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Antiplasmodial Activity of Aporphine Alkaloids and Sesquiterpene Lactones from *Liriodendron tulipifera* L

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Abstract

Aim of the study—The objective of this study was to isolate and characterize the active constituents of the traditionally used antimalarial plant *Liriodendron tulipifera* by antiplasmodial-assay guided fractionation.

Materials and methods—Bark and leaves were extracted with solvents of increasing polarity. Fractions were generated using flash chromatography, counter current chromatography and preparative HPLC and subjected to *in vitro* antiplasmodial and cytotoxicity assays. Active fractions were subjected to further fractionation until pure compounds were isolated, for which the IC₅₀ values were calculated.

Results and discussion—Six known aporphine alkaloids, asimilobine (**1**), norushinsunine (**2**), norglaucine (**3**), liriodenine (**4**), anonaine (**5**) and oxoglaucine (**6**) were found to be responsible for the antiplasmodial activity of the bark. Leaves yielded two known sesquiterpene lactones, peroxyferolide (**7**) and lipiferolide (**8**) with antiplasmodial activity. The antiplasmodial activity of (**2**) (IC₅₀ = 29.6 µg/ml), (**3**) (IC₅₀ = 22.0 µg/ml), (**6**) (IC₅₀ = 9.1 µg/mL), (**7**) (IC₅₀ = 6.2 µg/ml) and (**8**) (IC₅₀ = 1.8 µg/ml) are reported for the first time.

Conclusion—This work supports the historical use of *Liriodendron tulipifera* as an antimalarial remedy of the United States and characterizes its antiplasmodial constituents.

Keywords

Antimalarial; Aporphine alkaloids; Sesquiterpene lactones; *Liriodendron tulipifera* L.; Magnoliaceae; Cytotoxicity

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1. Introduction

Nearly half of the world's population lives in malaria endemic areas where over two hundred and fifty million people are infected and more than one million die each year from the disease (World Health Organization, 2008). Malaria treatment relies on a handful of accepted, affordable and effective drugs, many of which are chemically similar (World Health Organization, 2006). However, an increasing incidence of drug resistant strains of *Plasmodium* spp. highlights the need for novel antimalarial compounds.

Plants represent an important source of novel antimalarial compounds, as most famously evidenced by the antimalarial agents quinine and artemisinin initially isolated from *Cinchona* spp. (Smith, 1976) and *Artemisia annua* (Graziose et al., 2010), respectively. New compounds with antimalarial activity continue to be isolated from plant sources (Bero et al., 2009; Kaur et al., 2009) often representing the culmination of ethnobotanical investigations of plants used to treat malaria in endemic regions (Bourdy et al., 2008; Willcox et al., 2004). However, malaria was once much more widespread than it is today (Hay et al., 2004), and although often overlooked, many plants native to the United States were important sources of antimalarial treatments before the disease was eradicated from the country.

Liriodendron tulipifera L. (Magnoliaceae), known as the tulip tree or yellow poplar, is a majestic tree often reaching heights upwards of one hundred feet, that is endemic to the eastern United States (Keeler, 1902). The bark of *L. tulipifera* was used by the Native Americans as a tonic, stimulant and febrifuge, and likely was used to treat the intermittent fevers associated with malaria (Rafinesque et al., 1828). It was also adopted by American settlers as a suitable replacement for the imported and often scarce Peruvian bark (*Cinchona* bark) (Thacher, 1967). During the United States Civil War, when the confederate troop's quinine supplies were limited, army surgeons turned to *L. tulipifera* as a substitute (Hasegawa, 2007). During World War II, a U.S. government-directed program focused on developing quinine replacements confirmed that a crude extract of the tulip tree bark was effective in treating avian malaria (Spencer et al., 1947). However, while numerous phytochemical constituents, including sesquiterpene lactones (Doskotch and el-Feraly, 1969, 1970; Doskotch et al., 1976; Doskotch et al., 1977; Doskotch et al., 1975; Muhammad and Hufford, 1989) and aporphine alkaloids (Buchanan and Dickey, 1960; Chen-Loung and Hou-Min, 1978; Chen-Loung et al., 1976; Chen et al., 1976; Cohen et al., 1961; Taylor, 1961) have been isolated from this species, the antiplasmodial constituents have not been described until now. Although malaria is no longer a pressing issue in the native range of *L. tulipifera*, the antiplasmodial compounds from this species may be of value to future antimalarial drug development as well as to the preservation of traditional knowledge. The goal of this study was to describe the antiplasmodial components of *Liriodendron tulipifera*.

