of leishmaniasis through oral administration of the crude extracts, while the cutaneous

infections have been treated with topical preparations of the same. Therefore, the development of new antileishmanial therapies could involve the use of compounds present in plants of the endemic zones of leishmaniasis.

The majority of drug candidates with promising *in vitro* activity are either toxic or not efficient in experimental animal models. Those models are widely used to evaluate the antileishmanial efficacy of a new treatment with regard to the route of drug administration in correlation to the site of infection, and the treatment regimen. Among a pool of animal models, mostly mice are used for antileishmanial drug studies [31]. Some of the advantages of using inbred mice are the high reproducibility of the infection, the need of only few amounts of the tested drugs due to the small weight of the mice, and the contemporary evaluation of the drug efficacy within 2-5 weeks. *L. major* infection studies in BALB/c mice are most commonly chosen in drug discovery studies against experimental CL, as BALB/c mice are highly susceptible to the infection with no spontaneous recovery [32]. The cutaneous manifestation of a *L. major* infection is accompanied by footpad swelling at the site of infection. A reduction of swelling after drug administration is frequently associated with therapeutic elimination of parasites in the footpad and therefore used as first clinical readout for drug efficacy. However, foot pad swelling characterizes only indirectly the therapeutic effect. Theoretically, the reduction of inflammation can be uncoupled from the parasite burden in the infected footpad. Therefore, it is very important to determine the parasite burden directly within the infected foot pad and the inner organs of mice by LDA assay.

During this study we showed for the first time that phenylpropanoic acid bornyl ester (**2**) was efficient to treat *Leishmania* infections *in vivo*. Compound **2** tested against *L. major* in BALB/c mice was highly efficient, as the susceptible BALB/c mice showed a healing phenotype with reduced footpad swelling and parasite burdens. After a promising healing phenotype was observed in mice treated with compound **2** and the lack of apparent side effects *in vivo*, we further investigated the antileishmanial mechanism of action by *in vitro* approaches.

We first investigated cell morphological changes in promastigotes of *L. major* upon treatment with the compounds **1** and **2** and compared them with the effect induced by miltefosine. Presence of vacuole-like structures was evident for *L. major* promastigotes treated with the tested compounds and a massive cell death was visible after 24 h of incubation as compared to miltefosine-treated parasites.

Moreover, TEM revealed that the vacuole-like structures present in promastigotes of *L. major* after treatment with compound **1** and **2** corresponded to swollen mitochondria containing the kinetoplast DNA. Previous studies have shown that after treatment of *L. major* promastigotes and amastigotes with licochalcone A, a structurally similar constituent of roots and rhizomes of different plants, mitochondria of the cells were swollen as observed by electron microscopy [33]. Recent studies demonstrated that the effect of many antileishmanial drug candidates led to mitochondrial destabilization of the *Leishmania* parasite [34,35]. These findings support the approach of the identification and evaluation of mitochondria-targeting drug candidates, as mitochondrial integrity and functionality is essential for *Leishmania* survival [36,37].

The two compounds tested in the present study were found to induce cell death in *L. major* promastigotes through a combination of multiple features, such as shrinking of cells, mitochondrial swelling and loss of cell membrane integrity. Cell death is mainly defined by either the loss of cell membrane integrity as demonstrated by the incorporation of the vital dye PI or the degradation into distinct apoptotic bodies and vesicles [38]. The loss of membrane integrity has been shown by PI incorporation into compound **1-** and **2-**treated *L. major* promastigotes whereas apoptotic body formation within the parasites was not observed by TEM. Regulated cell death mechanism like apoptosis is described for mammalian cells based on the specificity of AV to its ligand PS [39], consequently serving as a specific marker for apoptosis. It is controversially discussed whether AV is a suitable marker for regulated cell death in *Leishmania* parasites [40,41]. One reason for this concern is that PS was not yet identified in membrane fractions and lipid extracts, if at all in very low levels of *L. major* promastigotes [42]. Furthermore, it has been shown that PS is not the only ligand for AV, but also phospholipids like phosphatidylethanolamine and phosphatidylinositol are reported to be recognized by AV on the cell surface of *Leishmania* [42]. Additional investigations on molecular mechanisms could define appropriate *Leishmania*-specific readouts for regulated or unregulated cell death mechanisms [40]. Our flow cytometry analysis data suggested that compound **1**- and **2**- treated *Leishmania* promastigotes underwent apoptosis and necrosis, respectively. A similar result with both kinds of cell death was observed after treatment with miltefosine, which is a well-known apoptosis inducer in *Leishmania* parasite [30]. However, there is an ample discussion if an apoptotic cell death really exists in *Leishmania* parasites. Regarding this controversy it has been suggested by Proto and colleagues [40] to classify protozoan cell death as

unregulated cell death as long as no molecular signaling mechanism proves regulated cell death processes. Taking these concerns into account, the antileishmanial activity of the compounds **1** and **2** leading to cell death is definitely based on severe mitochondrial alterations and the loss of the mitochondrial transmembran potential ($\Delta\Psi_m$).

Beside target-based drug design, plant-derived compounds with antileishmanial activity have been described. Extracts from various plants like *Croton cajucara* [43,44], *Zanthoxylum chiloperone* [45], *Chinese licorice* [46], and *Pera benensis* [47] have proven *in vitro* and *in vivo* activities against different strains of *Leishmania*. Apparently, the cytotoxicity of naturally derived compounds against host cells represents one obstacle of drug development [48]. *V. wallichii*-derived extracts possess a broad range of antimicrobial activity against e.g. *Staphylococcus aureus, Bacillus subtilis*, and *Escherichia coli* [49,50]. Antiparasitic activity of *V. wallichii* chloroform root extracts was explored in studies against *Leishmania*, where significant antileishmanial activity against *L. donovani* and *L. major* promastigotes and intracellular *L. major* amastigotes was identified [10]. Leaf extracts from *Pluchea spp.*, belonging to the class of *Asterids* like *V. wallichii*, inhibited the proliferation of *L. amazonensis in vitro* and the i.p. administration of 100 mg/kg/day is sufficient to prevent lesion development and to reduce parasite burden *in vivo* [51].

Furthermore, plants synthesize chalcones which are structurally related to the cinnamic acid derivatives tested in our study. Chalcones are precursors for flavonoids which are part of the biological defense mechanisms against plant pathogens [52]. Chalcones show antibacterial [53], antimalarial [54] and antileishmanial [55] activities among others. *In vitro*, the antileishmanial activity of chalcones isolated from *Chinese licorice* against *L. major* and *L. donovani* is related to mitochondrial alterations [56]. Synthetic analogues of chalcone isolates of *Crotalaria ramosissima* show enhanced *in vitro* and *in vivo* activity as compared to the natural chalcone [57]. Antileishmanial *in vivo* activity of chalcones is demonstrated against *L. donovani* infections in hamster and against the predominant causative agent of leishmaniasis in Latin America, *L. braziliensis* [55,56]. Natural or synthesized compounds with structural similarities, like cinnamic acid derivatives and chalcones, show activity against a broad range of *Leishmania* parasites, indicating their potential as lead structures for the development of chemotherapeutic approaches against *Leishmania* infections. However, the structure of chalcones and

cinnamic acid derivatives comprises a reactive Michael system, which is prone to unselective attacks by nucleophilic groups of proteins e.g. thiol groups of the amino acid cysteine or glutathione, thereby being able to give false-positive activity which is regarded as a PAINS problem [58].

To summarize, the process of the identification and evaluation of the antileishmanial compounds **1** and **2** started with the fractionation of crude root extracts from *V. wallichii*, a well-known plant used in traditional medicine. A semi-pure fraction of a chloroform extract was found to exhibit leishmanicidal *in vitro* activity against *L. major* and *L. donovani* [10]. Subsequent studies identified caffeic acid bornyl ester with antileishmanial *in vitro* activity [11]. SAR studies prompted the synthesis of less toxic but leishmanicidal compounds relying on the originally extracted structure from *V. wallichii* [12]. To eliminate possible unselective effects due to the reactive Michael system of cinnamic acid, compound **2** was synthesized without this moiety. Thus, cytotoxicity was reduced while antileishmanial activity was preserved. The successful evaluation of this compound in an experimental mouse model against *L. major* infections is an important step in the lead optimization and development. Preliminary studies in *L. donovani* infected BALB/c mice treated with the compounds **1** and **2** showed also promising antileishmanial *in vivo* activities, but need further evaluation. Primarily the hydrocinnamic acid derivative (**2**) shows promising *in vitro* activity while affecting and altering the mitochondria of *Leishmania*, making it a potent lead structure for the development of antileishmanial chemotherapeutic approaches.

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References

1. Leishmaniasis fact sheet N°375. World Health Organization. **2015**; Available: http://www.who.int/mediacentre/factsheets/fs375/en/.

2. Alvar, J.; Vélez, I. D.; Bern, C.; Herrero, M.; Desjeux, P.; Cano, J.; Jannin, J.; den Boer, M., Leishmaniasis worldwide and global estimates of its incidence. *PLOS ONE* **2012**, *5*: e35671.
3. Singh, S.; Sivakumar R., Challenges and new discoveries in the treatment of leishmaniasis. *J. Infect. Chemother*. **2004**, *10*, 307-315.
4. Croft, S. L. ; Sundar, S. ; Fairlamb A. H., Drug resistance in leishmaniasis. *Clin. Microbiol. Rev*. **2006**, *19*, 111-126.
5. Sundar, S.; Chakravarty, J.; Paromomycin in the treatment of leishmaniasis. *Expert Opin. Investig. Drugs* **2008**, *17*, 787-794.
6. Croft, S.; Neal, R.; Thornton, E.; Herrmann, D., Antileishmanial activity of the ether phospholipid ilmofosine. *Trans. R. Soc. Trop. Med. Hyg*. **1993**, *87*, 217-219.
7. Masic, A.; Hurdayal, R.; Nieuwenhuizen, N. E.; Brombacher, F.; Moll, H., Dendritic cell-mediated vaccination relies on interleukin-4 receptor signaling to avoid tissue damage after *Leishmania major* infection of BALB/c mice. *PLoS Negl. Trop. Dis*. **2012**, *6,* e1721.
8. Nakayama, H.; Desrivot, J.; Bories, C.; Franck, X.; Figadere, B.; Hocquemiller, R.; Fournet, A.; Loiseau, P. M., *In vitro* and *in vivo* antileishmanial efficacy of a new nitrilquinoline against *Leishmania donovani*. *Biomed. Pharmacother*. **2007**, *61*,186-188.
9. Aponte, J. C.; Castillo, D.; Estevez, Y.; Gonzalez, G.; Arevalo, J.; Hammond, G. B.; Sauvain, M., *In vitro* and *in vivo* anti-Leishmania activity of polysubstituted synthetic chalcones. *Bioorg. Med. Chem. Lett*. **2010**, *20*, 100-103.
10. Ghosh, S.; Debnath, S.; Hazra, S.; Hartung, A.; Thomale, K.; Schultheis, M.; Kapkova, P.; Schurigt, U.; Moll, H., *Valeriana wallichii* root extracts and fractions with activity against *Leishmania* spp. *Parasitol. Res*. **2011**, *108*, 861-871.
11. Glaser, J.; Schultheis, M.; Moll, H.; Hazra, B.; Holzgrabe, U., Antileishmanial and cytotoxic compounds from *Valeriana wallichii* and identification of a novel nepetolactone derivative. *Molecules* **2015**, *20*, 5740-5753.
12. Glaser, J.; Schultheis, M.; Hazra, S.; Hazra, B.; Moll, H.; Schurigt, U.; Holzgrabe, U., Antileishmanial lead structures from nature: Analysis of structure-activity relationships of a compound library derived from caffeic acid bornyl ester. *Molecules* **2014**, *19*, 1394-1410.

13. Bogdan, C.; Moll, H.; Solbach, W.; Röllinghoff, M., Tumor necrosis factor-α in combination with interferon-γ, but not with interleukin 4 activates murine macrophages for elimination of *Leishmania major* amastigotes. *Eur. J. Immunol*. **1990**, *20*, 1131-1135.
14. Bringmann, G.; Thomale, K.; Bischof, S.; Schneider, C.; Schultheis, M.; Schwarz, T.; Moll, H.; Schurigt, U., A novel *Leishmania major* amastigote assay in 96-well format for rapid drug screening and its use for discovery and evaluation of a new class of leishmanicidal quinolinium salts. *Antimicrob. Agents Chemother*. **2013**, *57*, 3003-3011.
15. Hazra, S.; Ghosh, S.; Debnath, S.; Seville, S.; Prajapati, V. K.; Wright, C. W.; Sundar, S.; Hazra, B., Antileishmanial activity of cryptolepine analogues and apoptotic effects of 2, 7-dibromocryptolepine against *Leishmania donovani* promastigotes. *Parasitol. Res*. **2012**, *111*, 195-203.
16. Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* **1983**, *65*, 55-63.
17. Ponte-Sucre, A.; Vicik, R.; Schultheis, M.; Schirmeister, T.; Moll, H. Aziridine-2, 3-dicarboxylates, peptidomimetic cysteine protease inhibitors with antileishmanial activity. *Antimicrob. Agents Chemother*. **2006**, *50*, 2439-2447.
18. Solbach, W.; Forberg, K.; Röllinghoff, M., Effect of T-lymphocyte suppression on the parasite burden in *Leishmania major*-infected, genetically susceptible BALB/c mice. *Infect. Immun*. **1986**, *54*, 909-912.
19. Hazra, S.; Ghosh, S.; Sarma, M. D.; Sharma, S.; Das, M.; Saudagar, P.; Prajapati, V. K.; Dubey, V. K.; Sundar, S.; Hazra, B., Evaluation of a diospyrin derivative as antileishmanial agent and potential modulator of ornithine decarboxylase of *Leishmania donovani*. *Exp. Parasitol*. **2013**, *135*, 407-413.
20. Lima, H.; Bleyenberg, J.; Titus, R., A simple method for quantifying *Leishmania* in tissues of infected animals. *Parasitol. Today* **1997**, *13*, 80-82.
21. Schurigt, U.; Schad, C.; Glowa, C.; Baum, U.; Thomale, K.; Schnitzer, J. K.; Schultheis, M.; Schaschke, N.; Schirmeister, T.; Moll, H., Aziridine-2, 3-dicarboxylate-based cysteine cathepsin inhibitors induce cell death in *Leishmania major* associated with accumulation of debris in autophagy-related lysosome-like vacuoles. *Antimicrob. Agents Chemother*. **2010**, *54*, 5028-5041.

22. Vermes, I.; Haanen, C.; Reutelingsperger, C., Flow cytometry of apoptotic cell death. *J. Immunol. Methods* **2000**, *243*, 167-190.
23. Zhang, G.; Gurtu, V.; Kain, S. R.; Yan, G., Early detection of apoptosis using a fluorescent conjugate of annexin V. *BioTechniques* **1997**, *23*, 525-531.
24. Croft, S. L.; Seifert, K.; Yardley, V., Current scenario of drug development for leishmaniasis. *Ind. J. Med. Res*. **2006**, *123*, 399.
25. Guy, R.,; Belosevic, M., Response of SCID mice to establishment of *Leishmania major* infection. *Clin. Exp. Immunol*. **1995**, *100*, 440-445.
26. Corware, K.; Harris, D.; Teo, I.; Rogers, M.; Naresh, K.; Müller, I.; Shaunak, S., Accelerated healing of cutaneous leishmaniasis in non-healing BALB/c mice using water soluble amphotericin B-polymethacrylic acid. *Biomaterials* **2011**, *32*, 8029-8039.
27. Simpson, L.; Kretzer, F., The mitochondrion in dividing *Leishmania tarentolae* cells is symmetric and circular and becomes a single asymmetric tubule in non-dividing cells due to division of the kinetoplast portion. *Mol. Biochem. Parasitol*. **1997**, *87*, 71-78.
28. Haecker, G., The morphology of apoptosis. *Cell Tissue Res*. **2000**, *30*, 5-17.
29. Britta, E. A.; Scariot, D. B.; Falzirolli, H.; Ueda-Nakamura, T.; Silva, C. C.; Benedito Filho, P.; Borsali, R.; Nakamura, C. V., Cell death and ultrastructural alterations in *Leishmania amazonensis* caused by new compound 4-nitrobenzaldehyde thiosemicarbazone derived from *S*-limonene. *BMC Microbiol.* **2014**, *14*, 236.
30. Khademvatan, S.; Gharavi, M. J.; Rahim, F.; Saki, J., Miltefosine-induced apoptotic cell death on *Leishmania major* and *L. tropica* strains. *Korean J. Parasitol*. **2011**, *49*, 17-23.
31. Cruz, A. K.; de Toledo, J. S.; Falade, M.; Terrao, M. C.; Kamchonwongpaisan, S.; Kyle, D. E.; Uthaipibull, C., Current treatment and drug discovery against *Leishmania* spp. and *Plasmodium* spp.: a review. *Curr. Drug Targets* **2009**, *10*, 178-192.
32. Gumy, A., Louis, J. A.; Launois, P., The murine model of infection with *Leishmania major* and its importance for the deciphering of mechanisms underlying differences in Th cell differentiation in mice from different genetic backgrounds. *Int. J. Parasitol*. **2004**, *34*, 433-444.

33. Zhai, L.; Blom, J.; Chen, M.; Christensen, S. B.; Kharazmi , A., The antileishmanial agent licochalcone A interferes with the function of parasite mitochondria. *Antimicrob. Agents Chemother*. **1995**, *39*, 2742-2748.
34. Fernandes Rodrigues, J.C; de Souza, W., Ultrastructural alterations in organelles of parasitic protozoa induced by different classes of metabolic inhibitors. *Curr. Pharm. Des*. **2008**, *14*, 925-938.
35. Inacio, J. D.; Canto-Cavalheiro, M. M.; Menna-Barreto, R. F.; Almeida-Amaral, E. E., Mitochondrial damage contribute to epigallocatechin-3-gallate induced death in *Leishmania amazonensis*. *Exp. Parasitol*. **2012**, *132*, 151-155.
36. de Souza, W.; Attias, M.; Rodrigues, J. C., Particularities of mitochondrial structure in parasitic protists (Apicomplexa and Kinetoplastida). *Int. J. Biochem. Cell Biol*. **2009**, *41*, 2069-2080.
37. Sogin, M. L., Early evolution and the origin of eukaryotes. *Curr. Opin. Genet. Dev*. **1991**, *1*, 457-63.
38. Kroemer, G.; Galluzzi, L.; Vandenabeele, P.; Abrams, J.; Alnemri, E.; Baehrecke, E., Blagosklonny, M. V.; El-Deiry, W. S.; Golstein, P.; Green, D. R.; Hengartner, M.; Knight, R. A.; Kumar, S.; Lipton, S. A.; Malorni, W.; Nunez, G.; Peter, M. E.; Tschopp, J.; Yuan, J.; Piacentini, M.; Zhivotovsky, B.; Melino, G., Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell. Death. Differ*. **2009**, *16*, 3-11.
39. Kerr, J. F.; Wyllie, A. H.; Currie, A. R., Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **1972**, *26*, 239-257.
40. Proto, W. R.; Coombs, G. H.; Mottram, J. C., Cell death in parasitic protozoa: regulated or incidental? *Nat. Rev. Microbiol*. **2013**, *11*, 58-66.
41. Castanys-Muñoz, E.; Brown, E.; Coombs, G.; Mottram, J., *Leishmania mexicana* metacaspase is a negative regulator of amastigote proliferation in mammalian cells. *Cell. Death Dis*. **2012**, *3*, e385.
42. Weingärtner, A., Kemmer, G., Müller, F.D.; Zampieri, R.A.; Gonzaga dos Santos, M.; Schiller, J.; Pomorski, T. G., *Leishmania* promastigotes lack phosphatidylserine but bind annexin V upon permeabilization or miltefosine treatment. *PLOS ONE* **2012**, *7*, e42070.
43. Maria do Socorro, S. R.; Mendonça-Filho, R. R.; Bizzo, H. R., de Almeida Rodrigues, I.; Soares, R. M. A.; Souto-Padrón, T.; Alviano, C. S.; Lopes, A. H.,

Antileishmanial activity of a linalool-rich essential oil from *Croton cajucara*. *Antimicrob. Agents. Chemother*. **2003**, *47*, 1895-1901.

44. Rodrigues, I. A.; Azevedo, M. M.,, Chaves, F.C., Bizzo, H. R., Corte-Real, S., Alviano, D. S., Alviano, C. S.; Rosa, M. S. S.; Vermelho, A. B., *In vitro* cytocidal effects of the essential oil from *Croton cajucara* (red sacaca) and its major constituent 7-hydroxycalamenene against *Leishmania chagasi*. *BMC Complement. Altern. Med*. **2013**, *13*, 249.
45. Ferreira, M. E.; de Arias, A. R.; de Ortiz, S. T.; Inchausti, A., Nakayama, H., Thouvenel, C., Hocquemiller, R.; Fournet, A., Leishmanicidal activity of two canthin-6-one alkaloids, two major constituents of *Zanthoxylum chiloperone* var. *angustifolium*. *J. Ethnopharmacol*. **2002**, *80*, 199-202.
46. Christensen, S. B.; Ming, C.; Andersen, L.; Hjørne, U.; Olsen, C. E.; Cornett, C.; Theander, T. G.; Kharazmi, A., An antileishmanial chalcone from Chinese licorice roots. *Planta Med*. **1994**, *60*, 121-123.
47. Fournet, A.; Angelo, A.; Muñoz, V.; Roblot, F.; Hocquemiller, R.; Cavé, A., Biological and chemical studies of *Pera benensis*, a Bolivian plant used in folk medicine as a treatment of cutaneous leishmaniasis. *J. Ethnopharmacol*. **1992**, *37*, 159-164.
48. Kingston, D. G.; Newman, D. J., Natural products as drug leads: an old process or the new hope for drug discovery? *IDrugs* **2005**, *8*, 990-992.
49. Khuda, F.; Iqbal, Z.; Khan, A.; Nasir, F., Antimicrobial and anti-inflammatory activities of leaf extract of *Valeriana wallichii* DC. *Pak. J. Pharm. Sci*. **2012**, *25*, 715-719.
50. Sati, S.; Khulbe, K.; Joshi, S., Antibacterial evaluation of the Himalayan medicinal plant *Valeriana wallichii* DC. (Valerianaceae). *Res. J. Microbiol*. **2011**, *6*, 289-296.
51. García, M.; Perera, W. H.; Scull, R.; Monzote, L., Antileishmanial assessment of leaf extracts from *Pluchea carolinensis*, *Pluchea odorata* and *Pluchea rosea*. *Asian Pac. J. Trop. Med*. **2011**, *4*, 836-840.
52. Dao, T.; Linthorst, H.; Verpoorte, R., Chalcone synthase and its functions in plant resistance. *Phytochem. Rev*. **2011**, *10*, 397-412.
53. Božić, D. D; Milenković, M.; Ivković, B.; Cirković, I., Antibacterial activity of three newly-synthesized chalcones & synergism with antibiotics against clinical

isolates of methicillin-resistant *Staphylococcus aureus*. *Ind. J. Med. Res*. **2014***, 140*,130-137.

54. Narender, T.; Tanvir, K.; Rao, M. S.; Srivastava, K.; Puri, S., Prenylated chalcones isolated from *Crotalaria genus* inhibits *in vitro* growth of the human malaria parasite *Plasmodium falciparum*. *Bioorg. Med. Chem. Lett*. **2005**, *15*, 2453-2455.
55. de Mello, T. F.; Bitencourt, H. R.; Pedroso, R. B.; Aristides, S. M.; Lonardoni, M. V.; Silveira, T. G., Leishmanicidal activity of synthetic chalcones in *Leishmania* (Viannia) *braziliensis*. *Exp. Parasitol*. **2014**, *136*, 27-34.
56. Gupta, S.; Shivahare, R.; Korthikunta, V.; Singh, R.; Gupta, S.; Tadigoppula, N., Synthesis and biological evaluation of chalcones as potential antileishmanial agents. *Eur. J. Med. Chem*. **2014**, *81*, 359-366.
57. Narender, T.; Gupta, S., A convenient and biogenetic type synthesis of few naturally occurring chromeno dihydrochalcones and their *in vitro* antileishmanial activity. *Bioorg. Med. Chem. Lett*. **2004**, *14*, 3913-3916.
58. Baell, J.; Walters, M. A., Chemical con artists foil drug discovery. *Nature* **2014**, *513*, 481-483.

3.4. Antischistosomal Activity of Cinnamic Acid Esters: Eugenyl and Thymyl Cinnamate Induce Cytoplasmic Vacuoles and Death in Schistosomula of *Schistosoma mansoni*

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Abstract

Bornyl caffeate (**1**) was previously isolated by us from *Valeriana (V.) wallichii* rhizomes and identified as an antileishmanial substance. Here, we screened a small compound library of synthesized derivatives (**1**-**30**) for activity against schistosomula of *Schistosoma (S.) mansoni*. Compound **1** did not show any antischistosomal activity. However, strong phenotypic changes, including the formation of vacuoles, degeneration and death were observed after *in vitro* treatment with compounds **23** (thymyl cinnamate) and **27** (eugenyl cinnamate). Electron microscopy analysis of the induced vacuoles in the dying parasites suggests that **23** and **27** interfere with autophagy.

Introduction

Schistosomiasis, also known as bilharzia after its discoverer Theodor Bilharz [1], is one of the most common chronic diseases of poverty. The disease is found in sub-Saharan Africa, parts of Asia and South America [2,3], and is caused by trematodes of the genus *Schistosoma* which can survive in the human host for years or even decades [4]. Of the three principal species infecting humans, *S. mansoni* is the most prevalent [5] causing intestinal schistosomiasis. Infection occurs during contact with freshwater bodies that contain the appropriate species of infected snails (*Biomphalaria* spp. [6]). Free-swimming larvae (*cercariae*) penetrate the skin and reach the bloodstream where they develop into adult worms. The infection exhibits a mortality rate of approximately 300,000 deaths a year [7] but is more commonly chronic and morbidly debilitating. Disease caused by *S. mansoni* infection is associated with chronic hepatic and intestinal fibrosis, internal varices, which, if located close to the stomach or esophagus can result in hematemesis [8,9]. With *S. haematobium* infections, a variety of urinary system complications can result [10].

