DOI: 10.1002/cbdv.201800305

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CHEMISTRY & BIODIVERSITY

Evaluation of Lignans from *Piper cubeba* against *Schistosoma mansoni* Adult Worms: A Combined Experimental and Theoretical Study

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Six dibenzylbutyrolactonic lignans ((−)-hinokinin (**1**), (−)-cubebin (**2**), (−)-yatein (**3**), (−)-5-methoxyyatein (**4**), dihydrocubebin (**5**) and dihydroclusin (**6**)) were isolated from *Piper cubeba* seed extract and evaluated against *Schistosoma mansoni*. All lignans, except **5**, were able to separate the adult worm pairs and reduce the egg numbers during 24 h of incubation. Lignans **1**, **3** and **4** (containing a lactone ring) were the most efficient concerning antiparasitary activity. Comparing structures **3** and **4**, the presence of the methoxy group at position 5 appears to be important for this activity. Considering **1** and **3**, it is possible to see that the substitution pattern change (methylenedioxy or methoxy groups) in positions 3′ and 4′ alter the biological response, with **1** being the second most active compound. Computational calculations suggest that the activity of compound **4** can be correlated with the largest lipophilicity value.

Keywords: *Schistosoma mansoni*, *Piper cubeba*, dibenzylbutyrolactonic lignans, lipophilicity, natural products, biological activity, phytochemistry.

Introduction

Schistosomiasis is a neglected tropical disease, which is caused by trematode flatworms of the *Schistosoma* genus and affects more than 207 million people. It causes morbidity in nearly 120 million people and over 6.4 million deaths per year.[1] The infection is prevalent in tropical and subtropical regions and more than 700 million people live in endemic areas.[1−3] Praziquantel (PQZ) has been the drug of choice for schistosomiasis chemotherapy for over 30 years and recent studies have been developed to understand its mechanism of action.[4−7] PZQ does not prevent reinfection; indices

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbdv.201800305

of resistant strains existence and studies have shown that young parasites are less susceptible to PZQ than adult worms.[8] In addition, side effects of PZQ together with the concern regarding drug resistant strains development[8–10] justify the search for alternative agents against *Schistosoma* worms.

Lignans are a class of widespread natural products with expressive structural and biological diversity. They derive from oxidative dimerization of two phenylpropanoid units (C6–C3).[11] Phenylpropanoid dimerization, oxygen incorporation and skeletal functionalization may occur in different ways, leading to a great variety of structures.[12] *Piper cubeba* L. (Piperaceae) is known for its high lignans content and the *in vitro* and *in vivo* therapeutic properties of (−)-cubebin and (−)-hinokinin against *Trypanosoma cruzi* have already been reported by our group.[13–17] In addition, the essential oil from *Piper cubeba* L. fruits has been reported as exhibiting schistosomicidal activity.[18]

As part of the ongoing investigations on antiparasitic compounds by our research group, the *in vitro* activity of lignans (−)-hinokinin (**1**), (−)-cubebin (**2**), (−)-yatein (**3**), (−)-5-methoxyyatein (**4**), dihydrocubebin (**5**) and dihydroclusin (**6**) isolated from ethanolic extract of *P. cubeba* seeds against *S. mansoni* are being reported now. Computational calculations based on the Density Functional Theory (DFT) were performed to relate the biological activity of all assayed compounds with parameters such as molecular electrostatic potential (MEP) and lipophilicity (log*P*). In addition, molecular docking simulations using *S. mansoni* tubulin structure as biological target to provide a more in-depth discussion on the mechanism of action of these compounds was also performed.

Table 1. ^{13}C-NMR Spectral data for compounds **1**–**6** (δ in ppm)

Position	1	2	3	4	5	6
1	131.8	134.2	131.9	134.0	134.6	134.7
2	109.2	109.3	109.1	102.8	108.5	109.6
3	146.8	147.9	148.3	149.4	146.2	148.0
4	148.2	146.1	146.8	134.5	148.0	146.1
5	108.6	108.5	108.7	144.0	109.7	108.4
6	121.9	121.7	121.9	109.2	122.2	122.2
7	38.7	39.7	35.6	39.3	36.3	36.3
8	41.7	46.3	41.4	41.5	44.6	44.0
9	71.4	72.6	71.5	71.5	60.7	60.7
1′	132.1	133.7	133.7	132.5	134.6	136.7
2′	108.6	108.5	106.7	106.7	108.5	106.5
3′	146.7	147.9	153.7	153.8	146.2	153.7
4′	148.2	146.1	133.7	137.4	148.0	134.7
5′	109.8	108.4	153.7	153.8	109.7	153.7
6′	122.6	122.1	106.7	106.7	122.2	106.5
7′	35.2	38.8	38.7	35.3	36.3	36.9
8′	46.8	52.4	46.8	46.9	44.6	44.4
9′	178.7	103.7	178.8	178.7	60.7	60.7
OCH_2O	101.3	101.2	–	56.5	101.2	101.8
3′-MeO	–	–	56.5	56.5	–	56.5
4′-MeO	–	–	61.2	61.2	–	61.2
5′-MeO	–	–	56.5	56.5	–	56.5
5-MeO	–	–	–	101.8	–	–

Results and Discussion

Purification of the hydro-methanolic fraction of *Piper cubeba* seed extract led to the isolation of six compounds. ^{1}H- and ^{13}C-NMR spectral data of isolated compounds were in agreement with previous published data and identified compounds **1**,[19] **2**,[20] **3**,[21] **4**,[21] **5**,[22,23] and **6**[23,24] (*Table 1* and *Figure 1*). Structures were confirmed on the basis of NMR data.