2. Materials and Methods

2.1. Instrumentation

Solvents and reagents were of HPLC grade and purchased from VWR. Counter current chromatography was performed on a Bench Scale Fast Centrifugal Partition Chromatography (FCPC) Kromaton[®]v. 1.0. Flash chromatography was performed using Silica gel (230-400 mesh 60 Å Merck). Preparative HPLC was performed with an Integrity system consisting of a solvent delivery system including a W600E pump and W600 controller, W717 plus auto-sampler and W490E UV multi wavelength detector; Preparative HPLC columns: Waters RP-8 300×19.0 mm, 7 µm; Phenomenex Synergy Hydro-RP 80A 250 × 21.20 mm, 4 µm; Phenomenex Synergy Hydro-RP 80A 250 × 4.60 mm, 4 µm, Ultra High Pressure Liquid Chromatography- Mass Spectroscopy (UPLC-MS) was performed with a Dionex[®] Ultimate 3000 RSLC UPLC system and Varian 1200 L (Varian Inc., Palo

Alto, CA) triple quadrupole mass detector with electrospray ionization (ESI) interface using a Dionex[®] Acclaim[®] RSLC 120 C18 reverse phase column (150 × 2.1 mm, 2.2 μm). ¹H NMR and ¹³C NMR spectra were recorded on Varian 400, 500 MHz & Bruker Avance 950 MHz spectrophotometer.

2.2 Plant material

Leaves and bark of *Liriodendron tulipifera* L. (Magnoliaceae) were collected from a single, large tree growing on Rutgers University farm on Ryders Lane in New Brunswick, NJ, which was identified by Lena Struwe, Ph.D. A voucher specimen (RG16) has been deposited in the Chrysler Herbarium (CHRB).

2.3 Extraction and Isolation

2.3.1 Isolation of actives from bark—The dried and powdered bark of *L. tulipifera* (300 g) was defatted with hexane (3 L) at room temperature for 24 h, dried, and extracted with 95% ethanol (3 L, 2×) at room temperature for 48 h. The ethanolic extract was separated by filtration and concentrated under vacuum to yield 20 g of crude extract that showed an antiplasmodial activity of 10.9 μg/mL. This crude extract was acidified with 3% HCl solution and extracted with chloroform (200 mL, 3×) to remove phenolic compounds, and the remaining aqueous extract was basified with 5 N NH₄OH solution and extracted with chloroform (200 mL, 3×), which was concentrated under vacuum to yield 0.23 g of extract. This basic extract was purified by reverse phase HPLC (Waters RP-8 300 × 19.0mm, 7 μm, 30-95% methanol in water which contains 0.01% TFA, over 60 min, flow rate 10 mL/min; UV detector, 254 nm) to collect four fractions, Fr-I (3 mg), Fr-II (22 mg), Fr-III (42 mg) and Fr-IV (28 mg) respectively. Fr-II was further purified by reverse phase HPLC (Phenomenex Synergy Hydro-RP 80A 250 × 21.20 mm, 4 μm, 30-95% methanol in water with 0.01% TFA, over 60 min, flow rate 10 mL/min; UV detector, 254 nm) to give 3 sub fractions, Fr-II-1 (2 mg), Fr-II-2 (4 mg) and Fr-II-3 (5 mg) respectively. Fr-II-2 & 3 were further purified by reverse phase HPLC (Phenomenex Synergy Hydro-RP 80 Å 250 × 4.60 mm, 4 μm, 30-95% methanol in water with 0.01% TFA, over 60 min, flow rate 1 mL/min; UV detector, 254 nm) to give compound **1** (2 mg) from Fr-II-2, compounds **2** (2 mg) and **3** (1 mg) from Fr-II-3 respectively. Similarly, Fr-III gave 3 sub fractions, Fr-III-1 (3mg), Fr-III-2 (2mg) and Fr-III-3 (8mg) and Fr-IV gave 4 sub fractions, Fr-IV-1 (5mg), Fr-IV-2 (3 mg), Fr-IV-3 (2 mg) and Fr-IV-4 (2 mg) respectively. Compound **4** (4 mg) was isolated from Fr-III-3 and compounds **5** (2 mg) and **6** (1.5 mg) were isolated from Fr-IV-1 & 2 respectively.