In 2012, 249 million people worldwide were in need of preventive treatment for schistosomiasis with praziquantel (PZQ), the only drug available that is active against all species of *Schistosoma* [11,12]. Although there are no reports of clinically relevant resistance to PZQ, recent commitments to increase access to the drug mean that we should remain vigilant in the event resistance does arise [13]. It is, therefore, advisable to look for alternate drugs to fight this disease.

We recently discovered that bornyl caffeate (**1**) from the rhizomes of *Valeriana (V.) wallichii* possessed antileishmanial activity [14]. We followed up on this initial finding with the synthesis of a small compound library of 28 systematically varied derivatives [15]. Here, we screened the entire library (except the cytotoxic acetylated bornyl caffeate) plus three additional compounds (**18**, **28** and **29**) for *in vitro* activity against *S. mansoni* schistosomula (post-infective larvae) and compounds **23**, **27**-**30** for *in vitro* activity against adult worms.

Results and Discussion

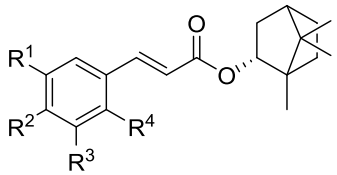
The compound library and the test results are summarized in Tables 1 and 2. Screens were performed at 5 μM and 10 μM against schistosomula and adults, respectively, over the course of 3 days. A previous constrained nomenclature is used to record

phenotypes [16] (every 24 h) that describe both motion and morphological changes. For schistosomula, the terms employed are ‘dead’ (D), ‘degenerate’ (Deg), ‘round’ (R), ‘overactive’ (O) and ‘dark’ (Dark). After incubation with **23** and **27**, we also noted the development of internal ‘vacuoles’ (V), which presaged the eventual death of the parasite.

Screen results

The bornyl caffeate **1** originally isolated and identified from *V. wallichii* was inactive, whereas some of its derivatives proved to be far more potent. In any case, compound **1** shows typical PAINS [17] characteristics, namely the catechol moiety and the α,β-unsaturated double bond that are known to give false positive results in multiple biochemical assays. As the catechol moiety is prone to oxidation that results in the formation of an *o*-benzoquinone [18], catechols can act as antioxidants or pro-oxidants, which can induce damage to DNA or proteins and therefore may result in antiparasitic activity. Omitting the vicinal phenolic hydroxyl groups improved anti-schistosomal activity and reduced cytotoxicity significantly. This was the case with bornyl cinnamate (**3**) as well as thymyl (**23**) and menthyl cinnamate (**26**), which produced stronger phenotypes than their respective catechol counterparts (**1**, **21** and **24**; s. Tables 1 and 2).

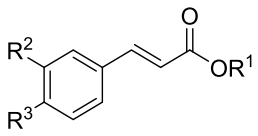
Table 1. Results of the screening of compounds **1-10** on schistosomula (Phenotypes: D = dead, Deg = degenerate, O = overactive, R = round, V = vacuoles; severe phenotypes are in bold). PZQ = Praziquantel.



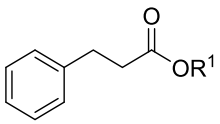
1-10

cmpd.	R^1	R^2	R^3	R^4	observed phenotype after (c = 5 µM) 24h	48h	72h	J774.1 [µM] [15]
1	-OH	-OH	-H	-H	-	-	-	8.3
2	-OH	$-OCH_3$	-H	-H	R	R, Dark	R, Dark	48.7
3	-H	-H	-H	-H	-	R, Dark	R, **Deg**	45.2
4	-H	-H	-Cl	-Cl	R	-	-	58.9
5	-H	-Cl	-H	-Cl	R	-	-	60.6
6	-H	$-OCH_3$	-H	-H	R	-	-	42.6
7	-H	-Br	-H	-H	R	-	Dark	54.6
8	-H	$-N(CH_3)_2$	-H	-H	R	-	-	> 100
9	-H	-OBn	-H	-H	R	-	-	> 100
10	-H	-Cl	-H	-H	-	Dark	-	49.5
PZQ	-	-	-	-	O, Dark	O, **Deg**	**Deg**	-

Table 2. Results of the screening of compounds **11-30** on schistosomula (Phenotypes: D = dead, Deg = degenerate, O = overactive, R = round, V = vacuoles; severe phenotypes are in bold). PZQ = Praziquantel.



11-27



28-30

observed phenotype after (c = 5 µM)

cmpd.	R^1	R^2	R^3	24h	48h	72h	J774.1 [µM] [15]
11	isoborneol	-OH	-OH	-	-	-	8.8
12	isoborneol	-OH	$-OCH_3$	-	R	-	44.3
13	isoborneol	-H	-H	-	-	-	46.0
14	isoborneol	-H	$-NO_2$	-	-	-	> 100
15	cyclohexanol	-H	-H	-	-	-	> 100
16	geraniol	-H	-H	-	-	-	> 100
17	adamantol	-H	-H	-	-	-	46.7
18	4-thujanol	-H	-H	-	-	-	> 100
19	naphthol	-H	-H	-	-	-	44.5
20	α-bisabolol	-H	-H	R	R	-	> 100
21	thymol	-OH	-OH	O	-	-	8.8
22	thymol	-OH	$-OCH_3$	-	R, Dark	Dark, R	45.6
23	thymol	-H	-H	**Deg**, **V**	**Deg**, **V**	**Deg**	44.8
24	menthol	-OH	-OH	-	R, Dark	Dark, R	2.0
25	menthol	-OH	$-OCH_3$	R	R, Dark	R, **Deg**	45.6
26	menthol	-H	-H	R	R, Dark	R, **Deg**	44.8
27	eugenol	-H	-H	**Deg**, **V**	**Deg**	**D**	32.2
28	thymol	-	-	-	-	-	> 100
29	eugenol	-	-	-	-	-	> 100
30	borneol	-	-	-	-	-	> 100
PZQ	-	-	-	O, R	O, **Deg**	**Deg**	-

The α,β-unsaturated double bond represents an potentially reactive Michael system. However, only the bornyl (**3**), menthyl (**25**, **26**), thymyl (**23**) and eugenyl cinnamates (**27**) induced the severest phenotypes. Accordingly, the existence of a Michael system does not necessarily lead to anti-schistosomal activity. Also the IC_{50} of the J774.1 cell line seemed not to be decreased by this Michael system.

The data also show that cytotoxicity appears not to correlate with anti-schistosomal activity. Compounds with highest cytotoxicity (**1, 11**, **21, 24**) have the lowest effect on

schistosomula. The most potent antischistosomal activity is achieved by maintaining the aromatic ring of the cinnamoyl in the absence of substituents.

Overall, the eugenyl cinnamate **27** is the most potent schistosomicidal compound. By 24 hours, the compound had caused degeneration of the schistosomula with the formation of vacuoles. After 72 hours all the parasites were dead. Similarly, the thymol cinnamate **23** also led to the formation of vacuoles (V) and degeneration (s. Table 2).

The most active cinnamates were again synthesized without the double bond (**28-30**). However, these were inactive. By comparison, the bornyl phenylpropionate (**30**) retained anti-leishmanial activity [15]. For schistosomula, it is possible that the Michael system is responsible for inducing the vacoules – as seen with **23** and **27** – and is an essential component to the pharmacophore. Finally, **23** and **27**-**30** were selected for screening against adult worms. No effect on the parasites was observed at a concentration of 10 µM over the course of 3 days (data not shown). The vulnerability of the schistosomula may be due to the major transcriptional, translational and structural changes taking place as part of the adaptive process to the mammalian host, in contrast to adult worms which are fully adapted.

Analysis of Vacuoles

Vacuoles are often indicators for autophagy [19] and contribute to the recycling of misfolded proteins and harmful cell products [20]. Interestingly, this mechanism seems to be induced by compounds **23** and **27**. The clear induction of the vacuoles by 24 h is followed by death. Light microscopic images (s. Fig. 1) reveal multiple large vacuoles inside the schistosomula. A similar phenotype was induced by **23** (data not shown). We further analyzed the vacuoles induced by the eugenyl cinnamate **27** and thymyl cinnamate **23** using transmission electron microscopy (TEM; Fig. 2). The images generated were compared to those previously reported [21]. The formation of large vacuoles in the parasite was promoted by both compounds in a similar fashion. Inside the vacuoles, resembling possible auto-phagosomes, onion-like multi-lamellar structures (b) and multiple internal membranes (a, b) were found. A fusion of two vacuoles is depicted in (B). These observations are consistent with the description of autophagy as a defense mechanism in *Mycobacterium tuberculosis* infected macrophages by Gutierrez et al. [22]. Autophagy is employed as a clean-up process to remove intracellular pathogens. The characteristics of autophagosomes include a

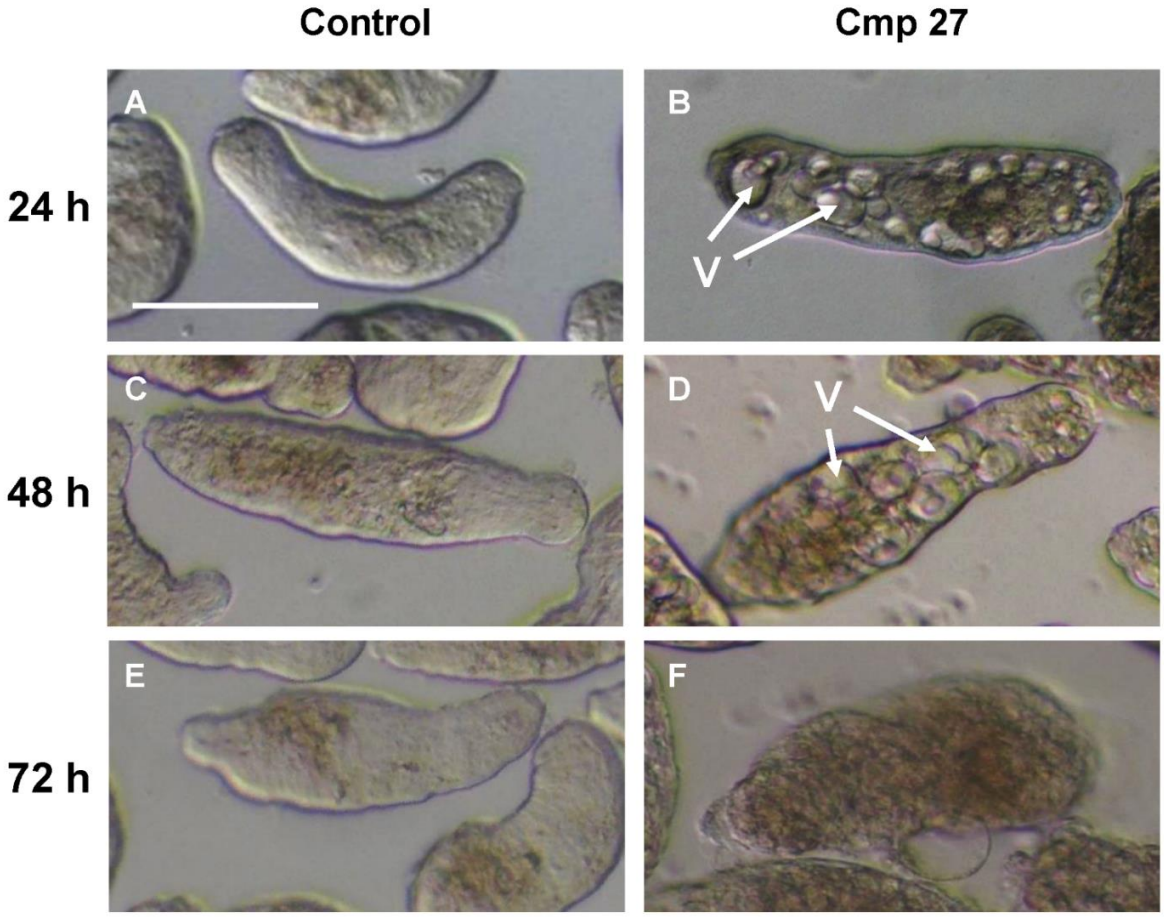


Fig.1. Microscopic images of schistosomula; control group (A, C, E) and treated with 5 μM **27** (B, D, F) after 24, 48 and 72 h. The vacuoles (V) are prominent after 24 and 48 h, whereas by 72 h the somules are dead. Scale bar = 0.2 mm.

double membrane (which is digested in autophagolysosomes during autophagic flux) and the presence of cytosolic content, e.g., membranes from captured organelles [23]. Therefore, the observed vacuoles in schistosomula may represent the late stage of autolysosomes, which occurs after the fusion of autophagosomes with lysosomes that contain multiple hydrolases (e.g. cysteine proteases) to degrade the enveloped cytosolic material. A downstream blockade of the autophagic flux, e.g. by inhibition of the hydrolases needed to degrade the vacuole content [24], may lead to the formation of the huge vacuoles observed in schistosomula after exposure to **23** and **27**. There are few reports on autophagy in *S. mansoni*. Autophagy is inducible in *S. mansoni* schistosomula upon starvation [25] and large acidophilic compartments [26,27] are detectable using the fluorescent dye monodansylcadaverine, a relatively specific dye

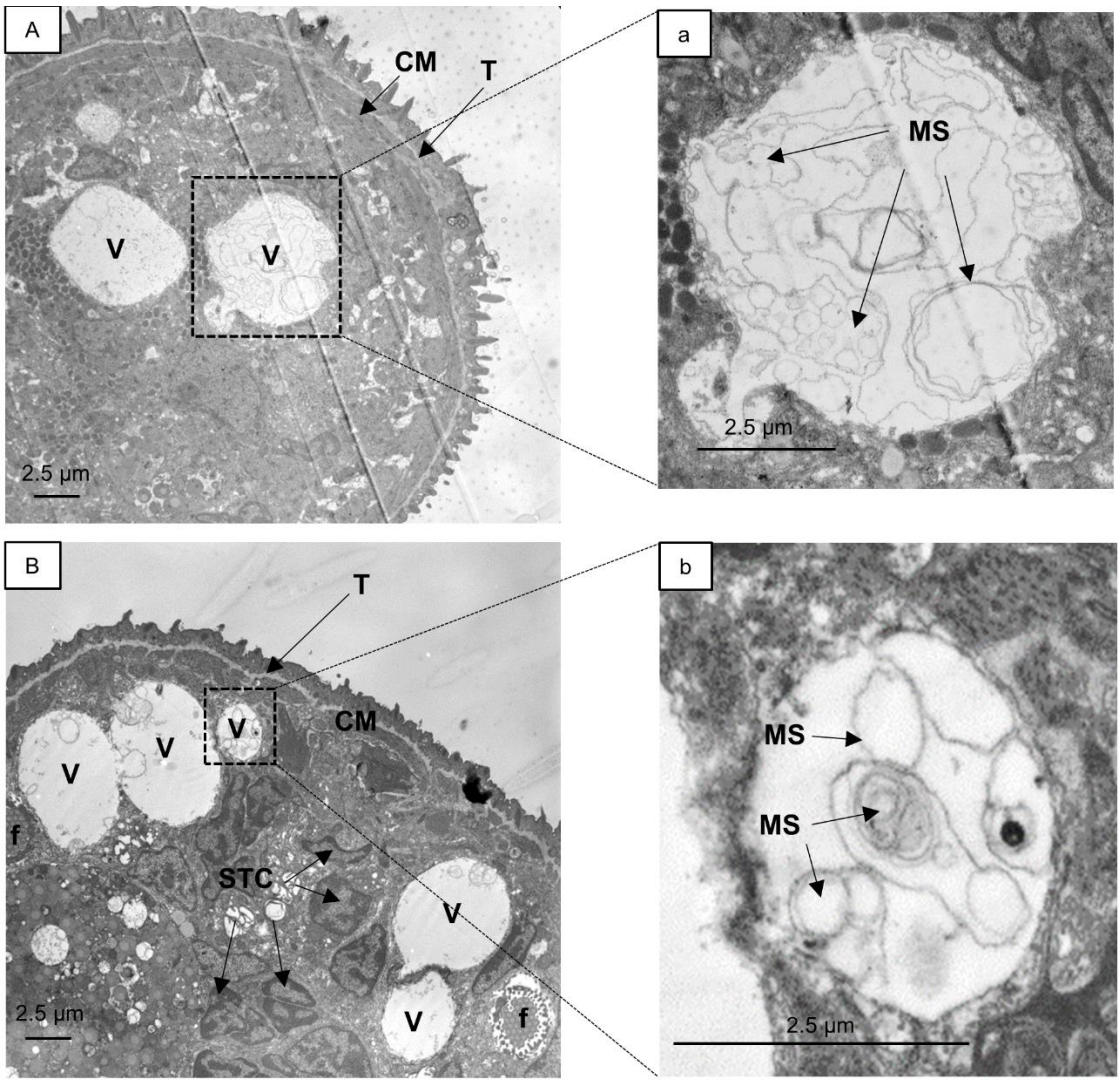


Fig.2. Transmission electron micrographs of *S. mansoni* schistosomula exposed for 24 h to 5 µM **27** (A) or **23** (B). Enlargements of marked areas in A and B are depicted in (a) and (b), respectively. A fusion event between vacuoles is pictured in B. Vacuoles with internal membranes (a, b) and onion-like multi-lamellar structures are visible (b). CM, circular muscle; f, flame cell; MS, multi-lamellar structures; STC, nucleus of subtegumental cell; T, tegument; V, vacuoles.

for autophagosomes [28]. Exposure of *S. mansoni* adult worms to astiban, lucanthone, hycanthone, and niridazole generate autophagic activities in the gastrodermis (gut) of *S. mansoni* and induce autophagic vacuoles [29]. There appear to be no reports on autophagy in schistosomes that is induced by cinnamic acid derivatives or terpenoids. Eugenol is considered to act as an antifungal [30] and antibacterial [31] as has also been suggested for thymol and other terpenoids [32], but the mechanisms of their respective actions have not been investigated. Interestingly, eugenol alone is also known to inhibit autophagy by preventing the dissociation of the Beclin1-Bcl2

heterodimer in A549 cells cotransfected with pMN-Bcl2 and pMC-Beclin1 [33]. As the Beclin1-Bcl2 complex is also present in *Schistosom*a [34] the eugenyl as well as the thymyl derivatives may influence the autophagy process in the same way. However, further studies are required.

Experimental Section

Synthesis and Cytotoxicity Assays

The synthesis and full spectroscopic data of compounds **1**-**17**, **19**-**27** and **30** were reported previously [15]. Compounds **18**, **28** and **29** were synthesized via Steglich esterification as explained there. The protocol for the cytotoxicity studies against J774.1 was as described [35].

Thujanyl cinnamate (**18**)*:* colorless syrup; IR (ATR): 2955, 2930, 2869, 1702, 1636, 1163 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ (ppm) *J* (Hz): 0.32 (dd;*3.7*;*5.2*;1H); 0.45 (m;1H); 0.91 (d;*6.8*;3H); 0.92 (d;*6.8*;3H); 1.27 (m;1H); 1.39 (m;1H); 1.60 (m;1H); 1.64 (s;3H); 1.79 (m;1H); 1.87 (m;1H); 2.10 (m;1H); 6.38 (d;*16.0*;1H); 7.37 (m;3H); 7.50 (m;2H); 7.60 (d;*16.0*;1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 13.3; 19.7; 20.0; 21.5; 25.6; 31.2; 32.2; 34.6; 34.8; 91.9; 120.2; 128.0 (2C); 128.8 (2C); 130.0; 134.7; 143.5; 166.5.

Thymyl phenylpropanoate (**28**): colorless liquid; IR (ATR): 3027, 2962, 2925, 2869, 1754, 1505, 1497, 1413, 1148 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ (ppm) *J* (Hz): 1.12 (d;*6.9*;6H); 2.30 (s;3H); 2.80 (sept;*6.9*;1H); 2.90 (m;2H); 3.10 (m;2H); 6.72 (s;1H); 7.01 (d;*7.9*;1H); 7.17 (d;*7.9*;1H); 7.24-7.35 (m;5H). ^{13}C NMR ($CDCl_3$) δ (ppm): 20.8; 23.0 (2C); 27.0; 31.0; 35.9; 122.6; 126.4 (2C); 127.1; 128.4 (2C); 128.6 (2C); 136.5; 137.0; 140.2; 147.8; 171.6.

Eugenyl phenylpropanoate (**29**): colorless liquid; IR (ATR): 2963, 2936, 2924, 1756, 1503, 1122 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ (ppm) *J* (Hz): 2.93 (m;2H); 3.12 (m;2H); 3.40 (m;2H); 3.80 (s;3H); 5.10 (m;1H); 5.14 (m;1H); 5.98 (m;1H); 6.78 (dd;*1.9*;*8.0*;1H); 6.80 (d;*1.9*;1H); 6.90 (d;*8.0*;1H); 7.23-7.36 (m;5H). ^{13}C NMR ($CDCl_3$) δ (ppm): 31.0; 35.6; 40.1; 55.8; 112.7; 116.1; 120.7; 122.5; 126.3; 128.4 (2C), 128.5 (2C); 137.1; 138.0; 139.0; 140.4; 150.9; 171.1.

Schistosoma Screens and TEM

Maintenance of the *S. mansoni* life cycle, preparation of schistosomula and adult worms, compound storage and treatment of schistosomula and adult worms were as described [16]. Compounds were dissolved in DMSO. Control assays contained the same amount of DMSO. The compound names, structures and phenotypes arising are listed in Tables 1 and 2. PZQ served as anti-schistosomal reference drug. TEM studies were performed as follows. After treatment with compound **27** for 24 h schistosomula were fixed for 45 min with 2.5% glutaraldehyde-50 mM cacodylate (pH 7.2; Sigma-Aldrich) at room temperature and then contrasted for 2 h at 4°C with 2% OsO_4 buffered with 50 mM cacodylate (pH 7.2). The material was washed with distilled water, and incubated overnight at 4 °C with 0.5% uranyl acetate in distilled water. The parasites were dehydrated and embedded in EPON. Ultrathin sections were mounted on 300-mesh grids, stained with uranyl acetate and lead citrate, and analyzed with an EM 900 transmission electron microscope (Carl Zeiss AG, Oberkochen, Germany).

Conclusions

We screened a library of 30 bornyl caffeate (**1**) derivatives for anti-schistosomal activity against schistosomula and adult worms of *S. mansoni*. Adults were refractory to all of the compounds. For schistosomula, the eugenyl cinnamate (**27**) was lethal. Prior to death, this compound and the thymyl cinnamate (**23**) induced the appearance of multiple internal vacuoles, which may indicate an induction of autophagy, although this hypothesis requires formal testing. No direct correlation between cytotoxicity (vs. J774.1 cells) and anti-schistosomal activity was found. Further studies with an expanded series of compounds and including dose-response relationships are necessary to determine whether these compounds represent a basis for future anti-schistosomal drugs

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Author Contributions

JG synthesized the compound library and prepared the manuscript in collaboration with US. BS, US, and CRC performed the assays and analyzed the results. UH, CRC, JG and US interpreted and discussed the results scientifically. UH and CRC contributed to writing and editing of the manuscript. All authors have read and approved the final version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

References and Notes

1. Bilharz, T.; Siebold, C. T. E., *Ein Beitrag zur Helminthographia humana*. W. Engelmann: Leipzig, **1852**; 1-77.
2. Chitsulo, L.; Engels, D.; Montresor, A.; Savioli, L., The global status of schistosomiasis and its control. *Acta Trop.* **2000**, *77*, 41-51.
3. Engels, D.; Chitsulo, L.; Montresor, A.; Savioli, L., The global epidemiological situation of schistosomiasis and new approaches to control and research. *Acta Trop.* **2002**, *82*, 139-146.
4. Hall, S. C.; Kehoe, E., Case reports. Prolonged survival of *Schistosoma japonicum*. *Calif. Med.* **1970**, *113*, 75-77.
5. Gryseels, B.; Polman, K.; Clerinx, J.; Kestens, L., Human schistosomiasis. *Lancet* **2006***, 368*, 1106-1118.
6. Morgan, J.; Dejong, R.; Snyder, S.; Mkoji, G.; Loker, E., *Schistosoma mansoni* and *Biomphalaria*: past history and future trends. *Parasitology* **2001**, *123*, 211-228.
7. van der Werf, M. J.; de Vlas, S. J.; Brooker, S.; Looman, C. W.; Nagelkerke, N. J.; Habbema, J. D. F.; Engels, D., Quantification of clinical morbidity associated with schistosome infection in sub-Saharan Africa. *Acta Trop.* **2003**, *86*, 125-139.