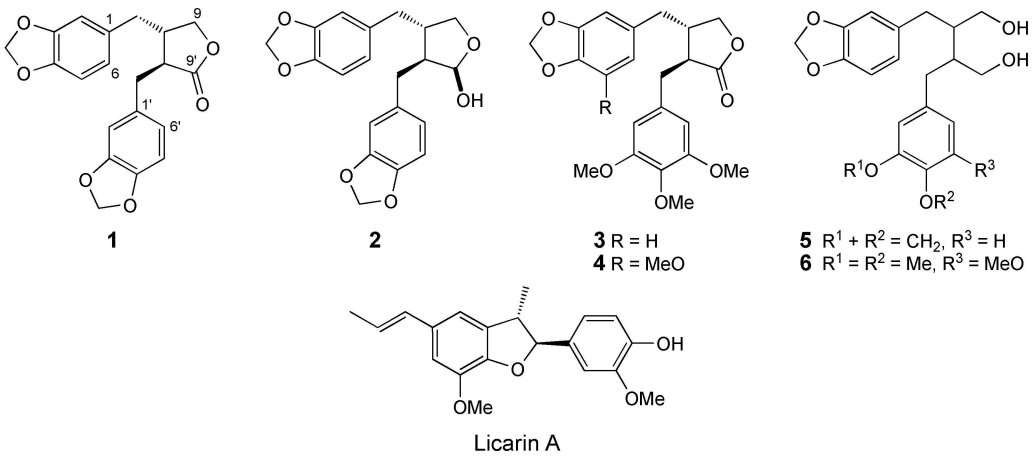

Figure 1. Structures of compounds **1**–**6** and licarin A (for comparison purposes).

Regarding the activity against *S. mansoni*, none of the assayed compounds caused alteration in adult worm couples at the concentration of 5 μM (data not shown). In addition, the assayed compounds did not cause mortality to male or female parasites at concentrations between 10 and 100 μM. However, decrease of significant motor activity of parasites was observed for compounds **1**–**4**, when compared to the negative control groups (parasite in RPMI 1640 medium or RPMI 1640 with 0.1% DMSO), as shown in *Table 2*. After 24 h of incubation of lignan **4** at concentrations of 10 to 100 μM, 100% of parasites presented motor activity decrease. The other lignans (**1**, **2** and **3**) caused a motor activity decrease between 75% and 100% at concentrations of 50 and 100 μM. Differences between motor activity changes of male or female parasites were not observed. Compounds **5** and **6** were inactive in decreasing the motor activity. On the other hand, incubation of worms with PZQ (1.56 μM) resulted in 100% parasite mortality within 24 h. Studies have shown that some lignans inflict lethal effect against adult *S. mansoni* worms. (±)-Licarin A (see *Figure 1* for structures comparison) for instance, which is a neolignan, displayed schistosomicidal activity with a lethal concentration (LC_{50}) value of 53.57 μM after 24 h of incubation,[25] while (−)-6,6′-dinitrohinokinin with a lethal concentration of 103.9 μM.[26]

Table 2. *In vitro* effects of lignans on adult *S. mansoni* worms' motor activity

Compound	% of motor activity decrease/ concentration ± SD[a][b]		
	10 μM	50 μM	100 μM
(−)-Hinoquinin (**1**)	0 ± 0	75 ± 12.5[c]	100 ± 0[c]
(−)-Cubebin (**2**)	0 ± 0	75 ± 12.5[c]	75 ± 12.5[c]
Yatein (**3**)	0 ± 0	75 ± 0[c]	100 ± 0[c]
5-Methoxyyatein (**4**)	100 ± 0[c]	100 ± 0[c]	100 ± 0[c]
Dihydrocubebin (**5**)	0 ± 0	0 ± 0	0 ± 0
Dihydroclusin (**6**)	0 ± 0	0 ± 0	0 ± 0

[a] Worms with decreased movement when compared to worms of the negative control group (RPMI 1640 medium with 0.1% DMSO). [b] % relative to 12 adult worms couples. Data is expressed as the mean ± SD of three independent experiments. RPMI 1640 medium with 0.1% DMSO (negative control) does not inflict alteration in worms' motor activity. PZQ at 1.56 μM (positive control) caused 100% parasite death in 24 h incubation. [c] $P < 0.001$ Statistical difference in relation to the negative control group.

In female schistosomes, pairing in the gynecophoric canal of the male parasite is a prerequisite for full development of female organs, such as ovary and vitellarium, that are involved in the production of oocytes and vitellocytes cells, important for egg production.[27 – 29] Eggs play a key role in transmitting the infection and are responsible for schistosomiasis pathogenesis, as they can be trapped in host tissues inducing immunologically mediated granulomatous inflammation and fibrosis leading to severe pathology such as hepatosplenomegaly. In this sense, there is significant interest in the search for new strategies and compounds, not only regarding parasite viability but also pairing and egg production.[29] For this reason, adult *S. mansoni* worm couples were incubated with the isolated lignans **1**, **2**, **3**, **4**, **5** and **6** from *P. cubeba* at the concentrations of 10, 50 and 100 μM for 24 h and pairing (*Figure 2*) and egg production was evaluated.