2.3.2 Isolation of actives from leaves—The dried powdered leaves (100 g) were defatted with hexane (1 L) at room temperature for 24 h, dried then extracted with 80% methanol (1L, 2×) at room temperature for 24 h. The combined aqueous methanolic extract was concentrated under vacuum to a volume of 300 mL, which was extracted with 300 mL chloroform three times and dried to yield 2.9 g of crude chloroform extract. This extract, which showed an antiplasmodial activity of 2.0 μg/mL, was separated by flash chromatography (80 g, 230-400 mesh, 60Å Merck, column dimensions 25 × 3cm) and eluted with CHCl₃:CH₃OH [100:0 (250 mL); 99.5:0.5 (375 mL); 99:1 (1 L); 98:2 (375 mL); 97:3 (375 mL); 95:5 (250 mL); 50:50 (250 ml)]; 125 mL fractions were collected. Fractions 16-19, which were the most active (IC₅₀ = 4.3 μg/mL), were combined (70 mg) and further separated by Counter Current Chromatography with a 2:1:2:1 HEMWat (Hexane:Ethyl Acetate:Methanol:Water) solvent system using the Elution-Extrusion method with the lower phase as the stationary phase following previously published protocol (Berthod et al., 2005). Eight fractions were collected according to peaks observed at 254 nm. Fractions I and II (mins 27-31 and 32-40) were further purified by silica gel column chromatography to yield compound **7** (6 mg) and compound **8** (10 mg) respectively.

2.4. *In vitro* assays

2.4.1 Antiplasmodial activity—A chloroquine-sensitive strain (D10) and a chloroquine-resistant strain (Dd2) of *Plasmodium falciparum* were continuously cultured according to a modified method (Trager and Jensen, 1976). The *in vitro* antiplasmodial assays were performed using the parasite lactate dehydrogenase (pLDH) activity assay to measure parasite viability. Chloroquine diphosphate (Sigma) was used as a positive control. Crude extracts and fractions were tested against the D10 strain at three concentrations: 20, 10, and 5 µg/mL. Pure compounds were tested in triplicate against both the D10 and Dd2 strains. IC₅₀-values were calculated from a ten point dose response curve (the samples were serially diluted 2× from 100 µg/mL to 0.2 µg/mL, CQ was serially diluted 2× from 1000 ng/mL to 2 ng/mL) using a non-linear dose-response curve fitting analysis via Graph Pad Prism v. 4.0 software.

2.4.2 Cytotoxicity—Cytotoxicity was assessed against a Chinese Hamster Ovarian (CHO) cell line using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay (Mosmann, 1983). Emetine hydrochloride (Sigma) was used as a positive control. Crude extracts and fractions were assayed in parallel with the antiplasmodial assay at three concentrations: 20, 10, and 5 µg/mL. IC₅₀ values of pure compounds were tested in triplicate and were calculated from a ten point dose response curve (samples were serially diluted 2× from 100 µg/mL to 0.2 µg/mL, emetine hydrochloride was serially diluted 2× from 100 µg/mL to 0.001 µg/mL) using a non-linear dose-response curve fitting analysis via GraphPad Prism v.4.0 software.