8. Warren, K. S., The pathology, pathobiology and pathogenesis of schistosomiasis. *Nature* **1978**, *273*, 609-612.
9. Boros, D., Immunopathology of *Schistosoma mansoni* infection. *Clin. Microbiol. Rev.* **1989**, *2*, 250-269.
10. Smith, J. H.; Christie, J. D., The pathobiology of *Schistosoma haematobium* infection in humans. *Hum. Pathol.* **1986**, *17*, 333-345.
11. Gönnert, R.; Andrews, P., Praziquantel, a new broad-spectrum antischistosomal agent. *Parasitol. Res.* **1977**, *52*, 129-150.
12. Fenwick, A.; Savioli, L.; Engels, D.; Robert Bergquist, N.; Todd, M. H., Drugs for the control of parasitic diseases: current status and development in schistosomiasis. *Trends Parasitol.* **2003**, *19*, 509-515.
13. Doenhoff, M. J.; Pica-Mattoccia, L., Praziquantel for the treatment of schistosomiasis: its use for control in areas with endemic disease and prospects for drug resistance. *Expert Rev. Anti. Infect. Ther.* **2006**, *4*, 199-210.
14. Glaser, J.; Schultheis, M.; Moll, H.; Hazra, B.; Holzgrabe, U., Antileishmanial and cytotoxic compounds from *Valeriana wallichii* and identification of a novel nepetolactone derivative. *Molecules* **2015**, *20*, 5740-5753.
15. Glaser, J.; Schultheis, M.; Hazra, S.; Hazra, B.; Moll, H.; Schurigt, U.; Holzgrabe, U., Antileishmanial lead structures from nature: analysis of structure-activity relationships of a compound library derived from caffeic acid bornyl ester. *Molecules* **2014**, *19*, 1394-1410.
16. Abdulla, M.-H.; Ruelas, D. S.; Wolff, B.; Snedecor, J.; Lim, K.-C.; Xu, F.; Renslo, A. R.; Williams, J.; McKerrow, J. H.; Caffrey, C. R., Drug discovery for schistosomiasis: hit and lead compounds identified in a library of known drugs by medium-throughput phenotypic screening. *PLoS Negl. Trop. Dis.* **2009**, *3*, e478 doi: 10.1371/journal.pntd.0000478.
17. Baell, J.; Walters, M. A., Chemistry: Chemical con artists foil drug discovery. *Nature News* **2014**, *513*, 481-483.
18. Schweigert, N.; Zehnder, A. J.; Eggen, R. I., Chemical properties of catechols and their molecular modes of toxic action in cells, from microorganisms to mammals. *Environ. Microbiol.* **2001**, *3*, 81-91.
19. Klionsky, D. J.; Abdalla, F. C.; Abeliovich, H.; Abraham, R. T.; Acevedo-Arozena, A.; Adeli, K.; Agholme, L.; Agnello, M.; Agostinis, P.; Aguirre-Ghiso, J. A.,

Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* **2012**, *8*, 445-544.

20. Yorimitsu, T.; Klionsky, D. J., Autophagy: molecular machinery for self-eating. *Cell Death Differ.* **2005**, *12*, 1542-1552.
21. Dorsey, C. H.; Cousin, C. E.; Lewis, F. A.; Stirewalt, M. A., Ultrastructure of the *Schistosoma mansoni* cercaria. *Micron* **2002**, *33*, 279-323.
22. Gutierrez, M. G.; Master, S. S.; Singh, S. B.; Taylor, G. A.; Colombo, M. I.; Deretic, V., Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. *Cell* **2004**, *119,* 753-766.
23. Deretic, V., Autophagy as an immune defense mechanism. *Curr. Opin. Immunol.* **2006**, *18*, 375-382.
24. Zhang, X.-J.; Chen, S.; Huang, K.-X.; Le, W.-D., Why should autophagic flux be assessed? *Acta Pharmacol. Sin.* **2013**, *34*, 595-599.
25. Bogitsh, B. J., Cytochemistry of gastrodermal autophagy following starvation in *Schistosoma mansoni*. *J. Parasitol.* **1975**, 237-248.
26. Al-Adhami, B.; Noble, C.; Sharaf, O.; Thornhill, J.; Doenhoff, M.; Kusel, J., The role of acidic organelles in the development of schistosomula of *Schistosoma mansoni* and their response to signalling molecules. *Parasitology* **2005**, *130,* 309-322.
27. Carneiro-Santos, P.; Thornhill, J.; Doenhoff, M.; Hagan, P.; Kusel, J., Acidic vesicles of *Schistosoma mansoni*. *Parasitol. Res.* **2001**, *87*, 1001-1006.
28. Biederbick, A.; Kern, H.; Elsässer, H., Monodansylcadaverine (MDC) is a specific *in vivo* marker for autophagic vacuoles. *Eur. J. Cell Biol.* **1995**, *66*, 3-14.
29. Clarkson, J.; Erasmus, D., *Schistosoma mansoni*: an *in vivo* study of drug-induced autophagy in the gastrodermis. *J. Helminthol.* **1984**, *58*, 59-68.
30. Rao, A.; Zhang, Y.; Muend, S.; Rao, R., Mechanism of antifungal activity of terpenoid phenols resembles calcium stress and inhibition of the TOR pathway. *Antimicrob. Agents Chemother.* **2010**, *54*, 5062-5069.
31. Pei, R. S.; Zhou, F.; Ji, B. P.; Xu, J., Evaluation of combined antibacterial effects of eugenol, cinnamaldehyde, thymol, and carvacrol against *E. coli* with an improved method. *J. Food Sci.* **2009**, *74*, 379-383.

32. Didry, N.; Dubreuil, L.; Pinkas, M., Activity of thymol, carvacrol, cinnamaldehyde and eugenol on oral bacteria. *Pharm. Acta Helv.* **1994**, *69*, 25-28.
33. Dai, J.-P.; Zhao, X.-F.; Zeng, J.; Wan, Q.-Y.; Yang, J.-C.; Li, W.-Z.; Chen, X.-X.; Wang, G.-F.; Li, K.-S., Drug screening for autophagy inhibitors based on the dissociation of beclin1-bcl2 complex using BiFC technique and mechanism of eugenol on anti-influenza A virus activity. *PLOS ONE* **2013**, *8*: e61026, doi:10.1371/journal.pone.0061026.
34. NCBI Protein database. Available online: http://www.ncbi.nlm.nih.gov/protein/CAJ19743.1 (accessed on 5th May, 2015).
35. Bringmann, G.; Thomale, K.; Bischof, S.; Schneider, C.; Schultheis, M.; Schwarz, T.; Moll, H.; Schurigt, U., A Novel Leishmania major amastigote assay in 96-well format for rapid drug screening and its use for discovery and evaluation of a new class of leishmanicidal quinolinium salts. *Antimicrob. Agents Chemother.* **2013**, *57*, 3003-3011.

Sample Availability: Samples of all synthesized compounds are available from the authors

3.5. Focus on PAINS: False Friends in the Quest for Selective New Anti-Protozoal Lead Structures from Nature?

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Abstract

Pan-assay interference compounds (PAINS) are molecules showing promising but deceptive activities in various biochemical screenings mainly due to unselective interactions with the target. Overall awareness of this problem has been raised recently. Many natural products inherently have PAINS characteristics but are nevertheless uncritically acclaimed as new antiprotozoal lead structures. However, a non-selective mode of action may be the cause of observed activity. This review describes the most common assay-interfering characteristics of antiprotozoal natural products, discusses significant examples from the recent literature and strategies to deal with these promiscuous structures.

Introduction

Protozoal diseases are a major global health problem affecting millions of people worldwide especially in Sub-Saharan Africa, India, and South America. The most prominent and widely distributed infections are due to protozoa of the genus *Leishmania*, *Trypanosoma* and *Plasmodium*, causing leishmaniasis, sleeping sickness as well as Chagas disease and malaria, respectively. Although these illnesses affect a high percentage of the world population, there is only little effort made to find new lead compounds which can substitute the drugs currently in use, even though these drugs exhibit severe adverse effects, and growing resistances are observed. [1,2] Hence, there is an urgent need for new drugs for each of these diseases.

Plant extracts have been providing blueprints for essential drugs for centuries. The antimalarial drug artemisinine (Nobel Prize Award 2015), a sesquiterpene lactone from *Artemisia annua* [3], quinine from the bark of the cinchona tree and anticancer drugs like the class of taxanes from *Taxus brevifolia* [4], but also such everyday drugs like aspirin are just a few examples.

Today the search for novel antiprotozoal lead structures is usually executed by either high-throughput screening (HTS) of commercially available large libraries of natural and/or synthetic compounds [5] (in industry) or, on a much smaller scale, by bioactivity-guided fractionation of a plant extract (often in academia). The thereby applied assays are mostly designed to find selective inhibitors of a predefined lethal target or whole cell assays.

Some members of the libraries may give false-positive results in the assays due to unselective interactions caused by reactive structural features. This often results in a (covalent) chemical modification of target proteins. Such substances match the definition of pan-assay interfering compounds (PAINS) [6]. Furthermore, for natural products Bisson et al. introduced the term “invalid metabolic panaceas” (IMPs) [7] with manifold ascribed bioactivities which cannot be explained by the phenomena observed in synthetic libraries. Not recognizing these artefacts may lead into a dead end when trying to optimize the promising structures synthetically. Indeed, commercially available libraries commonly used in HTS may consist of up to 5-12% of PAIN compounds. [8] With regard to natural products the situation can be even worse.

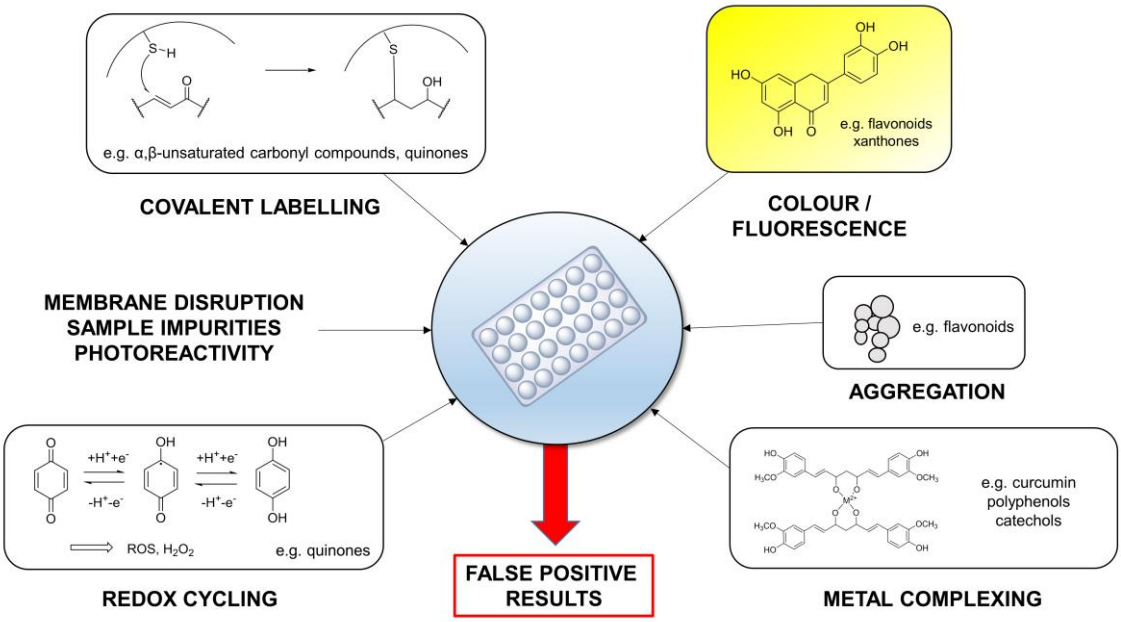


Fig.1: Possible mechanisms of interference of natural products.

In this review we focus on natural products with antiprotozoal activity which suffer from the PAINS problem. Beside the description of the different kinds of PAINS we will give representative examples of natural compounds which show antiprotozoal and cytotoxic activity in addition to typical compounds showing many activities. Finally, we would like to show how to avoid the PAINS problem or how to deal with it. The latter is of importance because PAIN compounds can be valuable hits in drug discovery and development.

Mechanisms of Interference

The modes of interference according to Baell et al. [6] are explained below and visualized in Fig. 1.

- **Redox cycling**. Redox cyclers like quinones and catechols can inactivate susceptible targets under certain conditions by producing (enzymatically or non-enzymatically) superoxide and reactive oxygen species (ROS), [9,10] leading to cysteine oxidation. Particularly the link between toxicity and the redox cycling ability of quinones is well established. [11,12] Furthermore, quinones form

Michael systems susceptible to nucleophilic attacks. A relatively high percentage of natural compounds consist of quinone and catechol moieties.

- **Covalent labelling**. Compounds with electrophilic functional groups (for example Michael acceptors) can form adducts with e.g. sulfhydryl containing amino acids of enzymes, simulating selective inhibition in vitro. [13,14] Michael systems are a typical substructure found in many natural products e.g. sesquiterpene(lactones) [15] or cinnamic acid derivatives.
- **Chelation**. Metal binding molecules, containing e.g. a catechol moiety [16], can complex toxic or reactive metal ions from the synthesis, the assay buffer or the central metal ion of the investigated protein or enzyme.
- **Membrane disruption**. Natural products may perturb cell membranes [17]. A possible result could be the leakage of inner cell components by physical disruption of the membrane.
- **Fluorescence/Colour**. False readouts in cell-based and biochemical assays can be caused by coloured or fluorescent compounds. [18]
- **Aggregation**. Many structurally diverse compounds can behave as aggregation-based promiscuous inhibitors [19] forming clusters (micelles or vesicles) in aqueous media [20] and thereby interfere with the assay outcome. A possible explanation of observed inhibition is the adsorption of an enzyme to the surface of the aggregate. These aggregates have a size of 50-400 nm and are visible by transmission electron microscopy (TEM). Amongst natural products especially flavonoids [21] are known to show this mechanism.

Additional possible causes for false positives in assays may be sample impurities or photoreactivity of the tested compound. [14]

Recently Reported Antiprotozoal Natural Products with PAIN Characteristics

A PubMed search (October 2015) for antiprotozoal natural products yields 4788 hits (1950-2015). 2532 (53%) of these publications have been issued in the last 10 years. Many of the reported natural products consist of typical PAIN related substructures [22] with Michael systems being most common. A structural search for PAIN related substructures using Scifinder showed that 37% of publications reporting antiprotozoal

natural products describe compounds with α,β-unsaturated carbonyl moieties, 6% quinones and 4% catechols.

Below we give selected examples of suspicious natural products showing antiprotozoal activity from the literature of the last decade (2005-2015). Ominous structural elements are highlighted in Fig. 2. It is of note, that for the following examples no data about the reactivity of the compounds were given.

Coumarins

The 2-chromenone skeleton of the coumarins consists of a Michael system characterized by a polarized double bond in 3,4-position in conjugation to the carbonyl

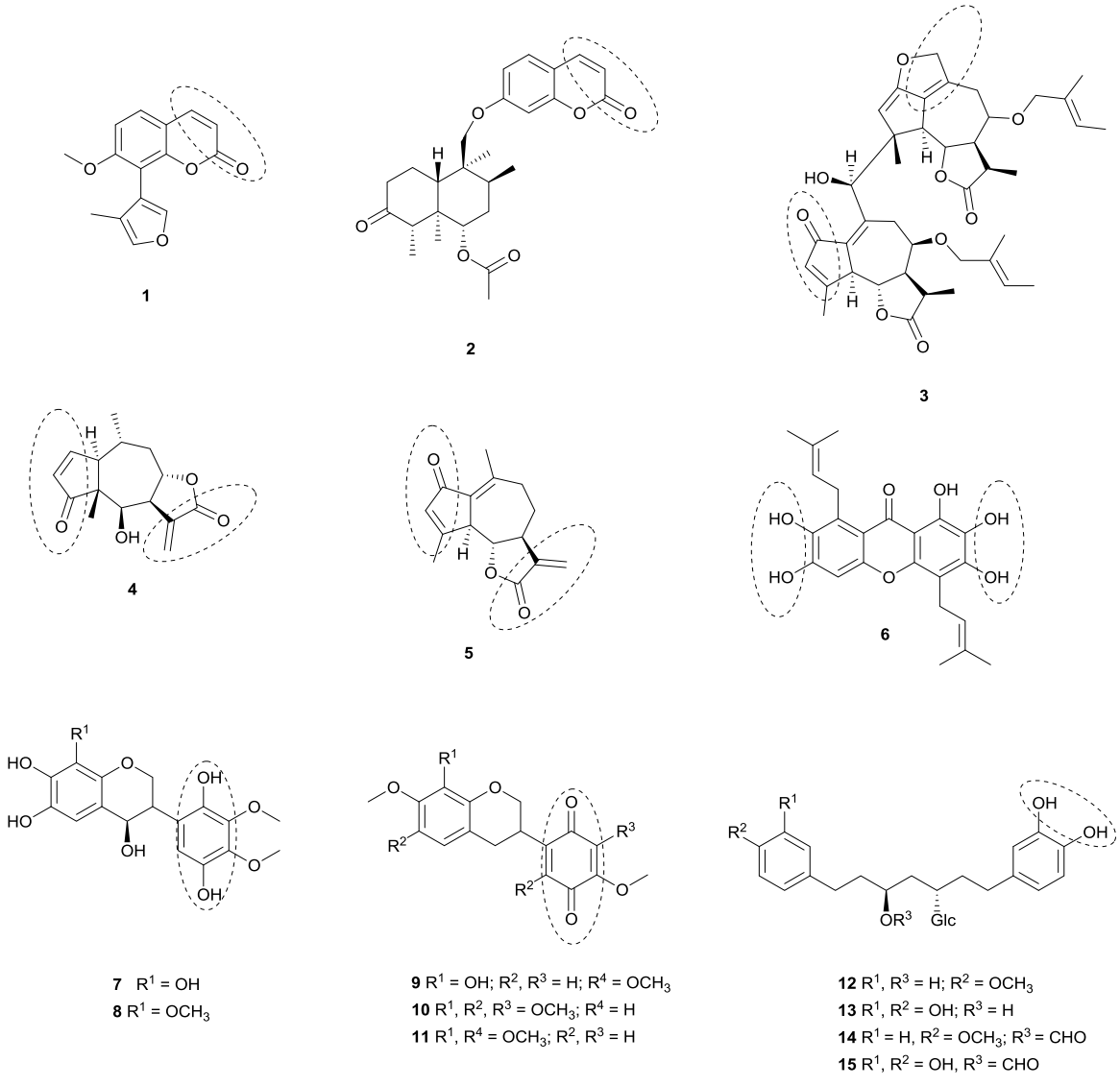


Fig.2: Structures of compounds **1**-**18**. PAIN-related substructures are highlighted.

(C=O) group. Coumarin compound **1** was isolated from the leaves of *Galipea panamensis* and its activity against axenic amastigotes of *Leishmania panamensis* was evaluated. [23] Coumarin **1** showed a 50% effective concentration (EC_{50}) of 9.9 µg/mL, cytotoxicity (IC_{50}) on human promonocytic U-937 cells was 9.7 µg/mL.
Novel sesquiterpene coumarins from *Ferula pseudalliacea* showing antimalarial activity were reported by Dastan et al. [24] Kamolonol acetate (**2**) exhibited an IC_{50} of 16.1 µM and cytotoxicity of 7.5 µM (L6 cells).

Terpenoids

From *Eupatorium perfoliatum* sesquiterpenes of the guaianolide type were isolated. [25] Among others the dimeric guaianolide (**3**) exhibited the highest antimalarial activity with an IC_{50} value of 2.0 µM against *Plasmodium falciparum*. The compound also showed antileishmanial (10.2 µM against *L. donovani*), antitrypanosomal (6.2 µM against *Trypanosoma brucei* and 30.3 µM against *T. cruzi*) activity. Cytotoxicity against L6 rat skeletal myoblasts was 16.2 µM. Cytotoxicity accounts for a low selectivity index which might be due to reactivity of the compound.
The sesquiterpene lactones mexicanin I (**4**) from *Gaillardia megapotamica* and dehydroleucodine (**5**) from *Artemisia douglasiana* were found to induce oxidative stress in *L. mexicana* promastigotes. [26] Especially the exocyclic Michael system will be highly reactive.

Xanthones

A review by Negi et al. [27] about naturally occurring xanthones collected a multitude of different activities for this compound class. δ-Mangostin (**6**) was isolated from *Garcinia mangostana* and found to have antimalarial activity *in vivo* and *in vitro* in the range of 12.4 µM against a chloroquine sensitive *P. falciparum* strain (3D7). Cytotoxicity was determined on U-937 cells with 262.6 µM (LD_{50}). [28]

Quinones

Five isoflavan quinones were found in *Abrus precatorius* ssp. *africanus* and investigated for their antiprotozoal activity. [29] Quinone **7** showed IC_{50} values of 8.0 µM (*P. falciparum*), 12.0 µM (*T. brucei rhodesiense*) and 122.8 µM (L6 cells). No activity against *L. donovani* was observed. Compound **8** was inactive against *P. falciparum*, but had an IC_{50} of 17.0 µM against *T. brucei rhodesiense*, 35.3 µM against *L. donovani* and 51.8 µM against L6 cells. For quinone **9** antiplasmodial activity of 20.4 µM, antitrypanosomal activity of 0.3 µM, antileishmanial activity of 3.4 µM and cytotoxicity of 22.1 µM was observed. The activity of **10** was determined to be 4.1 µM (*P. falciparum*), 0.2 µM (*T. brucei rhodesiense*), 2.9 µM (*L. donovani*) and 10.1 µM (L6). Compound **11** showed similar IC_{50} values: 8.9 µM against *Plasmodium*, 0.9 µM against *Trypanosoma*, 5.0 µM against *Leishmania* and cytotoxicity of 3.9 µM.

Curcumin analogs

Recently, from *Pyrostria major* four diarylheptanoid glucosides (**12**-**15**) with catechol moieties have been reported as anti-trypanosomal structures. [30] They all show only low antiplasmodial activity (> 20 µM) and the same minimum effective concentration against *T. brucei* with 25 µM (except for **12** with > 100 µM). All described glucosides exhibit similar antileishmanial activity with IC_{50} values in the 30 µM range of concentration, respectively.

Taking the results of this collection of natural PAINS compounds together, it can be stated that most of them are not very active (1-2 digit micromolar concentration) and most of them are rather toxic, resulting in a small selectivity index (SI). In addition they were mostly tested in one type of mammalian cell line only. Moreover, in most of these reports the possible reactivity is not discussed in depth.

Convicted Suspects

Whereas the compounds discussed in the last chapter are described to exhibit antiprotozoal activity only, there are natural products which are consistently reported to show activity in dozens of assays, even though their high reactivity is known and the

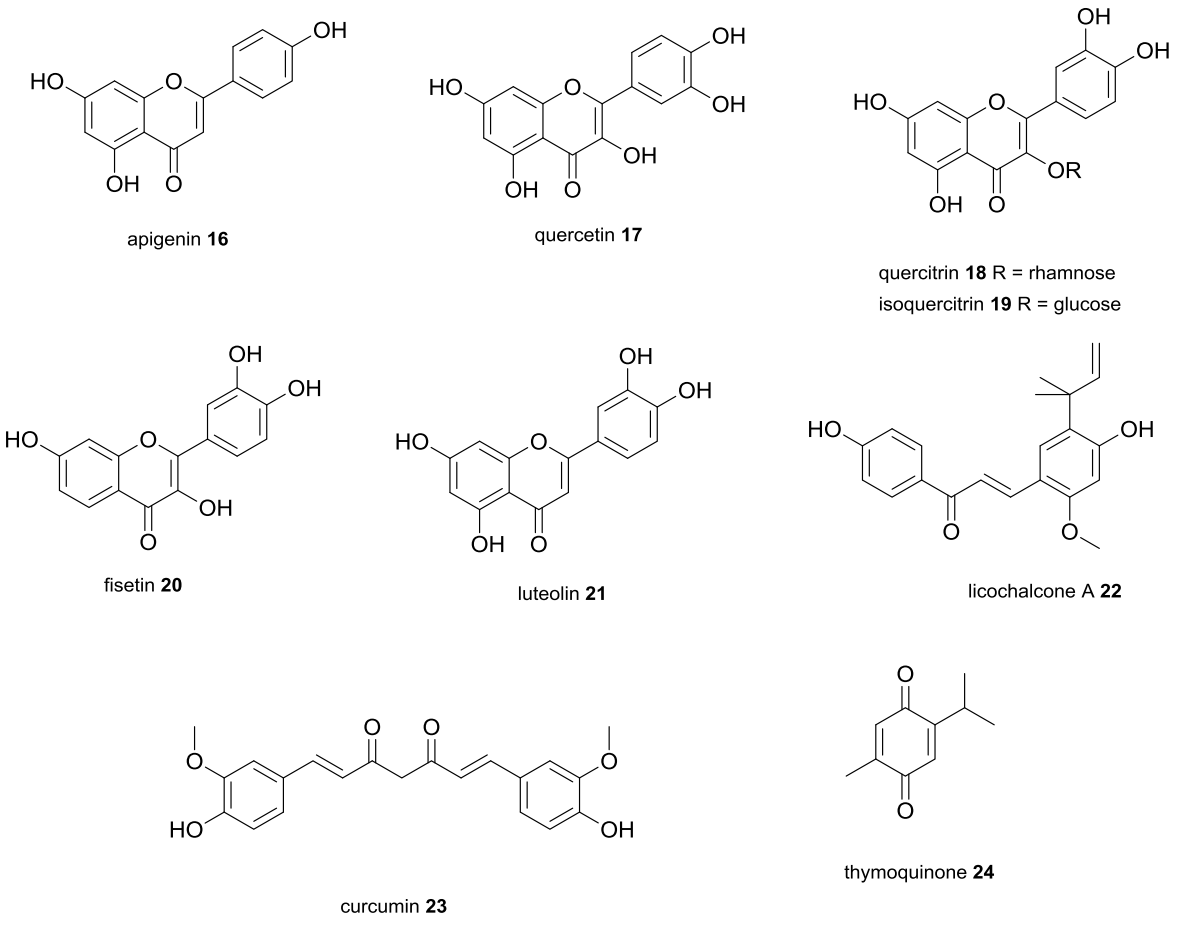


Fig.3. Chemical structures of compounds **16-24**.

unselective mode of action already proven. In the following paragraph we discuss some examples which are the most prominent from our point of view (see Fig.3).