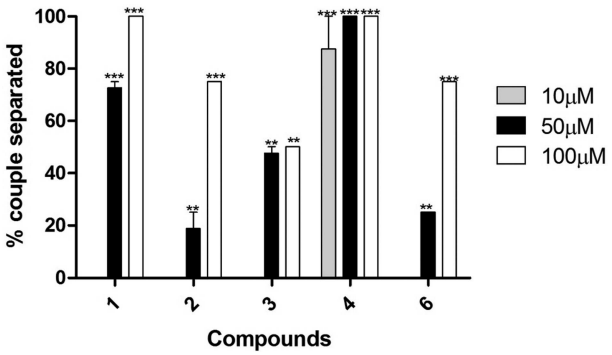

Figure 2. Effect of different concentrations of lignans **1**–**6** on *S. mansoni* pairing. *S. mansoni* couples were incubated with the lignans for 24 h and the number of couples were analyzed by inverted microscopy. RPMI 1640 medium and RPMI 1640 medium with 0.1% DMSO were used as negative control groups and did not induce couple separation. Data is expressed as the mean ± SD of three independent experiments. *** $P < 0.001$ and ** $P < 0.01$ indicate statistical difference in relation to the negative control group (RPMI 1640 medium with 0.1% DMSO).

Compound **4** was able to separate more than 85% of couples at 10 μM, while 100% of couples at the other assayed concentrations (50 or 100 μM). Lignan **1** was able to separate 75% and 100% of couples at concentrations of 50 μM and 100 μM, respectively. Moreover, lignans **2** and **6** promoted separation of 75% of coupled adult worms at a concentration of 100 μM, while less than 30% were separated at 50 μM. Lignan **3** was able to separate 50% of adult worm couples in both concentrations (50 or 100 μM). However, lignan **5** did not cause any adult worm couples

separation at any assayed concentrations. Furthermore, all lignans except **5** reduced the number of eggs laid during 24 h of incubation at concentrations that caused couple separation (data not shown). This result is probably due to *S. mansoni* adult worm couples separation and induced motor activity decrease by the lignans. A variety of natural compounds isolated from the *Piper* (Piperaceae) genus present biological activity within several organisms including *Trypanosoma* sp., *Leishmania* spp. and *Schistosoma mansoni*.[30] Among the major lignans found in *P. cubeba*, dibenzylbutyrolactolic lignan cubebin **2** and dibenzylbutyrolactonic lignans (−)-hinokinin (**1**), yatein (**3**) and isoyatein can be found. In this work, among the six evaluated lignans, dibenzylbutyrolactonic lignans **4**, **1** and **3** showed to be the most effective in motor activity decrease and adult worm couples separation. To the best of our knowledge, studies on biological evaluation of lignans **4** and **3** are rare, therefore, this work is an important contribution to the scientific literature, highlighting the antiparasitic potential of these substances. Lignan **1** has been studied by our group *in vitro* and *in vivo* for its trypanocidal potential,[16,17] as well as for its mutagenicity and antimutagenicity (evaluated by the Ames test).[31]

Based on both the experimental results, motor activity decrease and adult *S. mansoni* worms pairing, analyses to explore possible correlations between the chemical structure and biological activity of lignans **1**–**6** were conducted. A preliminary analysis suggests that lignans with a dibenzylbutyrolactonic skeleton (**1**, **3** and **4**) were more active than dibenzylbutyrolactolic lignan **2**, and dibenzylbutane lignans **5** and **6**. This observation is evident when comparing the scaffolds of compounds **1**, **2** and **5**, which only differ in relation to the presence and type of central ring in their chemical structure. In this sense, it is possible to verify the gradual biological activity decrease when going from compound **1** (that presents a lactonic ring; active) through **2** (that presents a lactolic ring; moderately active) and finally to compound **5** (that does not present a central ring; inactive). Moreover, comparison of structures of compounds **3** and **4** indicates that the presence of the methoxy group at position 5 seems to be important for activity, since substance **4** showed to be much more active than **3**. Concerning the two dihydro substituted lignans **5** and **6**, the latter containing methoxy groups at positions 3′, 4′ and 5′, only resulted in couple separation, while dihydrocubebin proved to be inactive.

Computational calculations were performed to find electronic molecular properties that could correlate with the biological activity of the studied compounds. Geometries of all six compounds were fully optimized (B3LYP/6-31 + G** level of theory) without any constraint, followed by vibrational analyses that ensure true minima identification. The molecular electrostatic potential (MEP) is able to provide information about the molecular shape and size, as well as indicates the reactive sites of a molecule.[32] The MEP was computed at the same theory level and the results are shown in *Figure 3*. The intensity of the negative electrostatic potential of compounds **1**, **3** and **4**, indicated by the extent of a particular red region (marked by arrows), makes them different from compounds **2**, **5** and **6**. Such difference may be the source of different biological activities of the former molecules group. Other red regions in all compounds are either less intense or surrounded by blue/green regions hindering interaction with charged particles. In general, the intensity of positive electrostatic potential (blue regions) is less intense, indicating electronically neural zones. The surface obtained with the COSMO-RS (Conductor-like Screening Model for Real Solvents) method (*Figure S1*, *Supporting Information*) corroborates the observations made by the MEP.

The biological activity of a compound can also be correlated with its lipophilicity (expressed by their log*P* values, which can be used to verify the distribution tendency of a substance between aqueous and lipid media).[33] The lipophilicity is traditionally estimated from the octanol-water partition coefficient (*P*).[34,35] However, octanol is partially miscible with water and leads to a more complex partitioning behavior than nonpolar solvents (hexane, for instance).[36] Compounds that are able to form hydrogen bonds, can have their log*P* values overestimated in the octanol-water model.[34,36] In this sense, it is appropriate to consider the log*P* value in different classes of lipophilic solvents.[34,36–38] Although the mechanism of action is unclear, studies have reported that the molecular lipophilicity plays an important role in schistosomicidal activity (in general, the more lipophilic compounds show higher activity indices).[39–42] In this work, the calculated lipophilicity values (*Table 3* and *Table S1*), obtained with the hexane/water model, suggest that the more active compounds **1**, **3** and **4** are also more lipophilic, corroborating the observations of previous studies.[39–42] It is also observed that the presence of hydroxy groups in the molecular structures (**2**, **5** and **6**) leads to decrease of log*P* values. A comparison between the biological activity profile and lipophilicity,