3. Results and Discussion

This research is the first systematic isolation and characterization of the antiplasmodial compounds from the tulip tree (*Liriodendron tulipifera*). Six known aporphine alkaloids and two known sesquiterpene lactones (Fig.1), which have been previously reported from *L. tulipifera*, were shown to be the antiplasmodial constituents of the bark and leaves, respectively. Asimilobine (**1**) (Chen-Loung et al., 1976; Leboeuf et al., 1982), norushinsunine (**2**) (Chen et al., 1976), norglaucine (**3**) (Cava et al., 1973), liriodenine (**4**) (Costa et al., 2009; Harrigan et al., 1994), anonaine (**5**) (Leboeuf et al., 1982), oxoglaucine (**6**) (Chen-Loung et al., 1976; Singh et al., 2007) were isolated from the bark of *L. tulipifera*, which were confirmed by comparison of spectral data with the literature values. Two sesquiterpene lactones, peroxyferolide (**7**) and lipiferolide (**8**) were isolated from the leaves of *L. tulipifera* and the structures were confirmed by comparison of spectral data previously published data (Doskotch et al., 1976; Doskotch et al., 1977; Doskotch et al., 1975; Muhammad and Hufford, 1989). ESI-MS and ¹H NMR spectral data for the compounds **1-8** is available in the supplementary data and further data can be obtained from the author of correspondence.

All the isolated compounds were evaluated for antiplasmodial activity using both CQ-sensitive (D10) and CQ-resistant (Dd2) plasmodium strains and cytotoxicity using the Chinese Hamster Ovarian (CHO) cell line (Table 1).

Several sesquiterpene lactones with antimalarial activity have been previously isolated from plants (Bero et al., 2009). However, with the exception of artemisinin and related compounds from *Artemisia annua*, none has proven therapeutically valuable. The antimalarial activity of artemisinin is thought to depend on the endoperoxide bridge (Cui and Su, 2009). The hydroperoxide moiety of peroxyferolide (**7**) is chemically related to the endoperoxide moiety of, artemisinin; a functionality group which is lacking in lipiferolide (**8**). The antiplasmodial activity of peroxyferolide (**7**) (IC₅₀=6.3 µg/mL) is not significantly different than that of lipiferolide (**8**) (IC₅₀=1.8 µg/mL), suggesting that the hydroperoxide

moiety does not contribute to the observed activity. Our results confirm the documented toxicity of α -methylene lactones (Chagonda et al., 1989) and suggest that the therapeutic value of both isolated sesquiterpene lactones may be counterbalanced by their relatively high cytotoxicity (Table 1).

The aporphine alkaloids isolated by antiplasmodial activity-guided fractionation of *L. tulipifera* bark display *in vitro* antiplasmodial activities with IC_{50} values ranging between 1.2 and 29.6 $\mu\text{g/mL}$ (Table 1). The antiplasmodial activity of compounds **1**, **4** and **5** showed considerable antiplasmodial activity against both the CQ sensitive D10 strain (IC_{50} values of 1.2, 4.1 and 1.2 $\mu\text{g/mL}$, respectively) and the CQ resistant Dd2 strain (IC_{50} values of 5.8, 7.9 and 5.2 $\mu\text{g/mL}$, respectively). These antiplasmodial values are consistent with previous reports for these compounds (del Rayo Camacho et al., 2000; Likhitwitayawuid et al., 1993; Wirasathien et al., 2006). The low resistance index (RI) (4.8, 1.9 and 4.2 for **1**, **4** and **5**, respectively) suggests a mechanism of resistance different from chloroquine. The favorable selectivity index (SI) for **1** and **5** suggests that these compounds show selective toxicity against *P. falciparum* when compared to mammalian cell lines. Compound **6** showed moderate antiplasmodial activity with an IC_{50} value of 9.1 $\mu\text{g/mL}$ with no observable cytotoxicity. Compounds **2** and **3** show much lower antiplasmodial activity with IC_{50} values of 29.6 and 22.0 $\mu\text{g/mL}$ respectively and no observable cytotoxicity.