Flavonoids

Besides their antiprotozoal effects apigenin (**16**) and quercetin (**17**) are described to have many other activities. With exception of **16** all flavonoids discussed below consist of both a Michael system in the chromone skeleton and a catechol moiety. Apigenin (**16**) is reported to have anti-inflammatory [31], antioxidative [32], antibacterial [33] and antitumor activity [34] amongst others and seems to inhibit many enzymes. [35-37] IC_{50} values are available for apigenin against *L. donovani* (1.9 µg/mL), *T. brucei rhodesiense* (5.1 µg/mL) and *T. cruzi* (21.8 µg/mL). Cytotoxicity was determined on L6 cells (18.1 µg/mL). Quercetin showed activity in the same concentration range (*L. donovani*: 1.0 µg/mL; *T. brucei rhodesiense*: 8.3 µg/mL; T. cruzi: > 30 µg/mL). [38]

It was recently reported that the effect of apigenin (**16**) on *L. amazonensis* is associated with ROS production followed by mitochondrial dysfunction. The same was found for quercetin (**20**) which causes death of L. amazonensis intracellular amastigotes by ROS production. [39]

Quercetin (**17**), quercitrin (**18**) and isoquercitrin (**19**) were found guilty being "promiscuous" drugs. Quercetin was shown to be a mixed inhibitor of the *L. amazonensis* arginase due to both interactions with the substrate L-arginin and with the cofactor Mn^{2+} (at pH 9.6). For quercitrin and isoquercitrin an interaction of the catechol moiety with Asp129 of the enzyme is described. [40] Quercitrin was reported to have antiprotozoal activity (*L. donovani*: 17.7 µg/mL; *T. brucei rhodesiense*: 27.9 µg/mL; *T. cruzi*: > 30 µg/mL; L6: > 90 µg/mL), [38] and non-dose-dependent activity of isoquercitrin against *L. amazonensis* amastigotes (0.78-20 µg/mL, 40-45%) has been described recently. [41]

Manjolin et al. found the same mixed mechanism of inhibition as described above for fisetin **20** (*L. donovani*: 0.6 µg/mL; *T. brucei rhodesiense*: 3.3 µg/mL; *T. cruzi*: > 30 µg/mL; L6: 38.5 µg/mL; [38] arginase inhibition: IC_{50} = 1.4 µM [42]), luteolin **21** (*L. donovani*: 0.8 µg/mL; *T. brucei rhodesiense*: 3.7 µg/mL; *T. cruzi*: 21.4 µg/mL; L6: 9.4 µg/mL; [38] IC_{50} = 9 µM [42]) and their derived analogues.

Licocalchone A

The chalcone licocalchone A (**22**) is widely discussed in the literature to be an antileishmanial [43,44] (IC_{50}: 0.9 µM, *L. donovani amastigotes*; 7.2 µM, *L. major promastigotes* [45]) and antimalarial [46,47] (IC_{50}: 1.4 µg/mL, *P. falciparum* [48]) agent as well as having antimycobacterial, antilegionella [49] and anti-inflammatory [50] activity to name a few applications only. Omitting the Michael system and without phenolic hydroxyl groups no activity against *P. falciparum in vitro* was observed. [51] It is suggested that the antiplasmodic activity of licochalcone A is due to its incorporation into the cell membrane as a membrane-active agent. [52] Despite this knowledge, multiple publications with "flat" SAR investigation results of chalcones have been published recently. [51,53,54]

Curcumin

Curcumin (**23**) is a yellow compound isolated from turmeric and is the best example for an intensively studied [55,56] substance with strong PAINS characteristics. It is described as a wonder drug, having a plethora of activities [57], most prominently anti-cancer properties. [58] It is a constituent of turmeric (*Curcuma longa*) powder which is traditionally used as a spice providing the characteristic gold-yellow colour to curry mixtures. The content of curcumin in powdered turmeric rhizome is usually around 3%, it is much less in curry powder for human consumption. [59]

Curcumin is known to be a membrane disruptor [60] as well as a covalent modifier due to its double activated Michael system (forms GSH adducts [61]). In addition, it can form strong complexes with metals [62] and generates ROS.[63] Therefore, curcumin has evidence of being a promiscuous inhibitor par excellence.

The generation of ROS results in antileishmanial [64] and antimalarialn [65] activity of curcumin. Glutathione assays confirmed the adduct formation with *T. brucei* trypanothione. [66] Curcumin is reported to have LD_{50} values of 37.6 µM against *L. major* promastigotes and 46.5 µM against procyclic forms of *T. bruci brucei*. [67] Antimalarial activity against different strains of *P. falciparum* is reported to be around 25 µM (3D7). [65]

Corroborating the PAINS concept, the hydrogenated derivatives of curcumin lacking the Michael acceptor show less antitumor and anti-inflammatory activity [68], and partially lose their leishmanicidal properties. [69]

Curcumin seems to be highly cytotoxic to normal human lymphocytes. [70] Further data on cytotoxicity towards normal cells is hardly available in the literature so we tested pure curcumin (Fisher Scientific, UK) ourselves. As expected, curcumin exhibited an IC_{50} value of 31 µM against a murine macrophage cell line (J774.1) and 15.2 µM against kidney cells (HEK 293T), respectively, the values being in the same range as the reported antileishmanial and antitrypanosomal activity. Additionally, we found an IC_{50} against *T. brucei* of 3.6 µM.

Curcumin is also investigated as an agent against Alzheimer´s disease, as it binds to small β-amyloid species and thereby blocks formation of oligomers and fibrils *in vitro* and *in vivo* by injection in rodents. [71] For this, the Michael systems are necessary. [72] However, a 24-week randomized, double blind, placebo-controlled Alzheimer´s

study with oral curcumin showed no effects. The authors blamed the very low plasma levels (max. 7.32 ng/mL). [73] Other studies were not successful either. [74]

The problem is its low water solubility of curcumin (max. 11 ng/mL [75]). It is only absorbed in very small quantities in the gastrointestinal tract upon oral administration. Thus, it belongs to class IV of the biopharmaceutical classification system. Additionally a very effective metabolism and a strong first-pass effect is observed. Therefore, only traces of curcumin are detectable in the plasma (e.g. of rats [76]) which is in line with the findings in ref. [74].

Thus, the uptake of curcumin as a part of the normal diet, especially in India, is hence negligible. This might also explain the safety to humans despite its cytotoxicity *in vitro*. Patients tolerated up to a single dose of 12 g of curcumin without adverse effects, no curcumin was detected in the plasma. [77] Scientifically based long-term toxicity studies for the safety of curcumin are not available.

The manifold reported *in vitro* activities were determined with concentrations of curcumin in the µM range. Taken above findings concerning bioavailability into account, it is not possible to reach these concentrations in plasma by oral administration. [78]

Hence, a lot of effort has been made to develop new application systems to increase the low oral bioavailability of curcumin. One suggestion is the simultaneous administration of piperine. The compound isolated from black pepper is a known inhibitor of hepatic and intestinal glucuronidation, and thereby improves bioavailability by reducing the first-pass effect and enables higher resorption rates. However, the accomplished plasma levels in humans were still found to be comparably low (0.18 µg/mL with piperine). [79] Additionally, this may be problematic when the patient takes other drugs at the same time.

Other approaches to improve bioavailability are nanoparticle curcumin, [80,81] curcumin phospholipid [88] or phosphatidyl choline complexes. [76]

In summary, a great effort is undertaken to improve the bioavailability of a cytotoxic covalent binder and ROS generator. Every increase of plasma levels would also increase the cytotoxic effects of curcumin. However, literature is flooded with references regarding curcumin (8202 hits for the term “curcumin” in PubMed in October 2015).

Thymoquinone

An emerging suspect is the quinone thymoquinone (**24**). This compound presents a similar picture as curcumin. A PubMed search results in 544 hits. Anti-inflammatory [82,83], antioxidant, [84] cardioprotective, [85] anti-fungal [86] and antileishmanial activities (IC_{50} = 1.16 µg/mL; *L. tropica* und 1.47 µg/mL; *L. infantum*) [87] are reported in the literature. The focus lies especially on the proposed anti-cancer activities. [88] However, only scarce data can be found on the cytotoxicity towards normal cells. Similar to the case of curcumin the bioavailability and water solubility of thymoquinone is poor. In a promising antitumor *in vivo* study on mice, the compound was directly injected into the tumor. [89] To solve the problem of bioavailability nanoemulsions [90] and liposome formulations [91] have been proposed. In one study, a maximum of around 0.4 µg/mL was found 2 h after oral administration of a thymoquinone suspension in rats and around 0.9 µg/mL after administration of a lipid nanoparticle preparation. [92] Cytotoxicity studies *in vivo* report no toxic effects in rats after oral administration, however the authors did not determine the plasma levels of the compound. [93] In contrast, hepatotoxic effects were found *in vitro*. [94]

There is evidence that the activity *in vitro* is caused by the generation of reactive oxygen species. [95] No reports on the possible reactivity to nucleophiles due to the Michael acceptor are available. However, for quinones in general this mechanism is well established. [12]

There is the possibility that this compound will be investigated further e.g. in clinical trials, inspiring numerous publications on its putative healing properties similar to the case of curcumin. Therefore, one needs to break the vicious circle of publications based on promising PAIN compounds with multiple “false friend” activities caused by unselective mechanisms.

Handling PAIN ridden natural products

How can we avoid to get caught in the trap of such seemingly promising compounds? First, the reactive substructures have to be identified and second, the reactivity has to be tested.

Some measures to cope with promiscuous behaviour in HTS have been compiled in the literature. Baell et al. [96] state that many PAINS can be readily identified by structure – others cannot be recognized at first glance. Promiscuous compounds can signal in biochemical assays as well as in cell assays and may even give the desired results *in vivo*. [97]

Therefore it is important to consult an experienced hit-to-lead medicinal chemist before or after the first screening. 1) Every hit should be assumed false until proven otherwise. 2) The screening sample should be pure, which has to be proven by LC/MS which is especially important for natural products isolated from a plant extract. 3) The activity must be confirmed under conditions where aggregation is not possible [98] 4) If activity is further confirmed in orthogonal assays, structure-activity relationship (SAR) investigations of a library of structurally related, newly synthesized compounds may be started to improve the activity. [97] 5) SAR results are suspicious, when both major and minor changes to the structure lead to minor changes in activity only. [99] This is referred to as structure-interference relationship (SIR).[14]

Dahlin et al. [14,100] give more elaborate advice for the identification of unselective reactivity in HTS. The authors differentiate between “knowledge-based methods” and “experimental-based methods”. Knowledge-based methods are the first step to identify possible PAIN candidates. 1) Literature research and screening of databases (e.g. Scifinder, PubMed, PubChem) for publications reporting multiple activities or discussing possible unselective mechanisms or assay interferences. 2) Application of computer programs using substructure filters (based e.g. on functional groups and/or physical properties like PAINS or REOS = Rapid Elimination Of Swill [101]) on compound databases to eliminate suspicious compounds from the collection before screening. 3) Computer calculations using fragment molecular orbital method (FMO) to investigate possible binding interactions. [102] 4) Following principles of medicinal chemistry.

To exclude possible pan-assay interferences at least two of the following experimental methods should be used. For aggregation use a counter screen: conduct assay with and without the addition of an aggregation suppressor (e.g. Triton X-100) or oxidation suppressor (e.g. dithiothreitol DTT), or conduct mechanistic experiments e.g. compound-target dilution; determine assay activity with and without compound dialysis; investigate if activity is time-dependent. For redox cycling: Test redox activity in apt redox assays e.g. using the detection reagent HRP-PR (horseradish peroxidase-phenol red). [103] For unspecific covalent labelling use counter screens: test the target´s

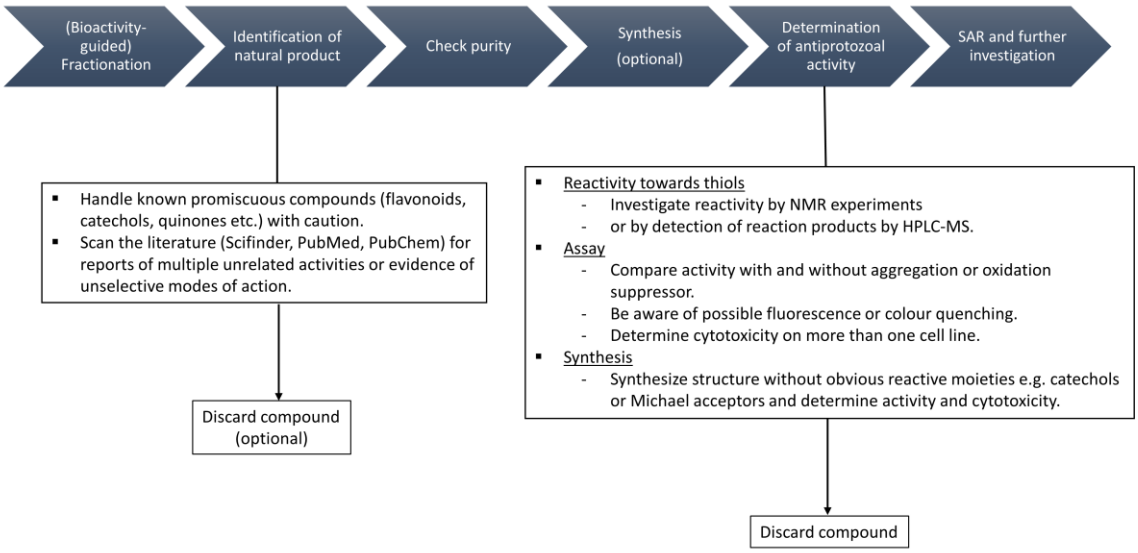


Fig.4. Selection of a minimum number of counter measures to be implemented into the process of antiprotozoal natural product discovery.

susceptibility to reactive compounds; investigate the compound´s activity against unrelated targets or, test sensitivity to nucleophilic attack with small-molecule thiol probes e.g. cysteamine or glutathione, or test susceptibility to nucleophilic attack with protein probes e.g. by ALARM NMR/MS. [104]

However, as the methods described above require time and resources, which may not always be available, we selected a minimum number of easy-to-follow measures and principles (s. Fig. 4) which should be implemented into the process of identification of new antiprotozoal natural products prior to publication. For example, simple NMR methods for the identification of thiol-trapping agents [105] or the determination of the reaction rate of Michael acceptors with sulfhydryl groups are available [106]. Another acceptable method could be the synthesis and subsequent testing of the compound without the obvious reactive moieties. At least determination of cytotoxicity on more than one mammalian cell line should be considered.

Covalent binding as desired mode of action

Some blockbuster drugs in use, e.g. β-lactamase inhibitors, aspirin, clopidogrel or omeprazole, [107] are covalent binding drugs. Despite their reactive mechanism these are highly selective. In contrast to promiscuous and very reactive compounds, these selective covalently binding drugs have weak electrophilic warheads only. [108] Some

of them are covalent reversible binding drugs e.g. avibactam [109] and related β-lactamase inhibitors. It goes without saying that they selectively bind to just one target. Obviously, the search for selective covalent drugs requires a different approach than the classic drug development for selective inhibitors. Such targeted covalent inhibitors (TCI) have to fulfil some requirements de-risking off-target effect: 1) high affinity and selectivity to one target and a tuned reactivity, 2) a necessary daily dose lower than 10 mg and 3) minimal formation of reactive metabolites. [108]
Dahlin et al. state that PAIN compounds cannot be developed into selective covalent binding drugs. It would be impossible to link activity to the target of interest. [99] However, not all compounds with PAIN characteristics should be discarded a priori. Just recently (-)-englerin A was reported as a selective activator of TRPC4 and TRPC5 calcium channels despite its Michael system. [110] Therefore, it must be pondered thoroughly for every single case if the compound is apt for further investigations.

Conclusion

In summary, natural products with PAIN characteristics can feign positive results in assays designed to find selective inhibitors for a defined target. If unrecognized they appear as new lead structures in the literature and are even patented or considered for clinical trials. However, these compounds may not be suitable for classical drug or ligand design, due to their unselective modes of action. The scientific community has been made aware of the concept of PAINS [6,111] but a lot of time and effort is still invested into the improvement of the same PAIN ridden natural phytochemicals. One has to keep in mind that most phytochemicals are mainly meant to be an unselective defence mechanism against all kinds of herbivores and are therefore always promiscuous by necessity. Nevertheless, these natural products may be a good starting point for drug development e.g. when it is possible to omit the “PAINS” moieties without the loss of activity, as has been recently demonstrated for antileishmanial caffeic acid esters [112] or to gain selectivity by structural modifications.

Notes and References

1. Takala-Harrison, S.; Laufer, M. K., Antimalarial drug resistance in Africa: key lessons for the future. *Ann. N.Y. Acad. Sci.* **2015**, 1342, *1*, 62-67.

2. Chakravarty, J.; Sundar, S., Drug resistance in Leishmaniasis. *J. Global Infect. Dis.* **2010**, *2*, 167-176.
3. Klayman, D. L., Qinghaosu (artemisinin): an antimalarial drug from China. *Science* **1985**, *228*, 1049-1055.
4. Wani, M. C.; Taylor, H. L.; Wall, M. E.; Coggon, P.; McPhail, A. T., Plant antitumor agents. VI. Isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *J. Am. Chem. Soc.* **1971**, *93*, 2325-2327.
5. Siqueira-Neto, J. L.; Song, O.-R.; Oh, H.; Sohn, J.-H.; Yang, G.; Nam, J.; Jang, J.; Cechetto, J.; Lee, C. B.; Moon, S., Antileishmanial high-throughput drug screening reveals drug candidates with new scaffolds. *PloS Negl. Trop. Dis.* **2010**, *4*, e675. doi:10.1371/journal.pntd.0000675.
6. Baell, J.; Walters, M. A., Chemistry: Chemical con artists foil drug discovery. *Nature News* **2014**, *513*, 481-483.
7. Bisson, J.; McAlpine, J. B.; Friesen, J. B.; Chen, S.-N.; Graham, J.; Pauli, G. F., Can invalid bioactives undermine natural product-based drug discovery? *J. Med. Chem.* **2015**. doi: 10.1021/acs.jmedchem.5b01009.
8. Baell, J. B.; Holloway, G. A., New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays. *J. Med. Chem.* **2010**, *53*, 2719-2740.
9. Rana, P.; Naven, R.; Narayanan, A.; Will, Y.; Jones, L. H., Chemical motifs that redox cycle and their associated toxicity. *Med. Chem. Commun.* **2013**, *4*, 1175-1180.
10. Schweigert, N.; Zehnder, A. J.; Eggen, R. I., Chemical properties of catechols and their molecular modes of toxic action in cells, from microorganisms to mammals. *Environ. Microbiol.* **2001**, *3*, 81-91.
11. Monks, T. J.; Hanzlik, R. P.; Cohen, G. M.; Ross, D.; Graham, D. G., Quinone chemistry and toxicity. *Toxicol. Appl. Pharmacol.* **1992**, *112*, 2-16.
12. O'Brien, P. J., Molecular mechanisms of quinone cytotoxicity. *Chem. Biol. Interact.* **1991**, *80*, 1-41.
13. Schultz, T. W.; Yarbrough, J. W.; Hunter, R. S.; Aptula, A. O., Verification of the structural alerts for Michael acceptors. *Chem. Res. Toxicol.* **2007**, *20*, 1359-1363.

14. Dahlin, J. L.; Nissink, J. W. M.; Strasser, J. M.; Francis, S.; Higgins, L.; Zhou, H.; Zhang, Z.; Walters, M. A., PAINS in the assay: chemical mechanisms of assay interference and promiscuous enzymatic inhibition observed during a sulfhydryl-scavenging HTS. *J. Med. Chem.* **2015**, *58*, 2091-2113.
15. Schmidt, T.; Nour, A.; Khalid, S.; Kaiser, M.; Brun, R., Quantitative structure – antiprotozoal activity relationships of sesquiterpene lactones. *Molecules* **2009**, *14*, 2062-2076.
16. Andjelković, M.; Van Camp, J.; De Meulenaer, B.; Depaemelaere, G.; Socaciu, C.; Verloo, M.; Verhe, R., Iron-chelation properties of phenolic acids bearing catechol and galloyl groups. *Food Chem.* **2006**, *98*, 23-31.
17. Ingólfsson, H. I.; Thakur, P.; Herold, K. F.; Hobart E. A.; Ramsey N. B.; Periole X.; de Jong D. H.; Zwama M.; Yilmaz D.; Hall K.; Maetzky T.; Hemmings H. C.; Blobel C.; Marrink S. J.; Koçer, A., Phytochemicals promiscuously alter membrane protein function and bilayer properties. *ACS Chem. Biol.* **2014**, *9*, 1788-1798.
18. Turek-Etienne, T. C.; Small, E. C.; Soh, S. C.; Xin, T. A.; Gaitonde, P. V.; Barrabee, E. B.; Hart, R. F.; Bryant, R. W., Evaluation of fluorescent compound interference in 4 fluorescence polarization assays: 2 kinases, 1 protease, and 1 phosphatase. *J. Biomol. Screen.* **2003**, *8*, 176-184.
19. McGovern, S. L.; Caselli, E.; Grigorieff, N.; Shoichet, B. K., A common mechanism underlying promiscuous inhibitors from virtual and high-throughput screening. *J. Med. Chem.* **2002**, *45*, 1712-1722.
20. Shoichet, B. K., Screening in a spirit haunted world. *Drug Discov. Today* **2006**, *11*, 607-615.
21. Pohjala, L.; Tammela, P., Aggregating behavior of phenolic compounds—a source of false bioassay results? *Molecules* **2012**, *17*, 10774-10790.
22. Ferrins, L.; Rahmani, R.; Baell, J. B., Drug discovery and human African trypanosomiasis: a disease less neglected? *Fut. Med. Chem.* **2013**, *5*, 1801-1841.
23. Arango, V.; Robledo, S.; Séon-Méniel, B.; Figadere, B.; Cardona, W.; Sáez, J.; Otálvaro, F., Coumarins from *Galipea panamensis* and their activity against *Leishmania panamensis*. *J. Nat. Prod.* **2010**, *73*, 1012-1014.
24. Dastan, D.; Salehi, P.; Gohari, A. R.; Zimmermann, S.; Kaiser, M.; Hamburger, M.; Khavasi, H. R.; Ebrahimi, S. N., Disesquiterpene and sesquiterpene

coumarins from *Ferula pseudalliacea*, and determination of their absolute configurations. *Phytochemistry* **2012**, *78*, 170-178.

25. Maas, M.; Hensel, A.; da Costa, F. B.; Brun, R.; Kaiser, M.; Schmidt, T. J., An unusual dimeric guaianolide with antiprotozoal activity and further sesquiterpene lactones from *Eupatorium perfoliatum*. *Phytochemistry* **2011**, *72*, 635-644.
26. Barrera, P.; Sülsen, V. P.; Lozano, E.; Rivera, M.; Beer, M. F.; Tonn, C.; Martino, V. S.; Sosa, M. A., Natural sesquiterpene lactones induce oxidative stress in *Leishmania mexicana*. *Evid. Based Complement. Alternat. Med.* **2013**. http://dx.doi.org/10.1155/2013/621459.
27. Negi, J.; Bisht, V.; Singh, P.; Rawat, M.; Joshi, G., Naturally occurring xanthones: Chemistry and biology. *J. Appl. Chem.* **2013**. http://dx.doi.org/10.1155/2013/621459.
28. Upegui, Y.; Robledo, S. M.; Gil Romero, J. F.; Quiñones, W.; Archbold, R.; Torres, F.; Escobar, G.; Nariño, B.; Echeverri, F., *In vivo* antimalarial activity of α-mangostin and the new xanthone δ-mangostin. *Phytother. Res.* **2015**, *29*, 1195-1201.
29. Hata, Y.; De Mieri, M.; Ebrahimi, S.; Zimmermann, S.; Melanie, R.; Naidoo, D.; Fouche, G.; Mokoka, T.; Maharaj, V.; Kaiser, U., Antiprotozoal isoflavan quinones from *Abrus precatorius*. *Planta Med.* **2012**, *79*, 492-498.
30. Beniddir, M. A.; Grellier, P.; Rasoanaivo, P.; Loiseau, P. M.; Bories, C.; Dumontet, V.; Guéritte, F.; Litaudon, M., Diarylheptanoid glucosides from *Pyrostria major* and their antiprotozoal activities. *Eur. J. Org. Chem.* **2012**, *2012*, 1039-1046.
31. Salem, M. M.; Capers, J.; Rito, S.; Werbovetz, K. A., Antiparasitic ativity of C-geranyl flavonoids from *Mimulus bigelovii*. *Phytother. Res.* **2011**, *25*, 1246-1249.
32. Burda, S.; Oleszek, W., Antioxidant and antiradical activities of flavonoids. *J. Agric. Food. Chem.* **2001**, *49*, 2774-2779.
33. Basile, A.; Giordano, S.; López-Sáez, J. A.; Cobianchi, R. C., Antibacterial activity of pure flavonoids isolated from mosses. *Phytochemistry* **1999**, *52*, 1479-1482.
34. Cárdenas, M.; Marder, M.; Blank, V. C.; Roguin, L. P., Antitumor activity of some natural flavonoids and synthetic derivatives on various human and murine cancer cell lines. *Biorg. Med. Chem.* **2006**, *14*, 2966-2971.

35. Tadera, K.; Minami, Y.; Takamatsu, K.; Matsuoka, T., Inhibition of α-glucosidase and α-amylase by flavonoids. *J. Nutr. Sci. Vitaminol.* **2006**, 52, (2), 149-153.
36. Mäkelä, S.; Poutanen, M.; Kostian, M.; Lehtimäki, N.; Strauss, L.; Santti, R.; Vihko, R., Inhibition of 17β-hydroxysteroid oxidoreductase by flavonoids in breast and prostate cancer cells. *Exp. Biol. Med.* **1998**, *217*, 310-316.
37. Liang, Y.-C.; Huang, Y.-T.; Tsai, S.-H.; Lin-Shiau, S.-Y.; Chen, C.-F.; Lin, J.-K., Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages. *Carcinogenesis* **1999**, *20*, 1945-1952.
38. Tasdemir, D.; Kaiser, M.; Brun, R.; Yardley, V.; Schmidt, T. J.; Tosun, F.; Rüedi, P., Antitrypanosomal and antileishmanial activities of flavonoids and their analogues: *in vitro*, *in vivo*, structure-activity relationship, and quantitative structure-activity relationship studies. *Antimicrob. Agents Chemother.* **2006**, *50*, 1352-1364.
39. Fonseca-Silva, F.; Canto-Cavalheiro, M. M.; Menna-Barreto, R. F.; Almeida-Amaral, E. E., Effect of apigenin on *Leishmania amazonensis* is associated with reactive oxygen species production followed by mitochondrial dysfunction. *J. Nat. Prod.* **2015**, *78*, 880-884.
40. da Silva, E. R.; do Carmo Maquiaveli, C.; Magalhães, P. P., The leishmanicidal flavonols quercetin and quercitrin target *Leishmania* (*Leishmania*) *amazonensis* arginase. *Exp. Parasitol.* **2012**, *130*, 183-188.
41. Cota, B. B.; Siqueira, E. P.; Oliveira, D. M. d.; Alves, T.; Sobral, M. E.; Rabello, A.; Zani, C. L., Chemical constituents and leishmanicidal activity from leaves of *Kielmeyera variabilis*. *Rev. Bras. Farmacogn.* **2012**, *22*, 1253-1258.
42. Manjolin, L. C.; dos Reis, M. B. G.; do Carmo Maquiaveli, C.; Santos-Filho, O. A.; da Silva, E. R., Dietary flavonoids fisetin, luteolin and their derived compounds inhibit arginase, a central enzyme in *Leishmania* (*Leishmania*) *amazonensis* infection. *Food Chem.* **2013**, *141*, 2253-2262.
43. Chen, M.; Christensen, S.; Theander, T. G.; Kharazmi, A., Antileishmanial activity of licochalcone A in mice infected with *Leishmania major* and in hamsters infected with *Leishmania donovani*. *Antimicrob. Agents Chemother.* **1994**, *38*, 1339-1344.