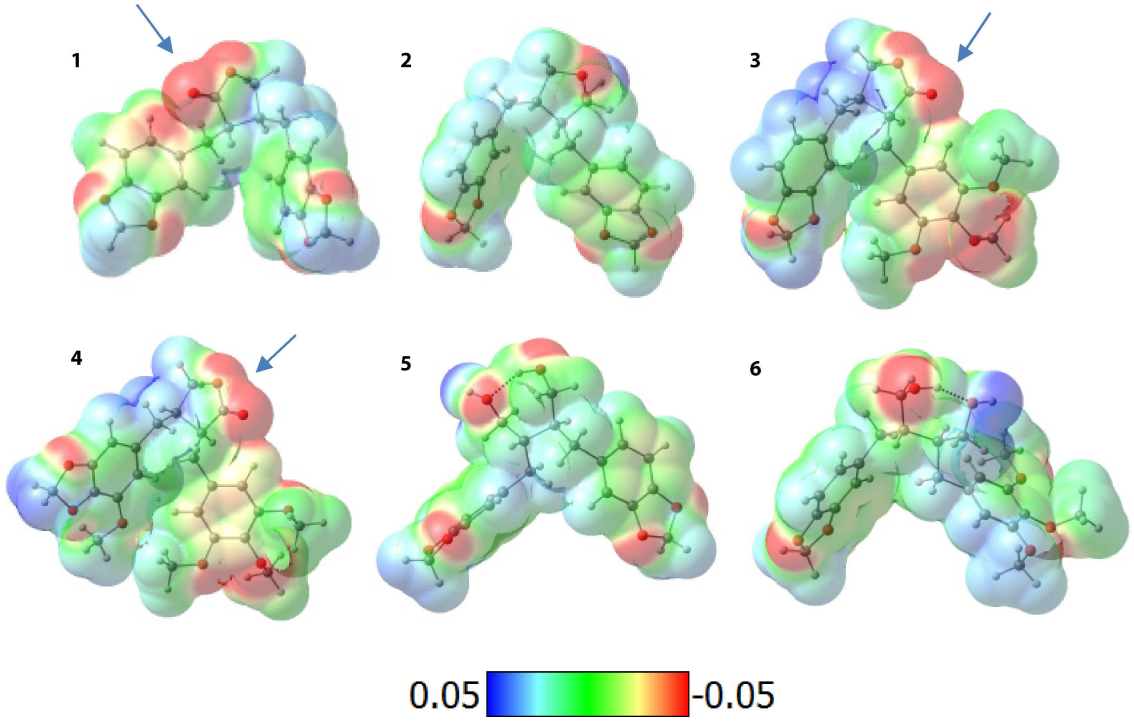

Figure 3. Molecular electrostatic potential (MEP) of compounds **1–6**, calculated at B3LYP/6-31+G** computational level. Red and blue colors indicate negative and positive MEP regions, respectively. Maxima and minima correspond to ±0.05 au. Most possible active (red) regions are indicated by an arrow.

Table 3. Calculated lipophilicities (expressed by their log*P* values - COSMO-RS methodology). Calculations performed at level BP/TZP theory level.

Compound	log*P* hexane/water	octanol/water
1	3.441	4.001
2	2.804	4.728
3	3.654	4.509
4	4.655	5.204
5	0.724	4.186
6	0.661	4.570

the last estimated by both hexane/water and octanol/water model, was also undertaken. The results of this comparison for the studied compounds suggest that the use of a lipophilic solvent such as hexane is more appropriate than octanol to estimate log*P* values.

In an attempt to provide a more in-depth discussion on the mechanism of action of these compounds, molecular docking simulations using the *S. mansoni* tubulin structure as biological target were also performed. It is important to address, however, that as tubulin is only one among several possible protein targets involved in parasite motility,[43] results from such predictions must be analyzed with some caution. The initial hypothesis was based on structural similarity between lignan **4** and the tubulin inhibitor colchicine, which could putatively share some pharmacophoric features necessary for target binding (*Figure 4*). In mammals, colchicine binds close to the α/β interface of tubulin, interacting mainly with the amino acid residues from β-tubulin.[44]

As no structures of α- and β-tubulin are available for *S. mansoni*, a homology model of α/β tubulin heterodimer was derived using the crystallographic

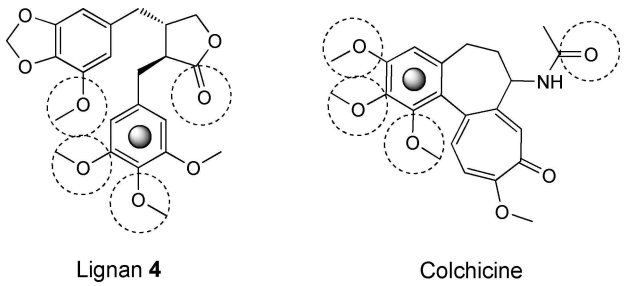

Figure 4. Two-dimensional representation structures of colchicine and lignan **4** highlighting possible common structural features. Putative common hydrogen acceptor groups are highlighted with dashed circles, while the aromatic features are displayed as solid gray circles.

structures from *Rattus norvegicus* as 3-D template (PDB code 4O2B). Considering the long phylogenetical distance between the sequences from the protein target and the crystallographic pivot,[45] alignment correction was performed using multiple tubulin sequences from *trematodes*, *tapeworms*, *nematodes*, *mollusca* and *chordata* (*Table S2*). Model validation was then performed based on normality indices, which describe some major geometrical aspects of the protein structure,[46] and on the Ramachandran plot, for which deviations from preferred, energetically favorable conformations of amino acid residues can be used as potential error indicators in the model.[47] In this regard, the homology model was considered appropriate for docking since the negative values for the Q-Mean (Qualitative Model Energy Analysis) score, −1.97 for α-tubulin and −0.83 for β-tubulin, indicate the model scores as lower than the experimental structures on average.[46] Additionally, 97.3% of the amino acid residues from the protein heterodimer were displayed in the most energetically favored regions of the Ramachandran plot and 2.3% were also in energetically allowed regions (*Figure S2*). Only three amino acid residues were identified as outliers (Lys^{43} and Asp^{45} from α-tubulin and Arg^{725} from β-tubulin), but as these are very distant from the colchicine-binding site (*Figure S3*), they are not expected to affect the docking simulation results.

