Antitrypanosomal Compounds from the Leaf Essential Oil of *Strychnos spinosa*

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Abstract

The composition of the essential oil obtained by hydrodistillation of the leaves of *Strychnos spinosa* (Loganiaceae) was analysed by GC-FID and GC-MS. Out of twenty-two compounds identified in the oil, the main constituents were palmitic acid (34.3%), linalool (16.0%), and (*E*)-phytol (6.7%). Since the leaves of this plant are used in African traditional medicine to treat African trypanosomiasis, we evaluated the *in vitro* activity of the essential oil as well as of 15 of its components on *Trypanosoma brucei brucei* bloodstream forms and on mammalian cells (J774 murine macrophages) to evaluate the selectivity of the antitrypanosomal effect. The essential oil was active on the parasites without a great selectivity [IC_{50} on *T. b. brucei* = 13.5 μg/mL with a selectivity index (SI) of 4.4]. (*E*)-Nerolidol, a minor component of the hydrodistillate, as well as linalool, were shown to have a more potent and selective effect on the trypanosomes [IC_{50} = 1.7 and 2.5 μg/mL (7.6 and 16.3 μM) with SI = 35.7 and > 40, respectively]. These two oxygenated terpenes have promising activity and it would be of interest to study their mechanism of action as well as their in *vivo* activity.

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African trypanosomes (e.g., *Trypanosoma brucei* subspecies) are protozoan parasites responsible for human African trypanosomiasis (sleeping sickness) or nagana in cattle and are causing major health and economic problems in rural sub-Saharan Africa, as they are fatal if left untreated. However, only few drugs with serious side effects are currently registered to treat this parasitic disease and the efficacy of these tends to decline. Therefore, there is an urgent need for new, safe, effective, and cheap antitrypanosomal molecules as well as for new leads with original mechanisms of action [1], [2], [3]. Among plant secondary metabolites, essential oils are very promising [3]. Indeed, Mikus et al. reported the interesting trypanocidal activity of some essential oils as well as of some terpenic constituents, such as terpinen-4-ol [4]. The leaf decoction of *Strychnos spinosa* (Loganiaceae) is traditionally used to treat African trypanosomiasis [5] and, in a previous study, we showed that the leaf dichloromethane extract had promising *in vitro* antitrypanosomal activity [6]. Hence, we thought it to be of interest to study the composition and the antitrypanosomal activity of the leaf essential oil of this plant as its lipophilic components may also be found in the dichloromethane extract. Additionally, we evaluated the antitrypanosomal activity of 15 constituents of the essential oil as well as the selectivity of this effect by comparing the antitrypanosomal activity to the cytotoxicity for a mammalian cell line (J774).

Twenty-one compounds, representing 74.3% of the hydrodistillate, were identified by GC-FID and GC-MS as well as linoleic acid contained in a composite peak (< 4.4%). The oil also contains more than 100 other constituents usually at low amounts or not well separated from neighbouring peaks which did not allow us to obtain unambiguous identifications. Nevertheless, we identified all major peaks (> 2%). The essential oil components and their percentage in the essential oil are presented in Table **1**.

At first, the high percentage of compounds with high boiling points (e.g., fatty acids, phytol) in this hydrodistillate was surprising for us but such compounds have already been found in a

Table **1** Main components (%) of the essential oil of the leaves of *S. spinosa*

Compounds	*LRI*[a]	*Percentage in oil (%)*
3-hexenol	858	0.5
benzaldehyde	981*	0.1
2-*n*-pentylfuran	996	0.5
phenylacetaldehyde	1062*	0.2
linalool	1116*	16.0
2,6-nonadienal	1180	1.6
2-nonenal	1189	0.6
α-terpineol	1213*	2.6
(*Z*)-nerol	1240*	1.5
(*E*)-geraniol	1271*	4.0
eugenol	1374*	1.1
(*E*)-geranylacetone	1465*	1.3
$C_{15}H_{24}$	1481	0.9
(*E*)-β-ionone	1497*	0.7
(*E*)-nerolidol	1569*	0.7
$C_{15}H_{26}O$	1648	0.7
isopropyl myristate	1835*	0.2
phytone	1856	0.5
(*E*,*E*)-farnesylacetone	1924	0.5
palmitic acid	2058*[b]	34.3
(*E*)-phytol	2127*	6.7
Composite peak containing linoleic acid	2165 – 2204	4.4
squalene	2804*	0.5
nonacosane	2900*	0.2

[a] LRI indices relative to C_8 to C_{30} *n*-alkanes on a DB-XLB column.
[b] Large fronting peak.
* Comparison of LRI and mass spectra obtained by injection of an authentic sample.

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series of other essential oils as reported in several publications [7], [8], [9], particularly in essential oil-poor plants.

This is the first time that the composition of the leaf essential oil of *S. spinosa* is reported. Furthermore, only four of the 22 identified compounds were already known to occur in other plant parts of this species: palmitic and linoleic acids in the seed oil [10] and eugenol and nonacosane in the fruit pericarp [11], [12] and two others (squalene and benzaldehyde) were already identified in other *Strychnos* species [13], [14]. The remaining 16 constituents are identified for the first time from the *Strychnos* genus.