44. Chen, M.; Christensen, S. B.; Blom, J.; Lemmich, E.; Nadelmann, L.; Fich, K.; Theander, T. G.; Kharazmi, A., Licochalcone A, a novel antiparasitic agent with potent activity against human pathogenic protozoan species of *Leishmania*. *Antimicrob. Agents Chemother.* **1993**, *37*, 2550-2556.
45. Zhai, L.; Chen, M.; Blom, J.; Theander, T. G.; Christensen, S. B.; Kharazmi, A., The antileishmanial activity of novel oxygenated chalcones and their mechanism of action. *J. Antimicrob. Chemother.* **1999**, *43*, 793-803.
46. Chen, M.; Theander, T. G.; Christensen, S.; Hviid, L.; Zhai, L.; Kharazmi, A., Licochalcone A, a new antimalarial agent, inhibits *in vitro* growth of the human malaria parasite *Plasmodium falciparum* and protects mice from *P. yoelii* infection. *Antimicrob. Agents Chemother.* **1994**, *38*, 1470-1475.
47. Sinha, S.; Medhi, B.; Sehgal, R., Chalcones as an emerging lead molecule for antimalarial therapy: a review. *J. Mod. Med. Chem.* **2013**, *1*, 64-77.
48. Awasthi, S. K.; Mishra, N.; Kumar, B.; Sharma, M.; Bhattacharya, A.; Mishra, L. C.; Bhasin, V. K., Potent antimalarial activity of newly synthesized substituted chalcone analogs *in vitro*. *Med. Chem. Res.* **2009**, *18*, 407-420.
49. Friis-Møller, A.; Chen, M.; Fuursted, K.; Christensen, S. B.; Kharazmi, A., In vitro antimycobacterial and antilegionella activity of licochalcone A from Chinese licorice roots. *Planta Med.* **2002**, *68*, 416-419.
50. Kolbe, L.; Immeyer, J.; Batzer, J.; Wensorra, U.; tom Dieck, K.; Mundt, C.; Wolber, R.; Stäb, F.; Schönrock, U.; Ceilley, R. I., Anti-inflammatory efficacy of Licochalcone A: correlation of clinical potency and *in vitro* effects. *Arch. Dermatol. Res.* **2006**, *298*, 23-30.
51. Tadigoppula, N.; Korthikunta, V.; Gupta, S.; Kancharla, P.; Khaliq, T.; Soni, A.; Srivastava, R. K.; Srivastava, K.; Puri, S. K.; Raju, K. S. R., Synthesis and insight into the structure–activity relationships of chalcones as antimalarial agents. *J. Med. Chem.* **2012**, *56*, 31-45.
52. Ziegler, H. L.; Hansen, H. S.; Stærk, D.; Christensen, S. B.; Hägerstrand, H.; Jaroszewski, J. W., The antiparasitic compound licochalcone A is a potent echinocytogenic agent that modifies the erythrocyte membrane in the concentration range where antiplasmodial activity is observed. *Antimicrob. Agents Chemother.* **2004**, *48*, 4067-4071.

53. Shivahare, R.; Korthikunta, V.; Chandasana, H.; Suthar, M. K.; Agnihotri, P.; Vishwakarma, P.; Chaitanya, T. K.; Kancharla, P.; Khaliq, T.; Gupta, S., Synthesis, structure–activity relationships, and biological studies of chromenochalcones as potential antileishmanial agents. *J. Med. Chem.* **2014**, *57*, 3342-3357.
54. Gupta, S.; Shivahare, R.; Korthikunta, V.; Singh, R.; Gupta, S.; Tadigoppula, N., Synthesis and biological evaluation of chalcones as potential antileishmanial agents. *Eur. J. Med. Chem.* **2014**, *81*, 359-366.
55. Priyadarsini, K. I., The chemistry of curcumin: From extraction to therapeutic agent. *Molecules* **2014**, *19*, 20091-20112.
56. Bairwa, K.; Grover, J.; Kania, M.; Jachak, S. M., Recent developments in chemistry and biology of curcumin analogues. *RSC Advances* **2014**, *4*, 13946-13978.
57. Goel, A.; Kunnumakkara, A. B.; Aggarwal, B. B., Curcumin as “Curecumin”: from kitchen to clinic. *Biochem. Pharmacol.* **2008**, *75*, 787-809.
58. Shanmugam, M. K.; Rane, G.; Kanchi, M. M.; Arfuso, F.; Chinnathambi, A.; Zayed, M.; Alharbi, S. A.; Tan, B. K.; Kumar, A. P.; Sethi, G., The multifaceted role of curcumin in cancer prevention and treatment. *Molecules* **2015**, *20*, 2728-2769.
59. Tayyem, R. F.; Heath, D. D.; Al-Delaimy, W. K.; Rock, C. L., Curcumin content of turmeric and curry powders. *Nutr. Cancer* **2006**, *55*, 126-131.
60. Barry, J.; Fritz, M.; Brender, J. R.; Smith, P. E.; Lee, D.-K.; Ramamoorthy, A., Determining the effects of lipophilic drugs on membrane structure by solid-state NMR spectroscopy: the case of the antioxidant curcumin. *J. Am. Chem. Soc.* **2009**, *131*, 4490-4498.
61. Awasthi, S.; Pandya, U.; Singhal, S. S.; Lin, J. T.; Thiviyanathan, V.; Seifert, W. E.; Awasthi, Y. C.; Ansari, G., Curcumin–glutathione interactions and the role of human glutathione *S*-transferase P1-1. *Chem. Biol. Interact.* **2000**, *128*, 19-38.
62. Jiao, Y.; Wilkinson Iv, J.; Christine Pietsch, E.; Buss, J. L.; Wang, W.; Planalp, R.; Torti, F. M.; Torti, S. V., Iron chelation in the biological activity of curcumin. *Free Radical Biol. Med.* **2006**, *40*, 1152-1160.
63. Fujisawa, S.; Atsumi, T.; Ishihara, M.; Kadoma, Y., Cytotoxicity, ROS-generation activity and radical-scavenging activity of curcumin and related compounds. *Anticancer Res.* **2004**, *24*, 563-570.

64. Das, R.; Roy, A.; Dutta, N.; Majumder, H. K., Reactive oxygen species and imbalance of calcium homeostasis contributes to curcumin induced programmed cell death in *Leishmania donovani*. *Apoptosis* **2008**, *13*, 867-882.
65. Cui, L.; Miao, J.; Cui, L., Cytotoxic effect of curcumin on malaria parasite Plasmodium falciparum: inhibition of histone acetylation and generation of reactive oxygen species. *Antimicrob. Agents Chemother.* **2007**, *51*, 488-494.
66. Alkhaldi, A. A.; Creek, D. J.; Ibrahim, H.; Kim, D.-H.; Quashie, N. B.; Burgess, K. E.; Changtam, C.; Barrett, M. P.; Suksamrarn, A.; de Koning, H. P., Potent Trypanocidal curcumin analogs bearing a monoenone linker motif act on *Trypanosoma brucei* by forming an adduct with trypanothione. *Mol. Pharmacol.* **2015**, *87*, 451-464.
67. Koide, T.; Nose, M.; Ogihara, Y.; Yabu, Y.; Ohta, N., Leishmanicidal effect of curcumin *in vitro*. *Biol. Pharm. Bull.* **2002**, *25*, 131-133.
68. Anand, P.; Thomas, S. G.; Kunnumakkara, A. B.; Sundaram, C.; Harikumar, K. B.; Sung, B.; Tharakan, S. T.; Misra, K.; Priyadarsini, I. K.; Rajasekharan, K. N., Biological activities of curcumin and its analogues (Congeners) made by man and Mother Nature. *Biochem. Pharmacol.* **2008**, *76*, 1590-1611.
69. Changtam, C.; de Koning, H. P.; Ibrahim, H.; Sajid, M. S.; Gould, M. K.; Suksamrarn, A., Curcuminoid analogs with potent activity against *Trypanosoma* and *Leishmania* species. *Eur. J. Med. Chem.* **2010**, *45*, 941-956.
70. Kuttan, R.; Bhanumathy, P.; Nirmala, K.; George, M., Potential anticancer activity of turmeric (*Curcuma longa*). *Cancer Lett.* **1985**, *29*, 197-202.
71. Yang, F.; Lim, G. P.; Begum, A. N.; Ubeda, O. J.; Simmons, M. R.; Ambegaokar, S. S.; Chen, P. P.; Kayed, R.; Glabe, C. G.; Frautschy, S. A., Curcumin inhibits formation of amyloid β oligomers and fibrils, binds plaques, and reduces amyloid *in vivo*. *J. Biol. Chem.* **2005**, *280*, 5892-5901.
72. Begum, A. N.; Jones, M. R.; Lim, G. P.; Morihara, T.; Kim, P.; Heath, D. D.; Rock, C. L.; Pruitt, M. A.; Yang, F.; Hudspeth, B., Curcumin structure-function, bioavailability, and efficacy in models of neuroinflammation and Alzheimer's disease. *J. Pharmacol. Exp. Ther.* **2008**, *326*, 196-208.
73. Baum, L.; Lam, C. W. K.; Cheung, S. K.-K.; Kwok, T.; Lui, V.; Tsoh, J.; Lam, L.; Leung, V.; Hui, E.; Ng, C., Six-month randomized, placebo-controlled, double-

blind, pilot clinical trial of curcumin in patients with Alzheimer disease. *J Clin Psychopharmacol.* **2008**, *28*, 110-113.

74. Ringman, J. M.; Frautschy, S. A.; Teng, E.; Begum, A. N.; Bardens, J.; Beigi, M.; Gylys, K. H.; Badmaev, V.; Heath, D. D.; Apostolova, L. G., Oral curcumin for Alzheimer's disease: tolerability and efficacy in a 24-week randomized, double blind, placebo-controlled study. *Alzheimers Res. Ther.* **2012**, *4*, 43-43.
75. Tønnesen, H. H.; Másson, M.; Loftsson, T., Studies of curcumin and curcuminoids. XXVII. Cyclodextrin complexation: solubility, chemical and photochemical stability. *Int. J. Pharm.* **2002**, *244*, 127-135.
76. Pan, M.-H.; Huang, T.-M.; Lin, J.-K., Biotransformation of curcumin through reduction and glucuronidation in mice. *Drug Metab. Dispos.* **1999**, *27*, 486-494.
77. Lao, C. D.; Ruffin, M. T.; Normolle, D.; Heath, D. D.; Murray, S. I.; Bailey, J. M.; Boggs, M. E.; Crowell, J.; Rock, C. L.; Brenner, D. E., Dose escalation of a curcuminoid formulation. *BMC Complement. Altern. Med.* **2006**, *6*. doi:10.1186/1472-6882-6-10.
78. Burgos-Morón, E.; Calderón-Montaño, J. M.; Salvador, J.; Robles, A.; López-Lázaro, M., The dark side of curcumin. *Int. J. Cancer* **2010**, *126*, 1771-1775.
79. Shoba, G.; Joy, D.; Joseph, T.; Majeed, M.; Rajendran, R.; Srinivas, P. S. S. R., Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. *Planta Med.* **1998**, *64*, 353-356.
80. Shaikh, J.; Ankola, D.; Beniwal, V.; Singh, D.; Kumar, M. R., Nanoparticle encapsulation improves oral bioavailability of curcumin by at least 9-fold when compared to curcumin administered with piperine as absorption enhancer. *Eur. J. Pharm. Sci.* **2009**, *37*, 223-230.
81. Kanai, M.; Imaizumi, A.; Otsuka, Y.; Sasaki, H.; Hashiguchi, M.; Tsujiko, K.; Matsumoto, S.; Ishiguro, H.; Chiba, T., Dose-escalation and pharmacokinetic study of nanoparticle curcumin, a potential anticancer agent with improved bioavailability, in healthy human volunteers. *Cancer Chemother. Pharmacol.* **2012**, *69*, 65-70.
82. El Gazzar, M.; El Mezayen, R.; Marecki, J. C.; Nicolls, M. R.; Canastar, A.; Dreskin, S. C., Anti-inflammatory effect of thymoquinone in a mouse model of allergic lung inflammation. *Int. Immunopharmacol.* **2006**, *6*, 1135-1142.

83. Chehl, N.; Chipitsyna, G.; Gong, Q.; Yeo, C. J.; Arafat, H. A., Anti-inflammatory effects of the *Nigella sativa* seed extract, thymoquinone, in pancreatic cancer cells. *HPB* **2009**, *11*, 373-381.
84. Mansour, M. A.; Nagi, M. N.; El-Khatib, A. S.; Al-Bekairi, A. M., Effects of thymoquinone on antioxidant enzyme activities, lipid peroxidation and DT-diaphorase in different tissues of mice: a possible mechanism of action. *Cell Biochem. Funct.* **2002**, *20*, 143-151.
85. Gonca, E.; Kurt, Ç., Cardioprotective effect of Thymoquinone: A constituent of *Nigella sativa* L., against myocardial ischemia/reperfusion injury and ventricular arrhythmias in anaesthetized rats. *Pak. J. Pharm. Sci.* **2015**, *28*, 1267-1273.
86. Al-Qurashi, A. R.; Akhtar, N.; Al-Jabre, S.; Omar, A.-A.; Randhawa, M. A., Anti-fungal activity of thymoquinone and amphotericine B against *Aspergillus niger*. *Scientific Journal of King Faisal University* **2007**, *8*, 137-145.
87. Mahmoudvand, H.; Tavakoli, R.; Sharififar, F.; Minaie, K.; Ezatpour, B.; Jahanbakhsh, S.; Sharifi, I., Leishmanicidal and cytotoxic activities of *Nigella sativa* and its active principle, thymoquinone. *Pharm. Biol.* **2014**, 1-6.
88. Gali-Muhtasib, H.; Roessner, A.; Schneider-Stock, R., Thymoquinone: a promising anti-cancer drug from natural sources. *Int. J. Biochem. Cell Biol.* **2006**, *38*, 1249-1253.
89. Ivankovic, S.; Stojkovic, R.; Jukic, M.; Milos, M.; Milos, M.; Jurin, M., The antitumor activity of thymoquinone and thymohydroquinone *in vitro* and *in vivo*. *Exp. Oncol.* **2006**, *28*, 220-224.
90. Tubesha, Z.; Bakar, Z. A.; Ismail, M., Characterization and stability evaluation of thymoquinone nanoemulsions prepared by high-pressure homogenization. *J. Nanomater.* **2013**. http://dx.doi.org/10.1155/2013/453290.
91. Odeh, F.; Ismail, S. I.; Abu-Dahab, R.; Mahmoud, I. S.; Al Bawab, A., Thymoquinone in liposomes: a study of loading efficiency and biological activity towards breast cancer. *Drug Deliv.* **2012**, *19*, 371-377.
92. Pathan, S. A.; Jain, G. K.; Zaidi, S. M. A.; Akhter, S.; Vohora, D.; Chander, P.; Kole, P. L.; Ahmad, F. J.; Khar, R. K., Stability-indicating ultra-performance liquid chromatography method for the estimation of thymoquinone and its application in biopharmaceutical studies. *Biomed. Chromatogr.* **2011**, *25*, 613-620.

93. Badary, O. A.; Al-Shabanah, O. A.; Nagi, M. N.; Al-Bekairi, A. M.; Elmazar, M., Acute and subchronic toxicity of thymoquinone in mice. *Drug Dev. Res.* **1998**, *44*, 56-61.
94. Khader, M.; Bresgen, N.; Eckl, P. M., *In vitro* toxicological properties of thymoquinone. *Food Chem. Toxicol.* **2009**, *47*, 129-133.
95. El-Najjar, N.; Chatila, M.; Moukadem, H.; Vuorela, H.; Ocker, M.; Gandesiri, M.; Schneider-Stock, R.; Gali-Muhtasib, H., Reactive oxygen species mediate thymoquinone-induced apoptosis and activate ERK and JNK signaling. *Apoptosis* **2010**, *15*, 183-195.
96. Baell, J. B.; Ferrins, L.; Falk, H.; Nikolakopoulos, G., PAINS: relevance to tool compound discovery and fragment-based screening. *Aust. J. Chem.* **2014**, *66*, 1483-1494.
97. Baell, J. B., Screening-based translation of public research encounters painful problems. *ACS Med. Chem. Lett.* **2015**, *6*, 229-234.
98. Thorne, N.; Auld, D. S.; Inglese, J., Apparent activity in high-throughput screening: origins of compound-dependent assay interference. *Curr. Opin. Chem. Biol.* **2010**, *14*, 315-324.
99. Dahlin, J. L.; Inglese, J.; Walters, M. A., Mitigating risk in academic preclinical drug discovery. *Nat. Rev. Drug Discov.* **2015**, *14*, 279-294.
100. Dahlin, J.; Baell, J.; Walters, M., Assay interference by chemical reactivity. **2015**. Available from: http://www.ncbi.nlm.nih.gov/books/NBK326709.
101. Walters, W. P.; Namchuk, M., Designing screens: how to make your hits a hit. *Nat. Rev. Drug Discov.* **2003**, *2*, 259-266.
102. Curpăn, R.; Avram, S.; Vianello, R.; Bologa, C., Exploring the biological promiscuity of high-throughput screening hits through DFT calculations. *Biorg. Med. Chem.* **2014**, *22*, 2461-2468.
103. Johnston, P. A., Redox cycling compounds generate H_2O_2 in HTS buffers containing strong reducing reagents - real hits or promiscuous artifacts? *Curr. Opin. Chem. Biol.* **2011**, *15*, 174-182.
104. Huth, J. R.; Mendoza, R.; Olejniczak, E. T.; Johnson, R. W.; Cothron, D. A.; Liu, Y.; Lerner, C. G.; Chen, J.; Hajduk, P. J., ALARM NMR: a rapid and robust experimental method to detect reactive false positives in biochemical screens. *J. Am. Chem. Soc.* **2005**, *127*, 217-224.

105.Avonto, C.; Taglialatela-Scafati, O.; Pollastro, F.; Minassi, A.; Di Marzo, V.; De Petrocellis, L.; Appendino, G., An NMR spectroscopic method to identify and classify thiol-trapping agents: Revival of Michael acceptors for drug discovery? *Angew. Chem. Int. Ed.* **2011**, *50*, 467-471.

106.Dinkova-Kostova, A. T.; Massiah, M. A.; Bozak, R. E.; Hicks, R. J.; Talalay, P., Potency of Michael reaction acceptors as inducers of enzymes that protect against carcinogenesis depends on their reactivity with sulfhydryl groups. *Proc. Natl. Acad. Sci.* **2001**, *98*, 3404-3409.

107.Potashman, M. H.; Duggan, M. E., Covalent modifiers: an orthogonal approach to drug design. *J. Med. Chem.* **2009**, *52*, 1231-1246.

108.Singh, J.; Petter, R. C.; Baillie, T. A.; Whitty, A., The resurgence of covalent drugs. *Nat. Rev. Drug Discov.* **2011**, *10*, 307-317.

109.Ehmann, D. E.; Jahić, H.; Ross, P. L.; Gu, R.-F.; Hu, J.; Kern, G.; Walkup, G. K.; Fisher, S. L., Avibactam is a covalent, reversible, non–β-lactam β-lactamase inhibitor. *Proc. Natl. Acad. Sci.* **2012**, *109*, 11663-11668.

110.Akbulut, Y.; Gaunt, H. J.; Muraki, K.; Ludlow, M. J.; Amer, M. S.; Bruns, A.; Vasudev, N. S.; Radtke, L.; Willot, M.; Hahn, S., (–)-Englerin A is a potent and selective activator of TRPC4 and TRPC5 calcium channels. *Angew. Chem.* **2015**, *127*, 3858-3862.

111.Erlanson, D. A., Learning from PAINful lessons. *J. Med. Chem.* **2015**, *58*, 2088-2090.

112.Glaser, J.; Schultheis, M.; Hazra, S.; Hazra, B.; Moll, H.; Schurigt, U.; Holzgrabe, U., Antileishmanial lead structures from nature: Analysis of structure-activity relationships of a compound library derived from caffeic acid bornyl ester. *Molecules* **2014**, *19*, 1394-1410.

4. DISCUSSION

The preparation and use of plant extracts for medicinal purposes has a longstanding tradition. The most basic preparation method is the heating of whole or pulverized parts of a plant with a solvent chosen to serve the purpose (commonly water or alcohol). The obtained extracts may then be consumed e.g. as tea or or in their dried form in capsules or tablets.

In a laboratory environment a soxhlet apparatus is standard equipment for the preparation of plant extracts. More modern and widely applied methods include microwave or ultrasound assisted extraction while less used methods are steam distillation, high pressure-liquid extraction and sorptive extraction [1].

For the isolation of certain compounds the solvent is chosen with respect to their polarity, e.g. water or alcohol for polar compounds like sugars and organic acids, chloroform or *n*-hexane for unpolar compounds like lipids or terpenoids. Solvent modifications in the form of additives, e.g. enzymes to ease the digestion of the plant matrix, organic acids or bases may increase the recovery of desired compounds.

The extract investigated in this work was prepared by standard soxhlet extraction of pulverized and dried rhizomes of *Valeriana* (*V.*) *wallichii* aquired from an herb shop in Kolkata. Chloroform was used as solvent, as this extract had shown the highest antileishmanial activity in previous reports [2].

The use of aequous and alcoholic extracts of *V. officinalis* is well established as the respective monographs in the European and United States Pharmacopoeia demonstrate. Despite the traditional use of *Valeriana* root extracts against sleeplessness and anxiety in folk medicine, recent investigations brought forward new and interesting properties. For instance, anti-inflammatory (*V. wallichii*) [3], anxiolytic (*V. officinalis*) [4], insecticidal (*V. jatamansi*) [5] and antidepressant (*V. fauriei*) [6] activities have been described.

To uncover the antileishmanial principle of the chloroform extract of *V. wallichii*, a purification process was initiated. The imperative underlying so-called bioactivity-guided fractionation is to find a suitable work-up method and an effective path of fractionation to isolate the desired active compounds. The unknown physicochemical properties of those substances may pose a problem in method development. Instability and limited amount of these compounds may eventually impede structure elucidation.

For the fractionation of the extract an assortment of techniques was available e.g. column chromatography, size-exclusion chromatography, preparative HPLC, flash chromatography, preparative TLC or liquid-liquid extraction.

In the case at hand, preparative HPLC with automated fraction collection seemed the method of choice providing better reproducibility in comparison to classic column chromatography. Reverse phase material (e.g. C_{18} modified silica gel as stationary phase) is standard here. Mixtures of H_2O with MeOH or CH_3CN are used as solvents, with or without additives.

Various fractionation schemes for the isolation of certain compounds from *V. wallichii* extracts can be found in the literature [7-9]. A number of HPLC methods has been developed for the determination of valtrates and valepotriates, typical *Valeriana* compounds, before [10-12]. However, as the chemical nature of the antileishmanial principle was unknown, a new method was developed and the antileishmanial activity was tracked throughout the fractionation process starting with the crude extract. This method was used to fractionate the extract (~ 10 g) into 12 fractions (see Fig. 1). All fractions were tested against *L. major* promastigotes *in vitro* by AlamarBlue© assays implemented in the SFB 630.

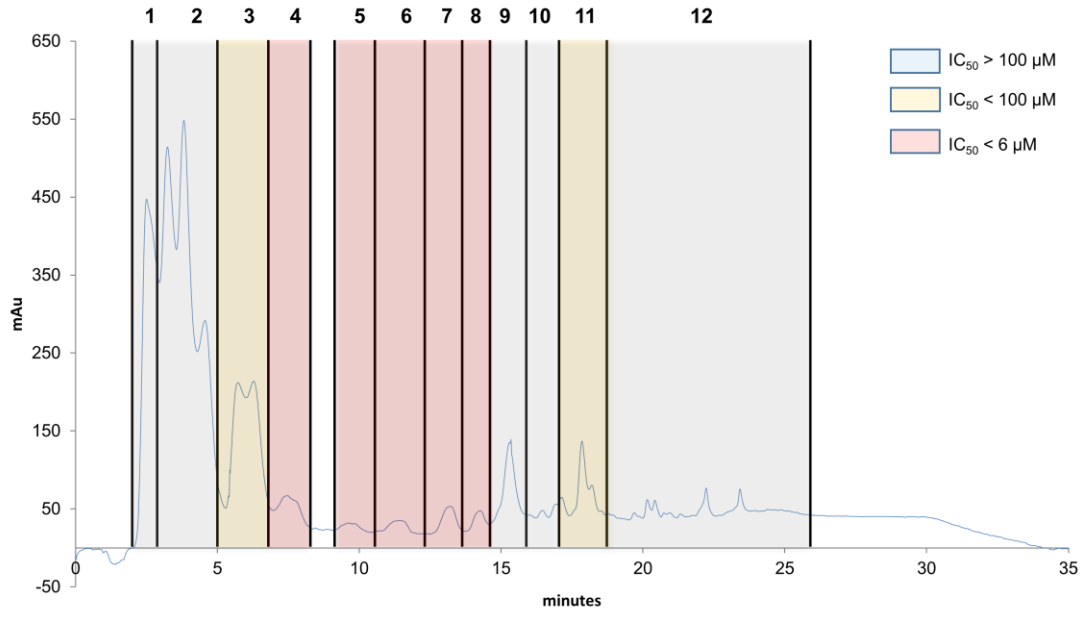


Fig.1: HPLC chromatogramm of first crude fractionation by preparative HPLC of *Valeriana wallichii* extract. Fractions 1-12 were collected and tested against *L. major* promastigotes *in vitro*. Different colours indicate the strength of found antileishmanial activity with < 6 µM being strongest and > 100 µM being inactive.