Comparisons of the most probable binding mode of lignan **4** (*Figure 5A* and *C*) with the bioactive pose of colchicine (*Figure 5A* and *B*) highlights the somewhat similar positions of their trimethoxybenzene rings, which are mainly involved in hydrophobic interactions with residues from β-tubulin. Although the carbonyl oxygens from lactone of compound **4** and from the amide in colchicine display approximately similar positions as originally envisioned (*Figures 4* and *5*A), these groups do not seem to be involved in target interactions. Another relevant aspect is that, while colchicine interacts only with residues from β-tubulin, the methylenedioxy group of **4** is expected to interact with residues from α-tubulin. As the structure of colchicine is more rigid than **4** and seems to tightly hold against the ligand site through several hydrophobic interactions coupled to a H-bond, the drug is expected to be a stronger inhibitor of tubulin assembly than **4**, as highlighted by the predicted $\Delta G_{binding}$ energies. Comparison of the predicted binding modes of lignan **4** with **3** also explains the importance of the additional methoxy group in the most active compound since it allows establishing an extra hydrogen bond. Generally speaking, lignans **3**, **1** and **2** displayed similar binding patterns, with a single hydrogen bond reinforced by hydrophobic or cation-π interactions around the entire structure, but a different scenario was observed for **6** and **5**, for which only part of the structure is expected to interact with the amino acid residues from tubulin and, accordingly, these compounds would be easier displaced from the ligand binding site.

Although this preliminary study does not allow taking final conclusions and requiring further studies, our findings give some insights into structure-activity relationships, since they suggest that the different chemical structures and substituent groups appeared to be important electronic and structural factors that determine schistosomicidal activity. Therefore, lignans are molecular prototypes, which can be used for the development of new antiparasitic drugs.[28,48]

Conclusions

In this work, lignans (−)-hinokinin (**1**), (−)-cubebin (**2**), (−)-yatein (**3**), (−)-5-methoxyyatein (**4**), dihydrocubebin (**5**) and dihydroclusin (**6**) were isolated from *Piper cubeba* seed extract and evaluated against adult *Schistosoma mansoni* worms. Lignans **4**, **1** and **3**, which have a dibenzylbutyrolactonic skeleton, showed most effect on motor activity decrease and adult worm couples separation. Although further studies are required, these results allow for some insights into structure-activity relationships. Accordingly, the presence of the methoxy group at position 5 increased the activity of compound **4** in relation to compound **3**. Comparing the predicted binding modes of lignans **4** and **3**, obtained by molecular docking analysis, also explains the importance of the additional methoxy group in the most active compound **4**, since it allows to establish an extra hydrogen bond with a probable target such as *S. mansoni* α/β-tubulin heterodimer. Finally, considering the six studied compounds, the molecular lipophilicity, calculated with the hexane/water model, suggests that the more active compounds are also more lipophilic.

Experimental Section

General

Separation of lignans **1**–**6** was accomplished on Shimadzu Shim-pack ODS (particle diameter 5 μm, 250×4.60 mm and 250×20 mm) columns equipped with a pre-column of the same material. MeOH used in