Our results suggest that the antiplasmodial activity of crude extracts of *Liriodendron tulipifera* bark ($IC_{50} = 10.9 \mu\text{g/mL}$) and leaves ($IC_{50} = 2.0 \mu\text{g/mL}$) can be partially explained by the isolated aporphine alkaloids and sesquiterpene lactones, respectively. However, the crude extract of the leaves showed a comparable antiplasmodial activity to the pure isolated compounds (**7**) or (**8**) (6.2 and 1.8 $\mu\text{g/mL}$, respectively), suggesting that these compounds may act in combination with other constituents found in the leaves (possibly aporphine alkaloids, which are known to occur throughout the plant).

4. Conclusions

The investigation of *L. tulipifera* bark and leaves, a traditional antimalarial remedy used in the United States, yielded six aporphine alkaloids and two sesquiterpene lactones with moderate *in vitro* antiplasmodial activity. The antiplasmodial activity of both isolated sesquiterpene lactones (**7**, **8**), along with three of the aporphine alkaloids (**2**, **3** and **6**) are reported here for the first time. While our results show high cytotoxicity of the isolated sesquiterpene lactones, long standing traditional use of *L. tulipifera* extracts may argue against toxicity in humans. It is also possible that the overall antimalarial efficacy and toxicology of the tulip tree extracts are derived from complex interactions of its antimalarial components, rather than from simple additive action. Species endemic to the United States, a region no longer troubled by malaria, are still important in the discovery of novel antimalarial botanical compounds. This work may serve as an example for future studies focused on American antimalarial plant species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig.1. Antiplasmodial compounds from leaves and bark of *L. tulipifera* L

Table 1
Antiplasmodial activity and cytotoxicity of the compounds from *L. tulipifera* L

Compounds	<i>Plasmodium falciparum</i> D10 (IC ₅₀)		<i>Plasmodium falciparum</i> Dd2: IC ₅₀		CHO Cell line(IC ₅₀)		SI	RI
	µg/mL	µM	µg/mL	µM	µg/mL	µM		
1	1.2 ± 0.1	4.5 ± 0.3	5.8 ± 1.3	21.7 ± 6.3	> 100	>300	> 83	4.8
2	29.6 ± 3.9	105.2 ± 13.9	30.9 ± 2.3	109.8 ± 8.1	> 100	>300	> 3	1.0
3	22.0 ± 2.8	64.4 ± 8.2	32.2 ± 1.3	94.3 ± 3.8	> 100	>300	> 5	1.5
4	4.1 ± 1.0	14.9 ± 3.6	7.9 ± 1.1	28.7 ± 4.0	8.1 ± 0.1	29.4 ± .4	2.0	1.9
5	1.2 ± 0.1	4.5 ± 0.3	5.2 ± 0.3	19.6 ± 1.1	>100	>300	> 83	4.2
6	9.1 ± 0.1	25.9 ± 0.2	20.8 ± 2.9	59.2 ± 8.3	>100	>300	> 11	2.3
7	6.2 ± 0.1	18.3 ± 0.3	4.3 ± 1.2	12.7 ± 3.5	3.2 ± 0.6	9.5 ± 1.8	0.5	0.7
8	1.8 ± 0.7	5.9 ± 2.3	2.3 ± 0.1	7.5 ± 0.3	1.4 ± 0.2	4.6 ± 0.7	0.8	1.3
Chloroquine	.01 ± .01	.03 ± .01	.07 ± .01	.23 ± .01	-	-	-	7.9
Emetine	-	-	-	-	0.2 ± 0.1	0.4 ± 0.2	-	-

Selectivity index (SI) = IC₅₀ CHO / IC₅₀ D10

Resistance index (RI) = IC₅₀ Dd2 / IC₅₀ D10