Further subfractionation was conducted by semi-preparative HPLC. For achieving good separation, an additional method was developed consisting of a full gradient using H_2O/MeOH on reverse phase material. For smaller fractions (> 100 mg) column chromatography on a small silica gel gravity column was more feasable. A mixture of $CHCl_3$/MeOH (4.6:0.4; v/v) was found to serve the purpose. Fractionation was continued with either one of these methods until the obtained fraction was pure enough to provide “interpretable” NMR data for structure elucidation.

In first experiments the 12 fractions yielded from preparative HPLC fractionation were investigated by TLC ($CHCl_3$/MeOH; 4.6:0.4; v/v) on silica gel (F_{254}). Staining reagents were applied to obtain information about functional groups. A mixture of vanillin in MeOH acidified with concentrated H_2SO_4 was used for the detection of higher alcohols and ketones. Especially terpenoid alcohols gave characteristically coloured spots. Dragendorff reagent (consisting of potassium bismuth iodide) was used for alkaloids. No positive reaction was found with Dragendorffs reagent, indicating the absence of alkaloids in the extract. Unsaturated compounds were detected by iodine vapours or staining with $KMnO_4$, UV-active compounds were made visible under UV-light (254 nm).

In previous versions of the European Pharmacopoeia a colour reaction (halazuchrom reaction [13]) for the identification of Valerian material is described. Heating of a fraction of the extract at hand with hydrochloric acid in MeOH gave indeed a deep blue color indicating the presence of iridoids of the valtrate diene type. The colour is caused by cyclopenta[*c*]pyryliumchlorid salts (s. Fig. 2) [14].

After multiple fractionation steps pure compounds were isolated from the *V. wallichii* extract and a full characterization by NMR was performed (^1H, ^{13}C, DEPT, ^1H-^1H COSY, NOESY, HMBC and HMQC experiments were applied). Low sensitivity due to very small

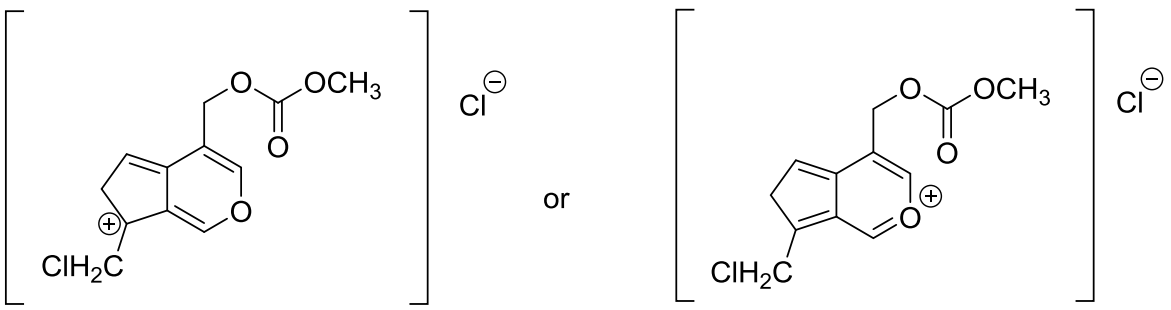


Fig.2: Cyclopenta[*c*]pyryliumchloride salts – cause of the blue colour in the halazuchromreaction after addition of hydrochloric acid and heating in the presence of valtrates of the diene type [14].

compound amounts (1-2 mg) could be improved by the use of Shigemi tubes. Based on this data the structures of the compounds were elucidated.

From the most active antileishmanial fractions various iridoids of the valtrate type were identified. The results of the antileishmanial *in vitro* assays proved these iridoids to be the most active antileishmanial principles of the extract. Valepotriates have been isolated for the first time by Thies et al. [15,16] from *V. wallichii*. Until today antifungal [17], anti-HIV and anticancer *in vitro* [7,18] activities are reported. However, valtrates are not only highly unstable in solution but also cytotoxic substances with a complex structure showing alkylating abilities [19]. Nevertheless, a few synthetic pathways have been proposed for the construction of the irdoid skeleton e.g. by means of a iridoid synthase [20], the conversion of limonene to nepetalactones [21] or the synthesis of valtrates from (+)-genipin [22,23].

Beside two iridoids of the valtrate type with a novel substitution pattern two other compounds were identified for the first time from *V. wallichii*, villoside aglycone and a novel nepetolactone derivative. Villoside aglycone (11-hydroxy-isodihydro-nepetalactone) has just once been mentioned before in the literature by Taguchi et al. [24]. Here, the molecule was prepared synthetically from villoside tetraacetate, the glycoside isolated from a methanol extract of the subterranean parts of *Patrinia villosa* Juss., a Valerianacae. Both compounds show structural similarities to nepetalactones, the active ingredients from catnip (*Nepeta cataria*) [25]. For this compounds feline attractant and insect repellant [26] activities are reported. However, both compounds exhibited no antileishmanial activity in this study.

Podophyllotoxin, 4-demethylpodophyllotoxin, bornyl isovalerate, linarin-*O*-2-methyl-butyrate and pinoresinol were also found in the extract. Interestingly podophyllotoxin and its derivate, isolated here for the first time from *Valeriana* and known for its strong cytotoxicity, did not show any leishmanicidal properties. It is originally prepared from the resin (podophyllin) of the rhizomes of *Podophyllum peltatum* [27] and can be found in higher amounts in *Juniperus virginiana* [28] as well. It has mitosis-inhibiting properties [29] and can be used by topical application against genital warts. Because of its high cytotoxicity it is discussed as potent anti-tumoral agent in the literature [30]. Pinoresinol is widely distributed in our diet and is already known from *V. officinalis* [31] and *V. microphylla* [32]. Bornyl isovalerate was even used as sleeping aid at the beginning of the 20th century (Bornyval). For linarin sleep enhancing and sedative properties are proposed [33]. All of these compounds did not show antileishmanial activity.

The following terpenoids were isolated and identified: β-bisabolol, valeranon and α-kessyl alcohol. β-Bisabolol was first isolated from cotton bud [34] (*Gossypium hirsutum*) and is a constitutional isomer of α-bisabolol from chamomile (*Matricaria chamomilla*). The latter compound is known for its anti-inflammatory properties amongst others. Valeranone can be found in *Nardostachys jatamansi* [35] and *V. officinalis* [36]. α-Kessyl alcohol was found in Japanese Valerian [37]. The antileishmanial activity of α-kessyl alcohol in our study was determined on a valtrate-contaminated fraction and was therefore not significant.

Finally, the isolated bornyl caffeate and derivatives showed good activity and were synthetically accessible. Therefore, this structure was chosen as scaffold for the synthesis of a compound library for SAR investigation.

To establish an ester bond basically an activated organic acid and an alcohol are needed. There are many synthetic procedures available e.g. 1) acid chloride and alcohol, 2) acid anhydride and alcohol, 3) Fischer esterification catalyzed by a Lewis or Brönstedt acid e.g. *p*-TsOH, H_2SO_4, 4) Steglich esterification using dicyclohexyl-carbodiimide (DCC) as activator and dimethylaminopyridine (DMAP) as catalyst, 5) using PPh_3 and diethyl azodicarboxylate (DEAD), under inversion of the stereocenter (Mitsunobu), or 6) Yamaguchi esterification using 2,4,6-trichlorobenzoylchloride and DMAP.

However, as phenolic hydroxyl groups are interfering in classic esterification reactions another approach for the synthesis of caffeic acid bornyl ester was necessary. One consideration included the protection of the phenolic groups using e.g. *t*-butyl ether, silyl ether, esterification or acetalization. However, in the last step of this hypothetical four-step synthesis, basic or acidic conditions are needed for deprotection which both may cleave the desired product. Nevertheless, literature describes a convenient one-pot reaction for the synthesis of caffeic acid ester derivatives based on a Knoevenagel-Doebner-condensation [38].

Derivatives without the catechol group of the caffeic acid showed lower cytotoxicity while retaining antileishmanial activity. Therefore, the focus changed to cinnamic acid derivatives and simple and effective Steglich esterifications could be applied. A disadvantage of this synthesis is the more elaborate purification process due to formation of poorly soluble dicyclohexylurea as by-product. The substitution of DCC with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC-HCl) which is converted into water soluble urea, improved the problem.

Synthesis confirmed the antileishmanial activity of bornyl caffeate. Interestingly, no activity was observed against schistosomula of *Schistosoma (S.) mansoni* and *L. mexicana* promastigotes despite good activity against *L. major* and *L. donovani* promastigotes *in vitro*. Additionally, preliminary results of a screening against *Toxoplasma gondii* showed that bornyl caffeate leads to the death of toxoplasma but also induces lysis of the host cells (human foreskin fibroblasts, HFF). This may be due to cytotoxicity caused by the catechol moeity because of the formation of *o*-quinones being reactive Michael systems. Bornyl caffeate is described in the literature as having anticancer [39], anti-inflammatory [40] and antibacterial activity [41]. Caffeic acid derivatives are generally known as antioxidants due to the phenolic hydroxyl groups [42]. Bornyl caffeate was also found to inhibit granulycote elastase [43] and HIV-1 integrase [38]. Except for the inhibition of trypanosomal cysteine protease [44], no antiprotozoal activities have been reported.

From the subsequent synthesized compound library especially the less toxic bornyl cinnamate showed the most promising activities against *Leishmania*. Additionally, it could reduce the number of toxoplasmas from 7 to 1-2 without lysis of the host cells, however, the host cells showed signs of morphological stress. There was again no activity against *L. mexicana* promastigotes. The compound additionally induced degeneration of schistosomula after 72 hours at a concentration of 20 µM.

Regarding bornyl cinnamate only a few references are available in the literature. One patent claims the successful use of the compound as melanin formation inhibitor fighting spots and freckles on the skin [45]. Others are reporting on larvicidal activity against the European corn borer [46] and inhibition of proinflammatory molecules through suppression of the redox-sensitive nuclear factor kB signaling pathway [47]. However, no antiparasitic activities have been reported until now.

Cinnamic acid esters in general are known for their antifungal [48], antimicrobial [49] and antioxidant [50] activity (s. Fig 3). Cinnamate derivatives are described as inhibitors of α-glucosidase [51], neutrophil elastase [52] and 17β-hydroxysteroid dehydrogenase [53]. Some cinnamic acid derivatives have anticancer properties [54]. Caffeic acid phenethylester (CAPE) is one prominent and much investigated example with e.g. antioxidant [55,56], antimicrobial [57], anti-inflammatory [58], anti-cancer [59,60] properties as well as the ability to inhibit HIV-1 integrase [61]. However, these activities may be caused by adduct formation with GSH [62] due to the Michael system and quinone formation or ion complexation of the catechol moeity. CAPE shows multiple

unrelated activities and has reactive substructures (catechol and Michael system) which hints to an unselective mode of action. This is evidence for a PAIN compound [63]. As caffeic acid bornyl ester shows the same structural features it should be considered suspicious.

SAR investigation proved borneol to be the best substituent together with other bulky or sterical demanding alcohols e.g. thymol, menthol or adamantol. Substitution at the aromatic ring with various groups e.g. halogen, methoxy, nitro groups in different positions could not improve the antileishmanial activity significantly. The catechol and the α,β-unsaturated carbonyl moiety of the caffeic acid were found to strongly contribute

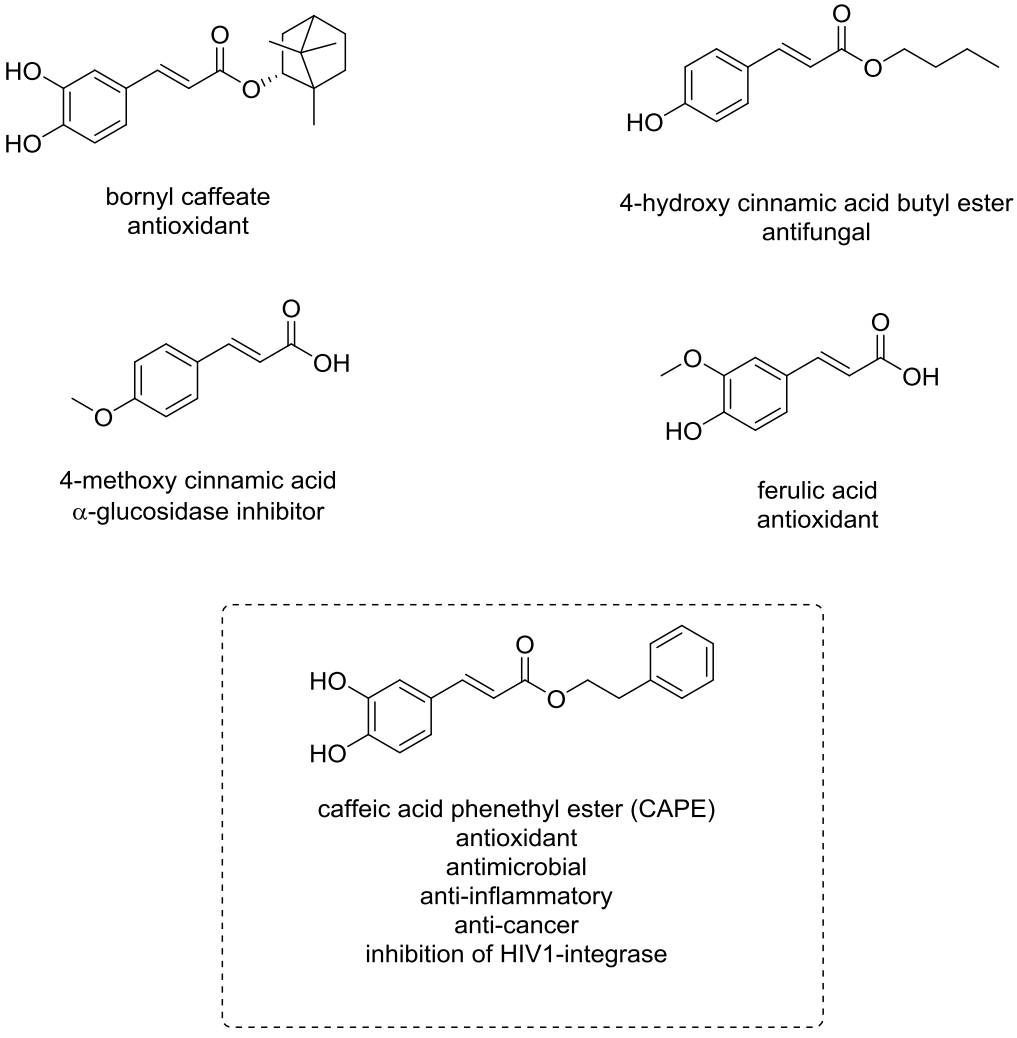


Fig.3. Reported activities of some cinnamic acids and derivatives. CAPE shows many unrelated activities and reactive structure elements – characteristics of PAIN compounds.

to cytotoxicity. However, it turned out that these structural elements were not necessary for antileishmanial activity.

The non-toxic bornyl phenylpropanoate lacking these structural PAIN characteristics exhibited good antileishmanial activity both *in vivo* and *in vitro*. There are no other activities known for this compound and only a few references can be found in the literature. Therefore, this is the first report of bornyl phenylpropionate being an antiprotozoal agent. The compound clearly fulfills Lipinski´s rule of five (logP < 5, M_R < 500 g/mol, ≤ 5 hydrogen bond donors, ≤ 10 hydrogen bond acceptors), a rule of thumb used to determine druglikeness and bioavailability of a lead structure. The amide derivative of bornyl phenylpropanoate was anticipated to be more stable against acidic and basic conditions and the cleavage by nonspecific esterases *in vivo*. The compound was found to be active against *L. donovani in vitro* and *in vivo*, however there was no activity against *L. major* promastigotes (s. Appendix).

The exact mechanism of activity still needs to be investigated. Mitochondrial membrane depolarization of *L. donovani* promastigotes was observed. Flow cytometry analysis showed that bornyl phenylpropionate time-dependently induced necrosis/late apoptosis cell death of *L. major* promastigotes similar to miltefosine, but in a faster way. Additionally, the formation of vacuoles and loss of membrane integrity was initiated. Preliminary screenings on *L. mexicana* cysteine protease (LmCPB2.8) hinted at possible inhibitory activity for bornyl caffeate (IC_{50} = 5.8 µM) and bornyl phenylpropionate (IC_{50} = 4.1 µM). However, the activity against *L. mexicana* promastigotes *in vitro* was higher than 100 µM for both compounds. A fluorescence proteinase activity assay showed no significant reduction of cathepsin B-like (CPA and CPB) and cathepsin L-like (CPC) enzymes from the cell lysates of *L. major* promastigotes after treatment with both compounds. Additionally, it was found that naphthyl cinnamate induces NO production but not respiratory burst in naive and infected (*L. amazonensis* and *L. mexicana* therapy resistant patient strains) macrophages in a way comparable to miltefosine.

If the mode of action is not the reversible inhibition of an enzyme, a natural product might still be effective e.g. like artemisinine (oxygen production), aspirin (selective covalent inhibitor) or taxols (selective binding to the spindel apparatus), as long as there is a certain mode and grade of selectivity. Regarding the main antileishmanial drugs in use, amphotericin B binds to 24-ergosterol, the main sterol of the *Leishmania* cell membrane, thereby changing the membrane permeability. The mode of action of

pentamidine is unknown and under debate [64]. Both drugs need to be administered intravenously due to their low bioavailability. The oral drug miltefosine seems to act as a protein kinase B inhibitor [65] but no definite mechanism has been confirmed.

At last, from the antischistosomal screening eugenyl and thymyl cinnamate emerged as most active compounds. The treatment led to formation of vacuoles in schistosomula of *S. mansoni*, a new phenotype in this type of assay. No such activities have been reported for these compounds before. They also exhibited activity against *L. mexicana* promastigotes (8 µM eugenyl cinnamate, 31 µM thymyl cinnamate). Only antifungal activity has been reported in the literature for both esters [48]. For the eugenyl ester weak antialopecic properties [66] are described. Cavier et al. found thymyl cinnamate to be inactive against *Rhabditis macrocerca* [67], another species of nematode worm. Unfortunately no activity was observed against juvenile and adult schistosoma without the Michael system.

Taken together, bioactivity-guided fractionation of an extract of *V. wallichii* rhizomes and SAR investigations led to the development of a non-toxic antileishmanial agent with good *in vitro* and *in vivo* activity. Further investigations regarding the elucidation of the mode of action are necessary.

References

1. Rostagno, M. A.; Prado, J. M., *Natural product extraction: principles and applications.* Royal Society of Chemistry: Cambridge, **2013**.
2. Ghosh, S.; Debnath, S.; Hazra, S.; Hartung, A.; Thomale, K.; Schultheis, M.; Kapkova, P.; Schurigt, U.; Moll, H.; Holzgrabe, U., *Valeriana wallichii* root extracts and fractions with activity against Leishmania spp. *Parasitol. Res.* **2011**, *108*, 861-871.
3. Subhan, F.; Karim, N.; Ibrar, M., Anti-inflammatory activity of methanolic and aqueous extracts of *Valeriana wallichii* DC rhizome. *Pak. J. Plant Sci.* **2007**, *13*, 103-108.
4. Murphy, K.; Kubin, Z. J.; Shepherd, J. N.; Ettinger, R. H., *Valeriana officinalis* root extracts have potent anxiolytic effects in laboratory rats. *Phytomedicine* **2010**, *17*, 674-678.

5. Dua, V.; Alam, M.; Pandey, A.; Rai, S.; Chopra, A.; Kaul, V.; Dash, A., Insecticidal activity of *Valeriana jatamansi* (Valerianaceae) against mosquitoes. *J. Am. Mosq. Control Assoc.* **2008**, *24*, 315-318.
6. Oshima, Y.; Matsuoka, S.; Ohizumi, Y., Antidepressant principles of *Valeriana fauriei* roots. *Chem. Pharm. Bull.* (Tokyo) **1995**, *43*, 169-170.
7. Lin, S.; Shen, Y.-H.; Li, H.-L.; Yang, X.-W.; Chen, T.; Lu, L.-H.; Huang, Z.-S.; Liu, R.-H.; Xu, X.-K.; Zhang, W.-D., Acylated iridoids with cytotoxicity from *Valeriana jatamansi*. *J. Nat. Prod*. **2009**, *72*, 650-655.
8. Marder, M.; Viola, H.; Wasowski, C.; Fernández, S.; Medina, J. H.; Paladini, A. C., 6-Methylapigenin and hesperidin: new valeriana flavonoids with activity on the CNS. *Pharmacol. Biochem. Behav*. **2003**, *75*, 537-545.
9. Wang, R.; Xiao, D.; Bian, Y.-H.; Zhang, X.-Y.; Li, B.-J.; Ding, L.-S.; Peng, S.-L., Minor Iridoids from the Roots of *Valeriana wallichii*. *J. Nat. Prod*. **2008**, *71*, 1254-1257.
10. Navarrete, A.; Avula, B.; Choi, Y.-W.; Khan, I. A., Chemical fingerprinting of valeriana species: simultaneous determination of valerenic acids, flavonoids, and phenylpropanoids using liquid chromatography with ultraviolet detection. *J. AOAC Int*. **2006**, *89*, 8-15.
11. Gränicher, F.; Christen, P.; Vuagnat, P., Rapid high performance liquid chromatographic quantification of valepotriates in hairy root cultures of *Valeriana officinalis* L. var. *sambucifolia* Mikan. *Phytochem. Anal*. **1994**, *5*, 297-301.
12. Hazelhoff, B.; Weert, B.; Denee, R.; Malingré, T. M., Isolation and analytical aspects of Valeriana compounds. *Pharm. Weekbl*. **1979**, *1*, 956-964.
13. Mannetstätter, E.; Gerlach, H.; Poethke, W., Über die Inhaltsstoffe von Valerianaceen. 1. Mitt.: Der Nachweis einiger Inhaltsstoffe von *Kentranthus ruber* DC. *Pharm. Zentralhalle* **1967**, *106*, 797-804.
14. Dingermann, T.; Schneider, G., *Arzneidrogen*. Elsevier, Spektrum Akad. Verlag: München, **2004**.
15. Thies, P.; Funke, S., Über die Wirkstoffe des Baldrians: 1. Mitteilung Nachweis und Isolierung von sedativ wirksamen Isovaleriansäureestern aus Wurzeln und Rhizomen von verschiedenen *Valeriana*-und *Kentranthus*-Arten. *Tetrahedron Lett*. **1966**, *7*, 1155-1162.

16. Thies, P., Über die Wirkstoffe des Baldrians: 2. Mitteilung Zur Konstitution der Isovaleriansäureester Valepotriat, Acetoxyvalepotriat und Dihydrovalepotriat. *Tetrahedron Lett.* **1966**, *7*, 1163-1170.
17. Fuzzati, N.; Wolfender, J. L.; Hostettmann, K.; Msonthi, J. D.; Mavi, S.; Molleyres, L. P., Isolation of antifungal valepotriates from *Valeriana capense* and the search for valepotriates in crude *Valerianaceae* extracts. *Phytochem. Anal.* **1996**, *7*, 76-85.
18. Bounthanh, C.; Bergmann, C.; Beck, J.; Haag-Berrurier, M.; Anton, R., Valepotriates, a new class of cytotoxic and antitumor agents. *Planta Med.* **1981**, *41*, 21-28.
19. Braun, R.; Dittmar, W.; Machut, M.; Weickmann, S., Valepotriate mit Epoxidstruktur - Beachtliche Alkylantien. *Dtsch. Apoth.* **1982**, *122*, 1109-1113.
20. Geu-Flores, F.; Sherden, N. H.; Courdavault, V.; Burlat, V.; Glenn, W. S.; Wu, C.; Nims, E.; Cui, Y.; O'Connor, S. E., An alternative route to cyclic terpenes by reductive cyclization in iridoid biosynthesis. *Nature* **2012**, *492*, 138-142.
21. SAKA, K., A New Conversion Method from (-)-limonene to nepetalactones. *Chem. Pharm. Bull.* **1988**, *36*, 172-177.
22. Oh, S.; Kwon, D.; Shin, W.-S.; Ham, J.; Lee, S., Synthesis of 7,8-*epi*-valtrate from (+)-genipin. *Notes* **2012**, *33*, 4215.
23. Tamura, S.; Fujiwara, K.; Shimizu, N.; Todo, S.; Murakami, N., Concise synthesis of 5,6-dihydrovaltrate leading to enhanced *Rev*-export inhibitory congener. *Biorg. Med. Chem.* **2010**, *18*, 5975-5980.
24. Taguchi, H.; Yokokawa, Y.; Endo, T., Studies on the constituents of *Patrinia villosa* Juss. *J. Pharm. Soc. Jap.* **1973**, *93*, 607.
25. Regnier, F.; Eeisenbraun, E.; Waller, G., Nepetalactone and epinepetalactone from *Nepeta cataria* L. *Phytochemistry* **1967**, *6*, 1271-1280.
26. Birkett, M. A.; Hassanali, A.; Hoglund, S.; Pettersson, J.; Pickett, J. A., Repellent activity of catmint, *Nepeta cataria*, and iridoid nepetalactone isomers against Afro-tropical mosquitoes, ixodid ticks and red poultry mites. *Phytochemistry* **2011**, *72*, 109-114.
27. Jackson, D. E.; Dewick, P. M., Aryltetralin lignans from *Podophyllum hexandrum* and *Podophyllum peltatum*. *Phytochemistry* **1984**, *23*, 1147-1152.