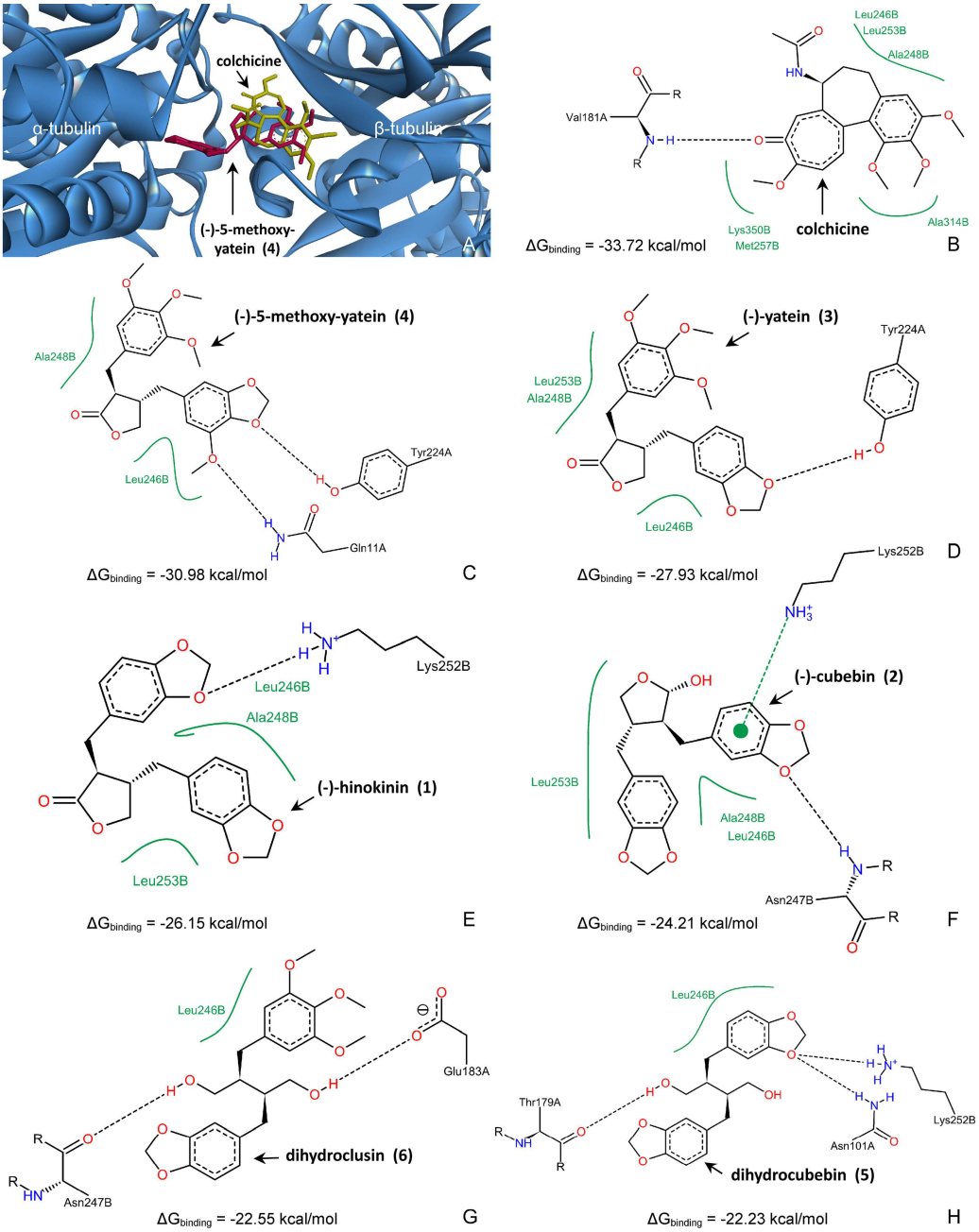

Figure 5. A) Comparison of the binding modes of colchicine (yellow) and lignan **4** (magenta) at the colchicine-binding site of α/β-tubulin heterodimer of *S. mansoni*. B)–H) 2-D diagrams of the docked poses of the ligands at the colchicine-binding site of the tubulin heterodimer from the parasite, highlighting the predicted Gibb's free binding energies ($\Delta G_{binding}$) and the main intermolecular interactions: black dashed lines correspond to H-bonds, the green dashed line to a cation-π interaction and solid green lines to hydrophobic interactions. B) Colchicine. C) Lignan **4**. D) Lignan **3**. E) Lignan **1**. F) Lignan **2**. G) Lignan **6**. H) Lignan **5**.

the experiments was HPLC grade, J. T. Baker. Ultrapure water was obtained by passing redistilled water through a Direct-Q UV3 system from Millipore. Silica gel 60 (60–230 mesh, Merck) was employed for column chromatography. Both analytical and preparative HPLC separation analyses were carried out using a Shimadzu LC-6AD system equipped with a degasser DGU-20 A5, a UV/VIS detector SPD-20 A series, a communication bus module CBM-20 A and a Reodyne manual injector. ^{1}H- and ^{13}C-NMR spectra were recorded on Bruker DRX-500 MHz with ^{1}H (500.13 MHz) and ^{13}C (125.77 MHz) in $CDCl_3$; δ in ppm

relative to Me_4Si as internal standard, *J* in Hz. The negative-ion mode HR-ESI-MS analysis was conducted on a Bruker Daltonics HR-MS ultrOTOF–Q-ESI-TOF (Billerica, MA-USA), employing electrospray ionization; in *m/z*.

Extraction and Purification of the Lignans

Dry seeds of *P. cubeba* were purchased from Floral Seed Company (Dehradun, India - Proc. FAPESP 05/01550-8), powdered and exhaustively extracted by maceration with ethanol at room temperature. After filtration, the solvent was removed under reduced pressure yielding the crude extract. A sample of 200.0 g of the obtained extract was then dissolved in MeOH/H_2O (9:1 v/v, 100 mL) and successively partitioned with hexane. The solvents were removed under reduced pressure, resulting in a hexane (159.0 g) and a hydro-methanol (40.0 g) fraction. The hydro-alcoholic fraction was chromatographed over 600 g silica gel 60 by vacuum liquid chromatography (VLC), furnishing fractions: Fr. 1, 9.6 g (hexane/CH_2Cl_2 6:4 and 1:1 v/v); Fr. 2, 20.6 g (CH_2Cl_2/AcOEt 8:2, v/v); Fr. 3, 8.0 g (CH_2Cl_2/AcOEt 8:2, v/v) and Fr. 4, 0.84 g (CH_2Cl_2/AcOEt 8:2, v/v). Fr. 1 was purified by column chromatography over 220 g silica gel 60 using hexane and AcOEt as eluents, yielding compound **1** (1.15 g). Fr. 2 afforded compound **2** after recrystallization with acetone and a few drops of hexane. In addition, the mother liquor was dried under reduced pressure in a rotary evaporator, yielding 5.0 g of the dry fraction. This fraction was sequentially purified by semi-preparative reversed-phase HPLC using MeOH/H_2O (61:39, v/v), yielding compounds **3** (0.04 g) and **4** (0.03 g). Likewise, Frs. 3 and 4 were separately chromatographed over 300 g and 130 g silica gel 60 using hexane and AcOEt as eluents, and either by semi-preparative reversed-phase HPLC using MeOH/H_2O (72:28, v/v) to Fr. 3, and MeOH/H_2O (61:39, v/v) Fr. 4, yielding compounds **5** (0.06 g) and **6** (0.13 g), respectively.

NMR Data

(−)-Hinokinin (**1**). ^{1}H-NMR ($CDCl_3$, 500 MHz): 6.73 (1H, *d*, *J* = 8.0, H-5′), 6.70 (1H, *d*, *J* = 1.9, H-2′), 6.63 (1H, *d*, *J* = 1.9, H-5), 6.46 (2H, *d*, *J* = 1.9, H-2; *dd*, *J* = 8.0, H-6), 6.60 (1H, *dd*, *J* = 1.9, 8.0, H-6′), 5.94 (4H, *s*, OCH_2O), 3.88 (1H, *t*, *J* = 7.5, H-9b), 4.13 (1H, *t*, *J* = 7.5, H-9a), 2.99 (1H, *dd*, *J* = 14, 4.8, H-7′a), 2.85 (1H, *dd*, *J* = 14.0, 7.3, H-7′b), 2.59–2.44 (4H, *m*, H-7a, H-7b, H-8, H-8′). ^{13}C-NMR: *Table 1*. HR-ESI-MS: 355.1316 ([*M* + H]$^+$).

(−)-Cubebin (**2**). ^{1}H-NMR ($CDCl_3$, 500 MHz): 6.72 (2H, *d*, *J* = 1.5, H-2′, *dd*, *J* = 1.5; *J* = 8.0, H-6′), 6.71 (1H, *dd*, *J* = 1.5; *J* = 8.0, H-6), 6.67 (1H, *d*, *J* = 8.0, H-5′), 6.61 (1H, *d*, *J* = 1.5, H-2),6.58 (1H, *d*, *J* = 8.0, H-5), 5.91 (4H, *d*, *J* = 3.4, OCH_2O), 5.21 (1 H, *t*, *J* = 3.4, H-9′), 4.08 (1H, *t*, *J* = 8.0, H-9a), 3.56 (1H, *t*, *J* = 8.0, H-9b), 2.77–2.73 (2H, *m*, H-7′a), 2.60–2.56 (1H, *m*, H-7b), 2.45–2.39 (2H, *m*, H-7a, H-7b), 2.16–2.11 (1H, *m*, H-8), 2.02–1.97 (1H, *m*, H-8′), 1.66–1.61 (1H, *m*, OH). ^{13}C-NMR: *Table 1*. HR-ESI-MS: 357.1473 ([*M* + H]$^+$).

(−)-Yatein (**3**). ^{1}H-NMR ($CDCl_3$, 500 MHz): 6.70 (1H, *dd*, *J* = 1.2, 7.8, H-6), 6.48 (1H, *d*, *J* = 7.8, H-5), 6.46 (1H, *s*, H-2), 6.36 (2H, *s*, H-2′, H-6′), 5.93–5.94 (2H, *m*, OCH_2O), 4.23–4.18 (1H, *m*, H-9a), 3.86–3.79 (1H, *m*, H-9b), 3.83 (9H, *s*, MeO), 2.83–2.97 (2H, *m*, H-7, H-7′), 2.48–2.64 (4H, *m*, H-7a, H-7b, H-8, H-8′). ^{13}C-NMR: *Table 1*. HR-ESI-MS: 401.1629 ([*M* + H]$^+$).

(−)-5-Methoxyyatein (**4**). ^{1}H-NMR ($CDCl_3$, 500 MHz): 6.34 (1H, *s*, H-6), 6.30 (1H, *s*, H-2), 6.22 (2H, *s*, H-2′, H-6′), 5.94 (2H, *s*, OCH_2O), 4.19 (1H, *t*, *J* = 8.0, H-9a), 3.89 (1H, *t*, *J* = 8.0, H-9b), 3.86 (6H, *s*, H-4′, H-5), 3.81 (6H, *s*, H-3′, H-5′), 2.95 (1H, *dd*, *J* = 14.0, 4.7, H-7′a), 2.87 (1H, *dd*, *J* = 14.0, 6.7, H-7′b), 2.50–2.65(4H, *m*, H-7a, H-7b, H-8, H-8′). ^{13}C-NMR: *Table 1*. HR-ESI-MS: 431.1732 ([*M* + H]$^+$).

(−)-Dihydrocubebin (**5**). ^{1}H-NMR ($CDCl_3$, 500 MHz): 6.72 (2H, *d*, *J* = 7.9, H-5, H-5′), 6.64 (2H, *s*, H-2, H-2′), 6.62 (2H, *d*, *J* = 7.9, H-6, H-6′), 5.93 (4H, *s*, OCH_2O), 4.14 (2H, *s*, OH), 4.2–3.5 (4H, *m*, H-9a, H9b, H9′a, H9′b), 2.81–2.76 (2H, *m*, H-7a, H-7′a), 2.63–2.58 (2H, *m*, H-7b, H-7′b), 1.89 (2H, *s*, H-8, H-8′). ^{13}C-NMR: *Table 1*. HR-ESI-MS: 359.1493 ([*M* + H]$^+$).

(−)-Dihydroclusin (**6**). ^{1}H-NMR ($CDCl_3$, 500 MHz): 6.71 (1H, *d*, *J* = 7.7, H-6), 6.64 (1H, *s*, H-2), 6.61 (1H, *d*, *J* = 7.7, H-5), 6.37 (2H, *s*, H2′, H-6′), 5.91 (2H, *s*, OCH_2O), 3.82–3.78 (2H, *m*, H-9a, H-9′a), 3.82 (9H, *s*, MeO), 3.57–3.54 (2H, *m*, H-9b, H-9′b), 2.80–2.73 (2H, *m*, H-7a, H-7′a), 2.67–2.63 (2H, *m*, H-7b, H-7′b), 1.93–1.90 (2H, *m*, H-8, H-8′). ^{13}C-NMR: *Table 1*. HR-ESI-MS: 405.1907 ([*M* + H]$^+$).