28. Kupchan, S. M.; Hemingway, J. C.; Knox, J. R., Tumor inhibitors VII. Podophyllotoxin, the active principle of *Juniperus virginiana*. *J. Pharm. Sci.* **1965**, *54*, 659-660.
29. Keller-Juslen, C.; Kuhn, M.; Von Wartburg, A.; Stähelin, H., Mitosis-inhibiting natural products. 24. Synthesis and antimitotic activity of glycosidic lignan derivatives related to podophyllotoxin. *J. Med. Chem*. **1971**, *14*, 936-940.
30. Cheng, W.-H.; Cao, B.; Shang, H.; Niu, C.; Zhang, L.-M.; Zhang, Z.-H.; Tian, D.-L.; Zhang, S.; Chen, H.; Zou, Z.-M., Synthesis and evaluation of novel podophyllotoxin derivatives as potential antitumor agents. *Eur. J. Med. Chem*. **2014**, *85*, 498-507.
31. Zhou, Y.; Fang, Y.; Gong, Z.-F.; Duan, X.-Y.; Liu, Y.-W., Two new terpenoids from *Valeriana officinalis*. *Chin. J. Nat. Med.* **2009**, *7*, 270-273.
32. Bach, K. K.; Ghia, F.; Torssell, K. B. G., Valtrates and lignans in *Valeriana microphylla*. *Planta Med.* **1993**, *59*, 478-479.
33. Fernández, S.; Wasowski, C.; Paladini, A. C.; Marder, M., Sedative and sleep-enhancing properties of linarin, a flavonoid isolated from *Valeriana officinalis*. *Pharm. Biochem. Behav.* **2004**, *77*, 399-404.
34. Minyard, J. P.; Thompson, A. C.; Hedin, P. A., Constituents of the cotton bud. VIII. β-Bisabolol, a new sesquiterpene alcohol. *J. Org. Chem.* **1968**, *33*, 909-911.
35. Hörster, H.; Rücker, G.; Tautges, J., Valeranongehalt in den unterirdischen Teilen von *Nardostachys jatamansi* und *Valeriana officinalis*. *Phytochemistry* **1977**, *16*, 1070-1071.
36. Stoll, A.; Seebeck, E.; Stauffacher, D., Isolierung und Charakterisierung von bisher unbekannten Inhaltsstoffen aus dem Neutralteil des frischen Baldrians. 2. Mitteilung über Valeriana. *Helv. Chim. Acta* **1957**, *40*, 1205-1229.
37. Itô, S.; Kodama, M.; Nozoe, T.; Hikino, H.; Hikino, Y.; Takeshita, Y.; Takemoto, T., Structure and absolute configuration of α-kessyl alcohol and kessyl glycol. *Tetrahedron* **1967**, *23*, 553-563.
38. Xia, C.-N.; Li, H.-B.; Hu, W.-X., Synthesis of *trans*-caffeate analogues and their bioactivities against HIV-1 integrase and cancer cell lines. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 6553-6557.
39. Yang, C.-B.; Pei, W.-J.; Zhao, J.; Cheng, Y.-Y.; Zheng, X.-H.; Rong, J.-H., Bornyl caffeate induces apoptosis in human breast cancer MCF-7 cells via the ROS- and JNK-mediated pathways. *Act. Pharm. Sin.* **2014**, *35*, 113-123.

40. Maldonado, E.; Ramírez, A. M.; Pérez-Castorena, A. L., Anti-inflammatory activity of phenyl propanoids from *Coreopsis mutica* var. *mutica*. *Planta Med*. **1998**, *64*, 660-661.
41. Setzer, W. N.; Setzer, M. C.; Bates, R. B.; Nakkiew, P.; Jackes, B. R.; Chen, L.; McFerrin, M. B.; Meehan, E. J., Antibacterial hydroxycinnamic esters from *Piper caninum* from Paluma, north Queensland, Australia. The crystal and molecular structure of (+)-bornyl coumarate. *Planta Med*. **1999**, *65*, 747-749
42. Chen, J. H.; Ho, C.-T., Antioxidant activities of caffeic acid and its related hydroxycinnamic acid compounds. *J. Agric. Food. Chem*. **1997**, *45*, 2374-2378.
43. Melzig, M.; Löser, B.; Lobitz, G.; Tamayo-Castillo, G.; Merfort, I., Inhibition of granulocyte elastase activity by caffeic acid derivatives. *Die Pharmazie* **1999**, *54*, 712-712.
44. Ogungbe, I. V.; Crouch, R. A.; Haber, W. A.; Setzer, W. N., Phytochemical investigation of *Verbesina turbacensis* Kunth: trypanosome cysteine protease inhibition by (-)-bornyl esters. *Nat. Prod. Comm*. **2010**, *5*, 1161-1166.
45. Suzuki, Y. K., Ichiro; Ochiai, Michio, Melanin formation inhibitors comprising bornyl cinnamates and skin preparations containing them. *Jpn. Kokai. Tokkyo Koho* **1995**, JP 09124474.
46. Lee, S.; Tsao, R.; Coats, J. R., Influence of dietary applied monoterpenoids and derivatives on survival and growth of the European corn borer (*Lepidoptera*: *Pyralidae*). *J. Ecol. Entomol*. **1999**, *92*, 56-67.
47. Kumar, K. J. S.; Li, J.; Vani, M. G.; Hsieh, Y.-H.; Kuo, Y.-H.; Wang, S.-Y., Bornyl cinnamate inhibits inflammation-associated gene expression in macrophage cells through suppression of nuclear factor-κB signaling pathway. *Planta Med*. **2015**, *81*, 39-45.
48. Tawata, S.; Taira, S.; Kobamoto, N.; Zhu, J.; Ishihara, M.; Toyama, S., Synthesis and antifungal activity of cinnamic acid esters. *Biosci. Biotech. Biochem.* **1996**, *60*, 909-910.
49. Narasimhan, B.; Belsare, D.; Pharande, D.; Mourya, V.; Dhake, A., Esters, amides and substituted derivatives of cinnamic acid: synthesis, antimicrobial activity and QSAR investigations. *Eur. J. Med. Chem.* **2004**, *39*, 827-834.
50. Natella, F.; Nardini, M.; Di Felice, M.; Scaccini, C., Benzoic and cinnamic acid derivatives as antioxidants: structure−activity relation. *J. Agric. Food. Chem*. **1999**, *47*, 1453-1459.

51. Adisakwattana, S.; Sookkongwaree, K.; Roengsumran, S.; Petsom, A.; Ngamrojnavanich, N.; Chavasiri, W.; Deesamer, S.; Yibchok-anun, S., Structure–activity relationships of *trans*-cinnamic acid derivatives on α-glucosidase inhibition. *Bioorg. Med. Chem. Lett*. **2004**, *14*, 2893-2896.
52. Loser, B.; Kruse, S. O.; Melzig, M. F.; Nahrstedt, A., Inhibition of neutrophil elastase activity by cinnamic acid derivatives from *Cimicifuga racemosa*. *Planta Med*. **2000**, *66*, 751-753.
53. Brožič, P.; Golob, B.; Gomboc, N.; Rižner, T. L.; Gobec, S., Cinnamic acids as new inhibitors of 17β-hydroxysteroid dehydrogenase type 5 (AKR1C3). *Mol. Cell Endocrinol.* **2006**, *248*, 233-235.
54. De, P.; Baltas, M.; Bedos-Belval, F., Cinnamic acid derivatives as anticancer agents - A review. *Curr. Med. Chem.* **2011**, *18*, 1672-1703.
55. Sud'ina, G. F.; Mirzoeva, O. K.; Pushkareva, M. A.; Korshunova, G. A.; Sumbatyan, N. V.; Varfolomeev, S. D., Caffeic acid phenethyl ester as a lipoxygenase inhibitor with antioxidant properties. *FEBS Lett*. **1993**, *329*, 21-24.
56. Russo, A.; Longo, R.; Vanella, A., Antioxidant activity of propolis: role of caffeic acid phenethyl ester and galangin. *Fitoterapia* **2002**, *73*, 21-29.
57. Lee, H. S.; Lee, S. Y.; Park, S. H.; Lee, J. H.; Ahn, S. K.; Choi, Y. M.; Choi, D. J.; Chang, J. H., Antimicrobial medical sutures with caffeic acid phenethyl ester and their in *vitro/in vivo* biological assessment. *MedChemComm* **2013**, *4*, 777-782.
58. da Cunha, F. M.; Duma, D.; Assreuy, J.; Buzzi, F. C.; Niero, R.; Campos, M. M.; Calixto, J. B., Caffeic acid derivatives: *In vitro* and *in vivo* anti-inflammatory properties. *Free Radical Res*. **2004**, *38*, 1241-1253.
59. Chuu, C.-P.; Lin, H.-P.; Ciaccio, M. F.; Kokontis, J. M.; Hause, R. J.; Hiipakka, R. A.; Liao, S.; Jones, R. B., Caffeic acid phenethyl ester suppresses the proliferation of human prostate cancer cells through inhibition of p70S6K and Akt signaling networks. *Canc. Prev. Res.* **2012**, *5*, 788-797.
60. Chen, M.-J.; Chang, W.-H.; Lin, C.-C.; Liu, C.-Y.; Wang, T.-E.; Chu, C.-H.; Shih, S.-C.; Chen, Y.-J., Caffeic acid phenethyl ester induces apoptosis of human pancreatic cancer cells involving caspase and mitochondrial dysfunction. *Pancreatology* **2008**, *8*, 566-576.

61. Fesen, M. R.; Pommier, Y.; Leteurtre, F.; Hiroguchi, S.; Yung, J.; Kohn, K. W., Inhibition of HIV-1 integrase by flavones, caffeic acid phenethyl ester (CAPE) and related compounds. *Biochem. Pharmacol*. **1994**, *48*, 595-608.
62. Ozturk, G.; Ginis, Z.; Akyol, S.; Erden, G.; Gurel, A.; Akyol, O., The anticancer mechanism of caffeic acid phenethyl ester (CAPE): review of melanomas, lung and prostate cancers. *Eur. Rev. Med. Pharmacol. Sci.* **2012**, *16*, 2064-2068.
63. Baell, J.; Walters, M. A., Chemistry: Chemical con artists foil drug discovery. *Nature News* **2014**, *513*, 481-483.
64. Balaña-Fouce, R.; Reguera, R. M.; Cubría, J. C.; Ordóñez, D., The pharmacology of leishmaniasis. General pharmacology: The vascular system **1998**, *30*, 435-443.
65. Ruiter, G. A.; Zerp, S. F.; Bartelink, H.; van Blitterswijk, W. J.; Verheij, M., Anti-cancer alkyl-lysophospholipids inhibit the phosphatidylinositol 3-kinase–Akt/PKB survival pathway. *Anticanc. Drugs* **2003**, *14*, 167-173.
66. Shin, J. S.; Park, J. H.; Park, H. G., Properties and antialopecic effect of eugenol-1-cinnamic acid derivative. *Kor. Soc. Appl. Pharm. Conf. Proc.* **1997**, *1*, 110-110.
67. Cavier R., C., M, Anthelmintic qualities of some cinnamic acid derivatives. I. *In vitro* tests of activity with *Rhabditis macrocerca*. *Ann. Pharm. Fr.* **1956**, *14*, 367-369

5. SUMMARY

The study is dealing with the bioactivity-guided fractionation of a chloroform extract from pulverized rhizomes of *Valeriana (V.) wallichii* with focus on isolation and structure elucidation of the antileishmanial active principles. The antileishmanial activity against *L. major* promastigotes *in vitro* was known from previous investigations.

Seventeen compounds (s. Fig. 1), namely linarin-*O*-2-methylbutyrate **1**, β-bisabolol **2**, α-kessyl alcohol **3**, valeranone **4**, bornyl isovalerate **5**, four iridoids of the valtrate type **6-9**, bornyl caffeate **10**, bornyl isoferulate **11**, bornyl *p*-hydroxycinnamate **12**, 4´-demethylpodophyllotoxin **13**, podophyllotoxin **14**, pinoresinol **15**, villoside aglycone **16** and a novel nepetolactone derivative **17** were isolated from the extract using chromatographic separation techniques. Thirteen of these compounds were already described in the literature being ingredients of various *Valeriana* species (**1**-**7**) or other plants (**10**-**15**). Hence, the latter were isolated from *V. wallichii* for the first time. Additionally, two iridoids (**8**, **9**) with a novel substitution pattern, as well as villoside aglycone **16** and the novel nepetolactone derivative **17** were described for the first time.

The iridoids of the valtrate type were found to be the main cause for the antileishmanial activity of the extract. However, these compounds were unstable in solution and exhibited strong cytotoxicity against a murine cell line (J774.1) which rendered them unapt for further investigations.

Bornyl caffeate and derivatives were also discovered to have leishmanicidal properties, in addition to lower cytotoxicity in comparison to the iridoids. Therefore, this structure was used as a scaffold for the synthesis of a compound library in order to derive structure-activity relationships (SAR). With regard to antileishmanial activity borneol proved itself to be the best substituent together with other bulky or sterically demanding alcohols e.g. thymol, menthol or adamantol. On the other hand, the catechol moiety and the α,β-unsaturated carbonyl moiety contributed significantly to cytotoxicity of the compounds. However, these structural elements turned out to be not necessary for good antileishmanial activity. This led to the development of the optimized structure of bornyl phenylpropionate **18** (s. Fig. 2), showing antileishmanial activity comparable to the activity of bornyl caffeate **10**, but without toxicity towards macrophages (J774.1 and BMDM), kidney cells (HEK 293T) and hepatocytes (HEP-2G) *in vitro* (s. Fig. 2). Flow

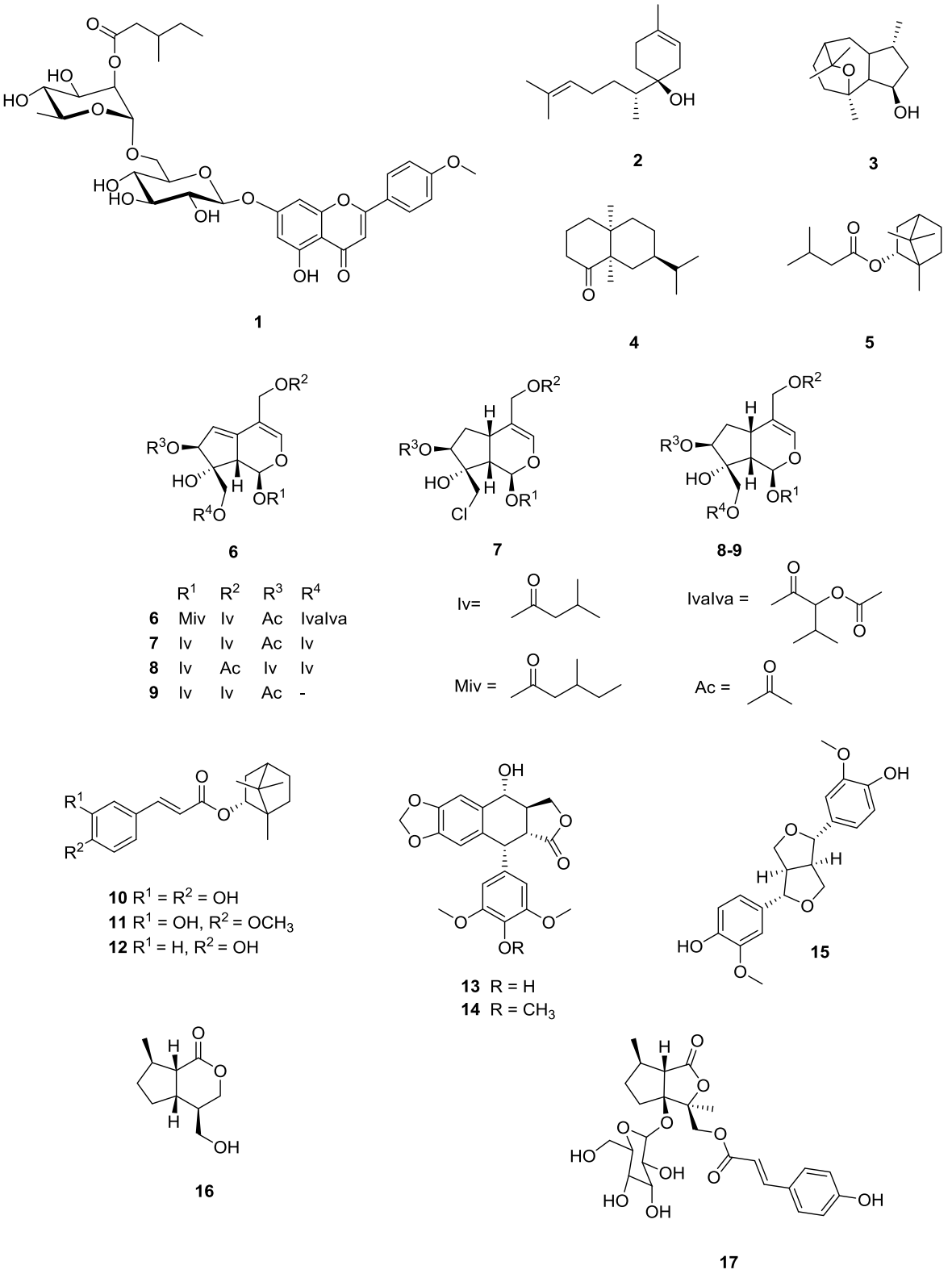


Fig.1. Structures of compounds **1**-**17** isolated from the *V. wallichii* chloroform extract.

cytometric analysis revealed a miltefosine-like cell death of *L. major* promastigotes, time-dependently for **18**. Additionally, the formation of vacuoles and loss of membrane integrity was observed. These findings prompted *in vivo* investigations in *L. major*-

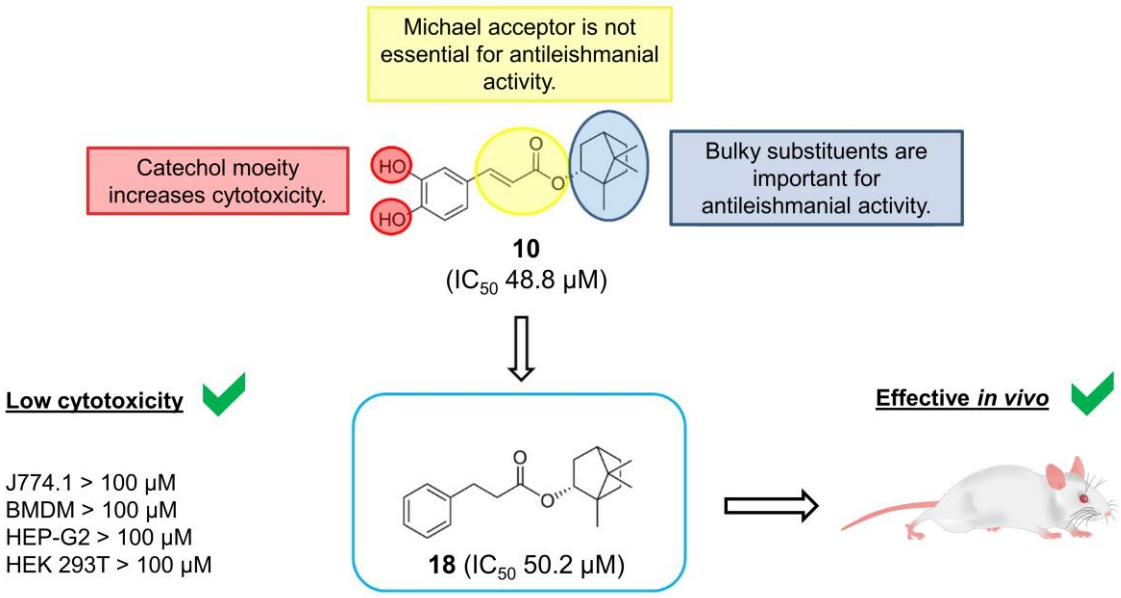


Fig.2. Summary of the results of the SAR investigations.

infected BALB/c mice. The mice tolerated the intraperitoneal (IP) application of compound **18** up to 50 mg/kg/day without any adverse effects. In this model for cutaneous leishmaniasis the application of bornyl phenylpropionate **18** gave a healing phenotype and a significant reduction of parasite burden of the infected footpads and of the infection site-draining popliteal lymph nodes after four weeks.

First *in vivo* experiments in a model for visceral leishmaniasis (*L. donovani*) were conducted in Kolkata. Intraperitoneal application of 2 mg/kg/day of **18** for five days gave a significant reduction of parasite burden in the liver.

A screening of the compound library against *L. mexicana* promastigotes revealed naphthyl cinnamate **19** and eugenyl cinnamate **20** (s. Fig. 3) to be the most active compounds (*cf.* page 159). **19** induced NO production in naive and infected macrophages in a way comparable to miltefosine.

From a screening of the compound library against schistosomula of *Schistosoma mansoni* (San Francisco) thymyl **21** and eugenyl cinnamate **20** (s. Fig. 3) emerged as effective antischistosomal agents *in vitro*. Both compounds led to either degeneration (**21**) or death (**20**) of the schistosomula, respectively, after 72 hours (c = 5 µM). These compounds also induced formation of vacuoles inside the parasites, which was a novel phenotype in this assay.

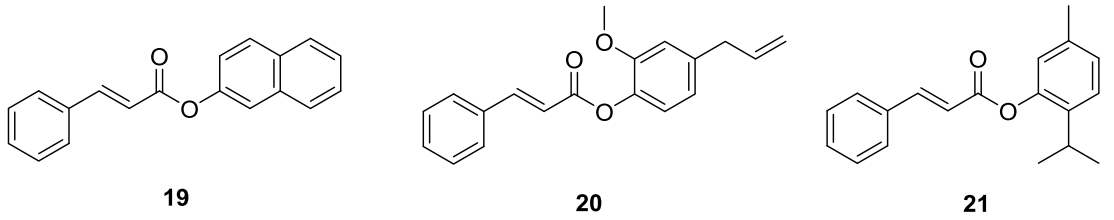


Fig.3. Napthyl cinnamate (**19**) active against *L. mexicana* promastigotes; eugenyl (**20**) and thymyl cinnamate (**21**) active against schistosomula of *S. mansoni*.

In summary, good leads against schistosomula and several strains of *Leishmania* were found, with bornyl phenylpropanoate standing out as a non-toxic, antileishmanial agent with *in vivo* activity. Further investigations regarding the elucidation of the mode of action are necessary.

6. ZUSAMMENFASSUNG

Die vorliegende Studie beschäftigt sich mit der bioaktivitätsgeleiteten Fraktionierung eines Chloroformextraktes aus pulverisierten Rhizomen von *Valeriana (V.) wallichii* mit Schwerpunkt auf der Isolierung und Strukturaufklärung des antileishmanialen Wirkprinzips. Die antileishmaniale *In-vitro*-Aktivität gegen *L. major*-Promastigoten war aus vorhergehenden Untersuchungen bekannt.

Aus dem Extrakt wurden siebzehn Substanzen (s. Abb. 1) mit Hilfe chromatographischer Auftrennungsmethoden isoliert, namentlich Linarin-*O*-2-methylbutyrat **1**, β-Bisabolol **2**, α-Kessylalkohol **3**, Valeranon **4**, Bornylisovalerat **5,** vier Iridoide des Valtrat-Typs **6-9,** Bornylkaffeat **10**, Bornylisoferulat **11**, Bornyl-*p*-hydroxycinnamat **12**, 4´-Demethylpodophyllotoxin **13**, Podophyllotoxin **14**, Pinoresinol **15,** Villosidaglycon **16** und ein neues Nepetolacton-Derivat **17**. Dreizehn dieser Substanzen waren bereits in der Literatur als Inhaltsstoffe verschiedener *Valeriana*-Spezies (**1**-**7**) oder anderer Pflanzengattungen beschrieben (**10**-**15**). Die Letzteren wurden folglich zum ersten Mal aus *V. wallichii* isoliert. Zusätzlich wurden sowohl zwei Iridoidester **8**, **9** mit neuen Substitutionsmustern als auch Villosidaglycon **16** und ein neues Nepetolacton-Derivat **17** erstmals beschrieben. Die Iridoide des Valtrat-Typs konnten als Hauptursache der antileishmanialen Aktivität des Extraktes identifiziert werden. Diese Substanzen waren jedoch in Lösung instabil und zeigten starke Zytotoxizität gegen eine murine Zelllinie (J774.1), was sie für weitere Untersuchungen ungeeignet machte.