Parasite Maintenance and Culture

LE (Luis Evangelista) strain of *S. mansoni* was maintained by passage through *Biomphalaria glabrata* snails and Balb/c mice. After 8 weeks, *S. mansoni* adult worms were recovered under aseptic conditions from mice, previously infected with 200 cercariae, by liver

and mesenteric veins perfusion.[49] The study protocol was approved by the Ethics Committee for Animal Care of the University of Franca (protocol 028/12). The worms were washed in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen), kept at pH 7.5 with 20 mM HEPES, and supplemented with penicillin (100 UI/ml), streptomycin (100 μg/ml) and 10% bovine fetal serum (Invitrogen). After washing, an adult worm couple was transferred to each well of a 24-well culture plate containing 2 mL of the same medium and incubated at 37 °C in a humid atmosphere containing 5% CO_2 prior to use. Compounds **1–6** were tested at a concentration of 5 μM for preliminary screening. The isolated compounds (**1**–**6**) were dissolved in dimethyl sulfoxide (DMSO) and added to RPMI 1640 medium containing the worms after a period of 24 h of adaptation. The parasites were kept in a constant temperature incubator at 37 °C in a 5% CO_2 atmosphere and monitored after 24 h under an inverted microscope (Leitz, Diavert) to evaluate motor activity, mortality rate, separation and egg production.[50] The worms were considered dead when no movement was observed for at least 2 min during examination and the motor activity was classified as decreased movement (when compared to negative control RPMI 1640 medium with 0.1% DMSO) and minimal movement (occasional movement of the head and body).[51] The compounds were further evaluated as previously described at concentrations of 10, 50 and 100 μM. These concentrations were selected based on previous reports on schistosomicidal activity of lignans and neolignans.[25,26] In addition, according to Ramirez et al., the screening of drugs against adult schistosomes worms should be performed at concentrations of about 12.5 μM.[52] Twelve adult worm couples were obtained for each employed concentration and three independent experiments were performed. RPMI 1640 medium and RPMI 1640 with 0.1% DMSO (the highest concentration of drug solvent) were used as negative control groups. Praziquantel (PZQ; 1.56 μM) was used as positive control group. Results were expressed as mean ± SD.

Computational Details

The B3LYP variant of density functional theory (DFT) was used to include correlation effects.[53–55] A double-ζ quality basis set augmented with polarized d-functions and diffuse sp-functions for all heavy atoms and polarization p-functions for hydrogen atoms (6-31 + G**) was used. All calculations were performed using the Gaussian 09 code.[56] Initial structures were obtained using the Chemcraft 1.7 software (http://www.chemcraftprog.com), which was also used to generate figures. The lipophilicity of the studied compounds (expressed by their log*P* values) was calculated with use of the COSMO-RS (Conductor-like Screening Model for Real Solvents) methodology,[57–59] which is implemented in the ADF (Amsterdam Density Functional) package (ADF2014 COSMO-RS, SCM, Theoretical Chemistry, Vrije Universiteit, Amsterdam, The Netherlands, http://www.scm.com).[60–62] For this purpose, the gas phase geometry optimization and ADF COSMO calculations were performed with a TZP small core basis set,[63] the Becke-Perdew exchange correlation functional (GGA:BP),[64] the relativistic scalar ZORA (zeroth order regular approximation to the Dirac equation) approach,[66–70] and an integration accuracy of 6. HOMO and LUMO orbitals were calculated and plotted with use of the ADF package.

The tridimensional model of *S. mansoni* α/β tubulin heterodimer was built via homology modeling[71] using the amino acid sequences from the parasite proteins (UniProtKB's accession number C4Q4 S5 and C4QIC0, respectively) and the crystal structures from *Rattus norvegicus* as 3-D templates (RCSB PDB code: 4O2B). Alignment correction[72] involved the use of multiple tubulin sequences from *trematodes*, *tapeworms*, *nematodes*, *mollusca* and *chordata* (*Table S2*). Model validation was based on the analyses of the QMEAN scoring function values and on the Ramachandran plot.[46][47] Docking simulations were performed in GOLD 5.4 as previously described,[73] using a 10 Å docking sphere defined from the colchicine centroid and by ranking the best poses using the ChemScore fitness function.[74] Redocking of the reference ligand yielded a heavy atom root mean square deviation (RMSD) value of 0.30 Å.

Acknowledgements

The authors thank São Paulo Research Foundation (FAPESP – Process numbers: 2009/15207-4, 2010/15332-0 and 2011/07623-8) and CNPq, Brazil, for financial support. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brazil (CAPES) – Finance Code 0082/2015 (CSF-PVE–S; Process number 88881.068346/2014-01).

Author Contribution Statement

The contributions of the respective authors are as follows: M. L. A. Silva and R. L. T. Parreira planned and conducted the project. M. L. A. Silva was responsible for obtaining the natural lignans and calculating the biological activity and R. L. T. Parreira for theoretical calculations. D. F. Kawano performed the homology modeling and docking simulations. E. S. Costa, G. V. Símaro, A. H. Januário and R. S. Laurentiz were responsible for isolation, purification and characterization of the lignans. R. L. T. Parreira, T. Kar and G. F. Caramori were responsible for all theoretical calculations as well as for data interpretation and results presentation. L. G. Magalhães, P. M. Pauletti and J. M. Souza were responsible for antileishmanial assays and results and methodology description. W. R. Cunha and J. K. Bastos interpreted the data and drafted the article. V. C. G. Heleno and R. L. T. Parreira carefully revised the article as well as all data interpretation presented.

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Received June 26, 2018
Accepted October 17, 2018