Bornylkaffeat und seine Derivate wiesen ebenfalls antileishmaniale Eigenschaften auf, zusammen mit geringerer Zytotoxizität im Vergleich zu den Iridoiden. Daher wurde diese Struktur als Grundlage für die Synthese einer Substanzbibliothek und zur Untersuchung von Struktur-Aktivitätsbeziehungen (SAR) herangezogen. Im Hinblick auf die antileishmaniale Aktivität erwies sich Borneol als bester Estersubstituent, zusammen mit anderen sperrigen oder sterisch anspruchsvollen Alkoholen wie z. B. Thymol, Menthol oder Adamantol. Die Brenzcatechinstruktur und die α,β-ungesättigte Carbonyleinheit trugen andererseits signifikant zur Zytotoxizität der Moleküle bei. Diese Strukturelemente erwiesen sich jedoch als nicht notwendig für eine gute antileishmaniale Aktivität. Dies führte zur Entwicklung der optimierten Struktur des Bornylphenylpropionates **18** (s. Abb. 2) mit antileishmanialer Aktivität, vergleichbar mit

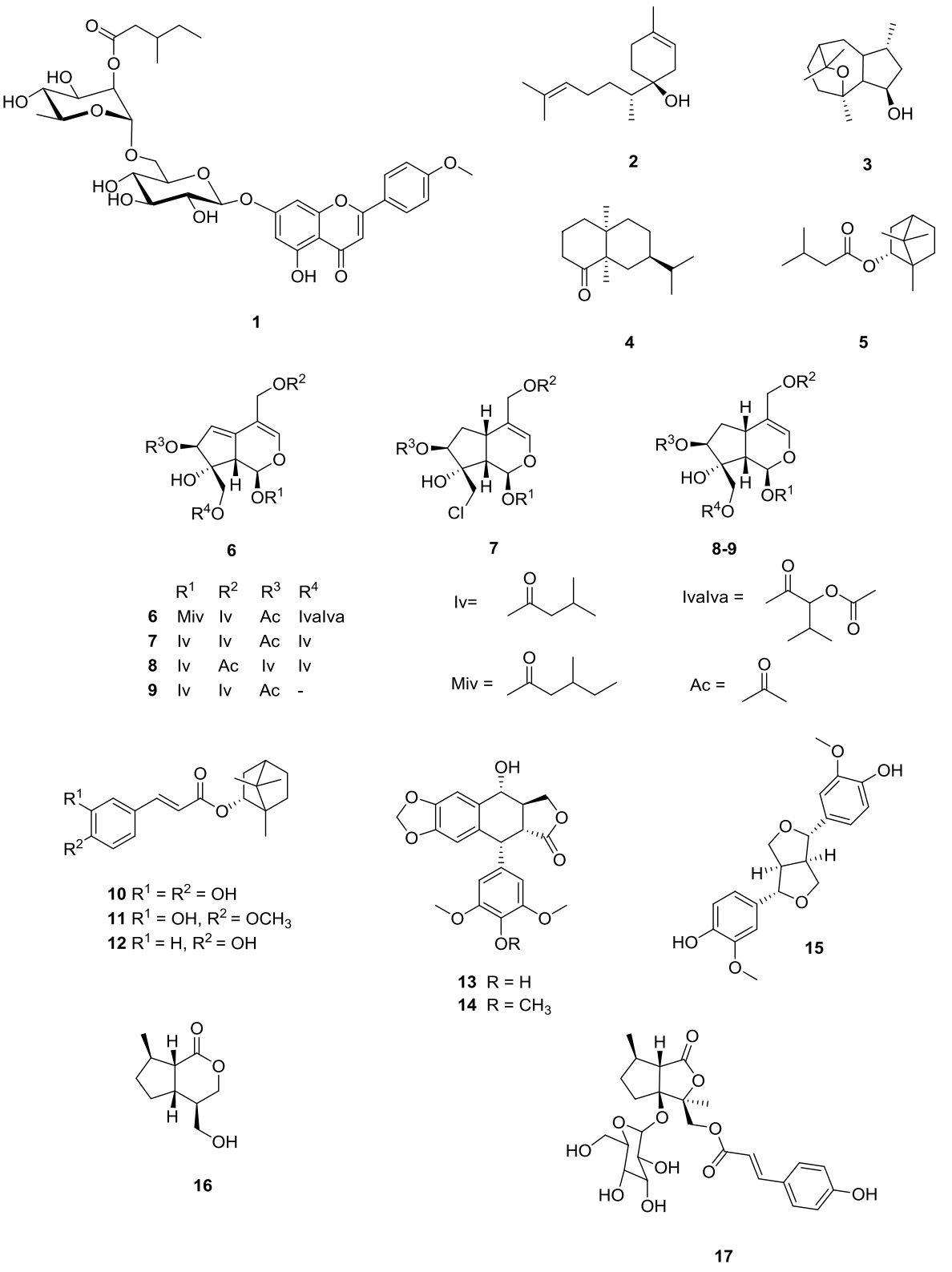


Abb.1. Strukturen der aus dem Extrakt isolierten Verbindungen **1**-**17**.

der Aktivität des Bornylkaffeats **10**, aber ohne Toxizität gegen Makrophagen- (J774.1 und BMDM), Nieren- (HEK 293T) oder Leberzellen (HEP-G2) (s. Abb. 2). Durchflusszytometrische Messungen zeigten, dass **18** in *L. major*-Promastigoten

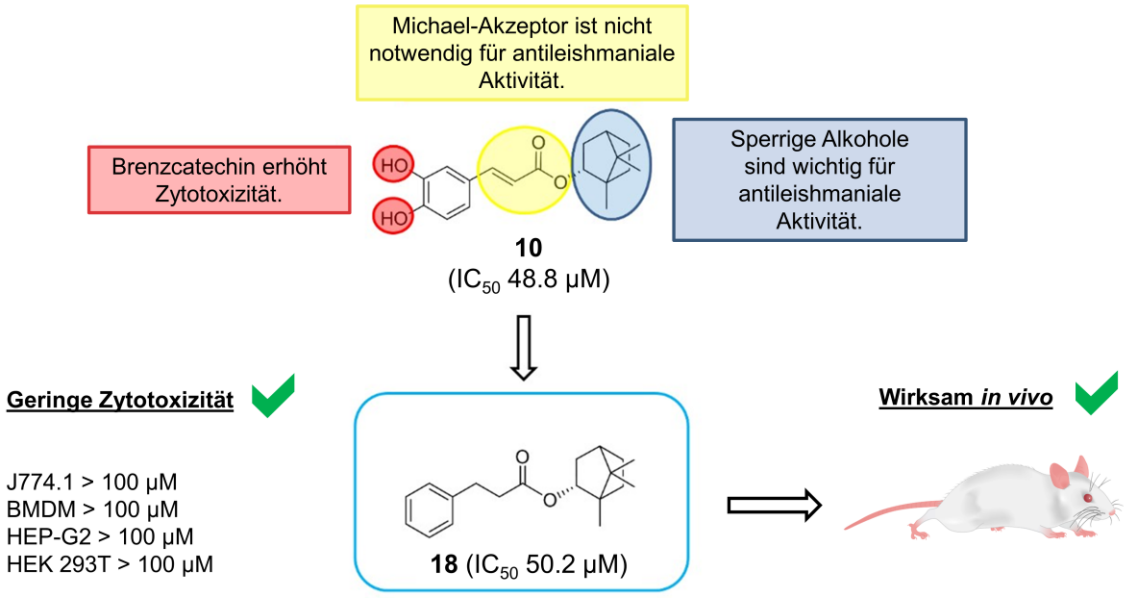


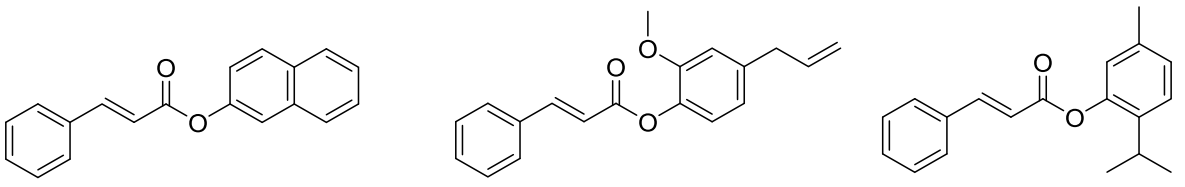
Abb.2. Zusammenfassung der Ergebnisse der SAR-Untersuchungen.

zeitabhängig einen ähnlichen Zelltod auslöst wie Miltefosin. Zusätzlich wurde die Bildung von Vakuolen und der Verlust der Membranintegrität beobachtet. Diese Ergebnisse veranlassten *In-vivo*-Experimente in *L. major*-infizierten BALB/c-Mäusen. Die Mäuse tolerierten die intraperitoneale (i.p.) Gabe von 50 mg/kg/Tag an **18** ohne nachteilige Effekte. In diesem Modell für kutane Leishmaniose ergab die Applikation von Bornylphenylpropionat **18** einen Heilungsphänotypen und eine signifikante Reduktion der Parasitenlast in den infizierten Pfoten und in den vom Ort der Infektion ableitenden Kniekehllymphknoten nach vier Wochen.

Erste *In-vivo*-Experimente in einem Modell für viscerale Leishmaniose (*L. donovani*) wurden in Kalkutta durchgeführt. Die Applikation (i.p.) von 2 mg/kg/Tag von **18** über fünf Tage ergab eine signifikante Reduktion der Parasitenlast in der Leber.

In einem Screening der Substanzbibliothek gegen *L. mexicana*-Promastigoten zeigten Naphthylcinnamat **19** and Eugenylcinnamat **20** (s. Abb. 3) die besten Aktivitäten (vgl. Seite 159). **19** induzierte die Produktion von NO in naiven und infizierten Makrophagen, vergleichbar mit Miltefosin.

In einem Screening der Substanzbibliothek gegen Schistosomula von *Schistosoma mansoni* (San Francisco) erwiesen sich Thymyl- und Eugenylcinnamat (**21**, **20**) (s. Abb. 3) als effektive antischistosomale Substanzen *in vitro*. Beide Verbindungen führten



19 **20** **21**

Abb.3. Napthylcinnamat (**19**) aktiv gegen *L. mexicana*-Promastigoten; Eugenyl- (**20**) und Thymylcinnamat (**21**) aktiv gegen Schistosomula von S. mansoni.

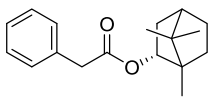
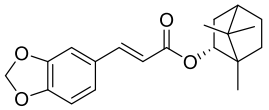
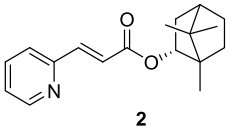
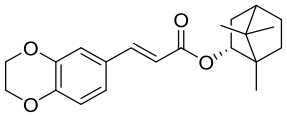
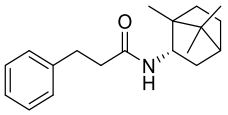
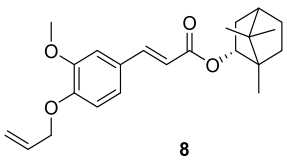
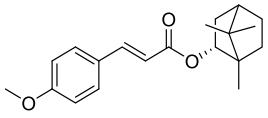
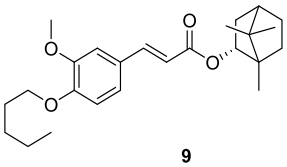
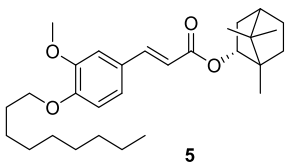
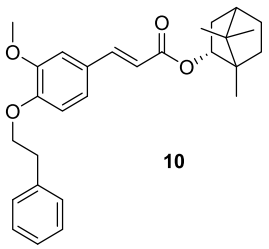
entweder zu Degeneration (**21**) oder zum Tod der Schistosomula (**20**) nach 72 Stunden (c = 5 µM). Diese Substanzen induzierten ebenfalls die Bildung von Vakuolen in den Parasiten, was einen neuen Phänotypen im Rahmen dieses Assays darstellte.

Zusammenfassend kann festgestellt werden, dass gute Leitstrukturen gegen Schistosomula und einige Leishmanienstämme gefunden wurden, wobei Bornylphenylpropanoat **18** als nicht toxischer, antileishmanialer Wirkstoff mit *In-vivo*-Aktivität hervorsticht. Weitere Untersuchungen, die Aufklärung des genauen Wirkmechanismus betreffend, sind notwendig.

7. APPENDIX

7.1. Unpublished Data

In vitro results (L. major promastigotes and J774.1) of unpublished compounds

Structure	IC_{50} [µM]	Structure	IC_{50} [µM]
 **1**	*L. major*: 57.3 J774.1: 52.3	 **6**	*L. major*: > 100 J774.1: > 100
 **2**	*L. major*: 97.0 J774.1: > 100	 **7**	*L. major*: 54.4 J774.1: 65.1
 **3**	*L. major*: > 100 *L. donovani*: 54.5*	 **8**	*L. major*: > 100 J774.1: > 100
 **4**	*L. major*: 54.0 J774.1: 86.9	 **9**	*L. major*: > 100 J774.1: > 100
 **5**	*L. major*: > 100 J774.1: > 100	 **10**	*L. major*: > 100 J774.1: > 100

*Reduction of parasite burden *in vivo* in *L. donovani* infected BALB/c mice after application of 1mg/kg/day for 5 days IP: 33.8%.

*Synthesis of compounds **1**, **3** and **4***

Compounds **1**, **3** and **4** were synthesized using the Steglich esterification method as described in P2 (chapter 3.2).

*Synthesis of precursor aldehydes for compounds **5**, **8**-**10***

Precursor aldehydes were synthesized starting from vanillin following the method described in the literature [1]. Briefly, vanillin, K_2CO_3 and tetrabutylammonium iodide (TBAI) were dissolved in acetone and the desired halogen alkane (e.g. 1-bromononane or benzyl bromide) was added. After 24 h stirring at room temperature the resulting precipitate was filtered off, the solvent was evaporated and the residue dissolved in 20 mL of $CHCl_3$. The solution was washed with saturated $NaHCO_3$, H_2O, NaCl-solution three times, respectively, and was dried over $MgSO_4$. Column chromatography (ethyl acetate/petrol ether, 60:40) yielded the desired aldehyde.

*Synthesis of precursor aldehydes for compounds **6** and **7***

According to the literature [2] 3,4-dihydroxybenzaldehyde was dissolved in DMSO and KF was added. After addition of the respective dihalogen alkanes (e.g. 1,2-dibromomethane or 1,2-dibromoethane) the solution was heated for 4-5 h at 110-120 °C. Extraction with diethyl ether and column chromatography (ethyl acetate/petrol ether, 60:40) yielded the desired aldehyde.

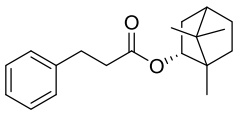
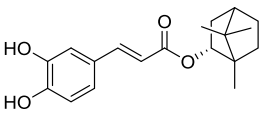
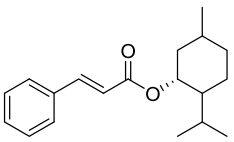
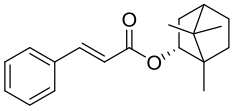
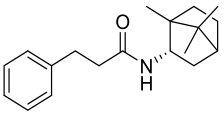
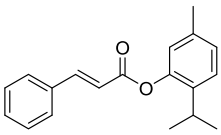
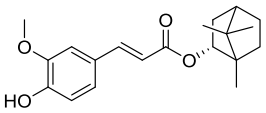
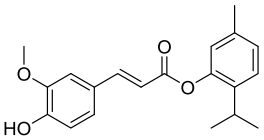
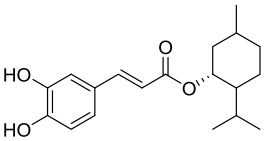
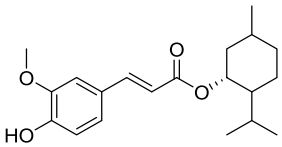
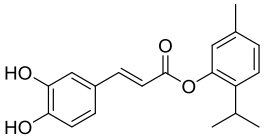
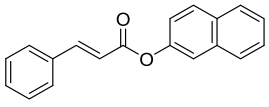
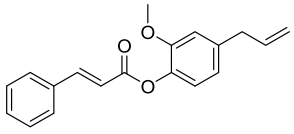
*Synthesis of compounds **2**, **5**-**10***

Compounds **2**, **5**-**10** were synthesized *via* a modified Knoevenagel-Doebner condensation as described in P2 (chapter 3.2).

References

1. Al-Douh, M.; Shafida, A. H.; Hasnah O., Efficient benzylation of *o*–vanillin using TBAI as catalyst and the crystal structure of the product. *Univ. Aden J. Nat. Appl. Sci*. **2008**, *12*, 79-92.
2. Luo, Y.; Zhang, S.; Qiu, K.-M.; Liu, Z.-J.; Yang, Y.-S.; Fu, J.; Zhong, W.-Q.; Zhu, H.-L. Synthesis, biological evaluation, 3D-QSAR studies of novel aryl-2*H*-pyrazole derivatives as telomerase inhibitors. *Bioorg. Med. Chem. Lett.* **2013**, *23*, 1091-1095.

*L. mexicana promastigotes in vitro results**

Structure	IC_{50} [µM]	Structure	IC_{50} [µM]
	> 100		no effect
	45.2		> 100
	> 100		31.4
	26.2		19.3
	10.6		12.9
	no effect		2.9
	7.6		

*We are grateful to Alicia Ponte-Sucre (Venezuela) and her co-workers for providing us with these data.

7.2. Experimental logP Values

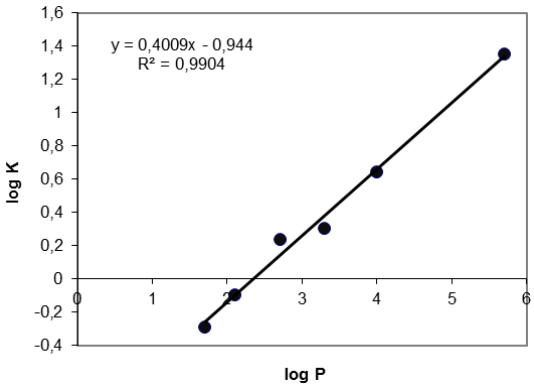
Experiments were conducted following the OECD guidelines for logP determination by HPLC [1] using following formula for the calculation of capacity factor k:

$$k = \frac{t_R - t_O}{t_O}$$

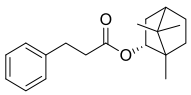
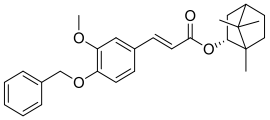
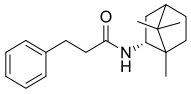
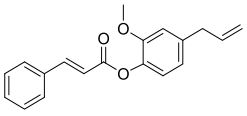
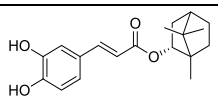
t_R = retention time; t_0= dead-time

Calibration line

reference	logk	logP
thiourea	0	0 [1]
acetophenon	-0.29	1.7 [1]
benzene	-0.10	2.1 [1]
toluene	0.24	2.7 [1]
thymol	0.30	3.3 [1]
biphenyl	0.65	4.0 [1]
anthracene	0.80	4.6 [2]
triphenylamine	1.35	5.7 [1]



By inserting the retention time of the measured substance into the linear equation the logP value can be extrapolated.

Compound	logP	Compound	logP
	3.2		6.2
	4.3		4.9
	6.2		

HPLC conditions

Isocratic: H_2O/MeOH 30:70 (v/v)

Column: Nucleodur Sphinx RP Macherey Nagel, 150 x 4,6 mm, 5 µm.

Flow: 1.0 mL/min

Inj.-Vol.: 10 µL

Wavelength: 254 nm

Temp.: 20 °C

References

1. OECD , *Test No. 117: Partition coefficient (n-octanol/water), HPLC method*, OECD Guidelines for the testing of chemicals, section 1, OECD Publishing: Paris, **2009**. DOI: http://dx.doi.org/10.1787/9789264069824-en
2. Pinsuwan, S.; Li, A.; Yalkowsky S. H., Correction of octanol/water solubility ratios and partition coefficients, *J. Chem. Eng. Data* **1995**, *40*, 623-626.

7.3. List of Publications and Documentation of Authorship

I. Antileishmanial and Cytotoxic Compounds from *Valeriana wallichii* and Identification of a Novel Nepetolactone Derivative.

Jan Glaser, Martina Schultheis, Heidrun Moll, Banasri Hazra and Ulrike Holzgrabe, *Molecules* **2015**, *20*, 5740-5753.
https://dx.doi.org/10.3390/molecules20045740.

II. Antileishmanial Lead Structures from Nature: Analysis of Structure-Activity Relationships of a Compound Library Derived from Caffeic Acid Bornyl Ester.

Jan Glaser, Martina Schultheis, Sudipta Hazra, Banasri Hazra, Heidrun Moll, Uta Schurigt and Ulrike Holzgrabe, *Molecules* **2014**, *19*, 1394-1410.
https://dx.doi.org/10.3390/molecules19021394.

III. Cinnamic Acid Bornyl Ester Derivatives from *Valeriana wallichii* Exhibit Antileishmanial I*n Vivo* Activity in *Leishmania major*-Infected BALB/c Mice.

Anita Masic, Ana Maria Valencia Hernandez, Sudipta Hazra, **Jan Glaser**, Ulrike Holzgrabe, Banasri Hazra, Uta Schurigt, *PLOS ONE* **2015***, 10,* e0142386. doi:10.1371/journal.pone.0142386.

IV. Antischistosomal Activity of Cinnamic Acid Esters: Eugenyl and Thymyl Cinnamate Induce Cytoplasmic Vacuoles in Schistosomula of *Schistosoma mansoni*.

Jan Glaser, Uta Schurigt, Brian Suzuki, Conor R. Caffrey and Ulrike Holzgrabe, *Molecules* **2015**, *20*, 10873-10883.
http://dx.doi.org/10.3390/molecules200610873.

V. Focus on PAINS: False Friends in the Quest for Selective Antiprotozoal Lead Structures from Nature?

Jan Glaser, Ulrike Holzgrabe; manuscript in revision, *MedChemComm* **2015**.

This section contains a list of the individual contribution for each author to the publications reprinted in this thesis. Unpublished manuscripts are handled, accordingly.

P1 **Glaser J, Schultheis M, Moll H, Hazra B, Holzgrabe U,** Antileishmanial and Cytotoxic Compounds from *Valeriana wallichii* and Identification of a Novel Nepetolactone Derivative. *Molecules* **2015**, *20*, 5740-5753.

Author	**1**	**2**	**3**	**4**	**5**
Fractionation of the extract	x				
Structure elucidation	x				
Isolation and identification of compounds	x				
Antileishmanial assays		x			
Supply of plant material and extract				x	
Study design/concept development	x				x
Data analysis and interpretation	x			x	x
Manuscript planning	x				x
Manuscript writing	x				
Correction of manuscript	x		x	x	x
Supervision of Jan Glaser					x

P2 **Glaser J, Schultheis M, Hazra S, Hazra B, Moll H, Schurigt U, Holgrabe U,** Antileishmanial Lead Structures from Nature: Analysis of Structure-Activity Relationships of a Compound Library Derived from Caffeic Acid Bornyl Ester. *Molecules* **2014**, *19*, 1394-1410.

Author	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Synthesis of the compound library	x						
Antileishmanial and cytotoxicity assays		x	x	x		x	
Study design/concept development	x						x
Data analysis and interpretation	x					x	
Manuscript planning	x						x
Manuscript writing	x						
Correction of manuscript	x			x	x		x
Supervision of Jan Glaser							x

P3 **Masic A, Hernandez AMV, Hazra S, Glaser J, Holzgrabe U, Hazra B, Schurigt U,** Cinnamic Acid Bornyl Ester Derivatives from *Valeriana wallichii* Exhibit Antileishmanial *In Vivo* Activity in *Leishmania major*-Infected BALB/c Mice. *PLOS ONE* **2015***, 10,* e0142386. doi:10.1371/journal.pone.0142386

Author	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Synthesis and development of the tested compounds				x			
In vivo experiments	x						
In vitro experiments	x	x	x			x	x
Study design/concept development	x				x	x	x
Data analysis and interpretation	x	x	x	x	x	x	x
Manuscript planning	x			x			x
Manuscript writing	x	x		x			
Correction of manuscript	x	x		x	x	x	x
Supervision of Jan Glaser					x		

P4 **Glaser J, Schurigt U, Suzuki BM, Caffrey CR, Holzgrabe U,** Antischistosomal Activity of Cinnamic Acid Esters: Eugenyl and Thymyl Cinnamate Induce Cytoplasmic Vacuoles in Schistosomula of *Schistosoma mansoni*. *Molecules* **2015**, *20*, 10873-10883.

Author	**1**	**2**	**3**	**4**	**5**
Fractionation of the extract	x				
Synthesis of compound library	x				
Isolation and identification of compounds	x				
Antischistosomal assays		x	x	x	
Study design/concept development		x		x	x
Data analysis and interpretation	x	x		x	x
Manuscript planning	x	x			x
Manuscript writing	x	x			
Correction of manuscript	x	x		x	x
Supervision of Jan Glaser					x

M1 **Glaser J, Holzgrabe U,** Focus on PAINS. False friends in the Quest for Selective Antiprotozoal Lead Structures from Nature ? *Manuscript in revision, MedChemComm,* **2015**.

Author	**1**	**2**
Study design/concept development	x	x
Literature analysis and interpretation	x	
Manuscript planning	x	x
Manuscript writing	x	
Correction of manuscript	x	x
Supervision of Jan Glaser		x

7.4 Conference Contributions

Glaser, J.; Gonzalez-Leal, I.; Schurigt, U.; Hazra, B.; Schirmeister, T.; Holzgrabe, U.
Antileishmanial Activity in Fractions of Chloroformic Extract from Valeriana wallichii Roots.
DPhG/OePhG-Jahrestagung, 2011, Innsbruck.

Glaser, J.; Schultheis, M.; Banasri, H.; Gonzalez-Leal, I.; Schurigt, U.; Schirmeister, T.; Moll, H.; Holzgrabe, U.
Valeriana wallichii Roots as a Source for New Drugs with Antileishmanial Activity.
22nd International Symposium of Medicinal Chemistry, 2012, Berlin;
Chem-SystM, 2012, Würzburg (Poster price).

Glaser, J.; Schultheis, M.; Hazra, S.; Gomez, C.; Alcazar, W.; Hazra, B.; Schurigt, U.; Ponte-Sucre, A.; Moll, H.; Holzgrabe, U.
Antileishmanial Lead Structure from Nature – Structure Activity Relationship Investigation of a Compound Library Based on Caffeic Acid Bornyl Ester.
DPhG-Jahrestagung, 2013, Freiburg im Breisgau;
3rd International Symposium SFB 630, 2013,Würzburg.

Glaser, J.; Schurigt, U.; Suzuki, B.; Caffrey, C.R.; Holzgrabe, U.
Antischistosomal Activity of Derivatives of Caffeic Acid Bornyl ester Isolated from Valeriana wallichii Rhizomes.
DPhG-Jahrestagung, 2014, Frankfurt am Main;
Chem-SystM, 2014, Würzburg;
SFB630 Final Symposium, 2015, Würzburg.