The IC_{50} values obtained with the leaf essential oil of *S. spinosa* as well as with 15 of its components when tested on the bloodstream forms of *T. b. brucei* and on a mammalian cell line (J774) are presented in Table 1 in the Supporting Information. The essential oil proved to be moderately active on the trypanosomes (IC_{50} = 13.5 μg/mL) without a great selectivity (SI = 4.4). As the content of the essential oil is very low (0.02%) and as it is much less active than the dichloromethane extract of the plant (IC_{50} = 1.5 μg/mL [6]), it is unlikely that essential oil components may be responsible for the activity of the dichloromethane extract.

Among the various components tested, 6 proved to have an IC_{50} towards the trypanosomes inferior to 10 μg/mL and to 50 μM: (*E*)-nerolidol, linalool, (*E*)-geranylacetone, β-ionone, (*E*)-phytol, and α-terpineol. The most active compound of the series, (*E*)-nerolidol, had an IC_{50} value of 7.6 μM (1.7 μg/mL). The second most active compound, linalool (IC_{50} = 16.3 μM, 2.5 μg/mL), was also the second most abundant substance identified in the essential oil (16.0%). Palmitic acid, the main component of the essential oil (34.3%), can be considered as inactive (IC_{50} > 100 μM) on the trypanosomes as well as some minor compounds of the essential oil such as (*Z*)-nerol, eugenol, squalene, phenylacetaldehyde, benzaldehyde, nonacosane, and isopropyl myristate while (*E*)-geraniol was weakly active (IC_{50} = 95 μM).

We included in our study (+)-terpinen-4-ol, which was reported by Mikus et al. to be highly active on *T. b. brucei* bloodstream forms, as a bench mark. In our study, we did not encounter for this substance such a high activity on the trypanosomes (IC_{50} = 6.5 μg/mL) as described by Mikus et al. (IC_{50} = 0.02 μg/mL) [4]. This compound was active in the same range of concentration as the 6 most active compounds of our study. Such discrepancies in the results obtained in different laboratories might be due to different experimental conditions or may be due to different stereoisomers tested [we tested the activity of (+)-terpinen-4-ol while Mikus et al. did not specify which isomer they used].

To evaluate the selectivity of the antitrypanosomal activity, we also evaluated the toxicity of the compounds on a mammalian cell line (J774). Palmitic acid, which accounts for one third of the essential oil composition, was more toxic for the mammalian cells than for the trypanosomes, certainly contributing to the low selectivity of the essential oil (SI = 0.6).

Among the 6 most active compounds on trypanosomes, the IC_{50} values obtained on mammalian cells were superior to 500 μM for 3 of them, linalool, (*E*)-geranylacetone, and α-terpineol, which had respectively selectivity indices superior to 40, 19, and 14. (*E*)-Phytol, accounting for 6.7% of the essential oil, showed a rather unselective activity towards trypanosomes (SI = 3.4) contributing also to the low selectivity of the essential oil. The most active compound of the series, (*E*)-nerolidol, possessed a rather selective antitrypanosomal effect (SI = 35.7).

Considering the antitrypanosomal activity, this is the first time, to our knowledge, that the 15 compounds included in this study, as well as the leaf essential oil of *S. spinosa*, were tested. Additionally, (*E*)-nerolidol and linalool have already shown interesting activities on other parasites, *Leishmania* and *Plasmodium* [15], [16], [17]. Nerolidol (*E*- and *Z*-mixture) was shown to block an undefined but early step of the mevalonate pathway leading to the inhibition of isoprenoid biosynthesis (e.g., dolichol, ergosterol, and ubiquinone) of *L. amazonensis* [17] while (*E*)-nerolidol and linalool were shown to interfere with the biosynthesis of isoprenoids in *Plasmodium* [16]. *In vivo* experiments on mice infected with *L. amazonensis* showed that nerolidol (topical or intraperitoneal administration) reduced significantly the lesions but long-term follow-up indicated that the disease was not cured [17].

In addition to providing significant phytochemical information on *S. spinosa*, this study reveals the interesting *in vitro* antitrypanosomal activity of some oxygenated terpenes, especially linalool and (*E*)-nerolidol. It would be of interest to study the action mechanisms of these molecules as well as their *in vivo* activity in animal models.

Materials and Methods

Plant material: Leaves of *S. spinosa* L. were collected in the Zou province (Savane de Dan, Benin) in September 2004 and identified by Prof. V. Adjakidjé (Université d'Abomey-Calavi, Benin). A voucher specimen was deposited at the Herbarium of the Belgian National Botanical Garden at Meise (BR S.P. 848 106).

Oil isolation: The essential oil was obtained by hydrodistillation using a Clevenger-type apparatus for 4 hours [yield: 0.02% (w/w)]. It was diluted in *tert*-butyl methyl ether (75 mg/mL) prior to GC-FID and GC-MS analyses.

Standards: Palmitic acid, phenylacetaldehyde, benzaldehyde, squalene were purchased from Acros Organics (Geel, Belgium); (+)-linalool, (*E*)-geraniol, (+)-α-terpineol, (*E*)-geranylacetone, eugenol, (*E*)-β-ionone, (*E*)-nerolidol, isopropyl myristate, (+)-terpinen-4-ol from Fluka (Bornem, Belgium); nerol and nonacosane from Aldrich (Bornem, Belgium). (*E*)-Phytol was isolated from the leaves of *Croton zambesicus* Muell. Arg. [18].

GC analyses: The GC-FID analyses were performed on a Focus GC (Thermo Finnigan; Milan, Italy) using the following operating conditions: capillary column, DB-XLB (15 m × 0.25 mm; film thickness: 0.25 μm) (J&W Scientific, Agilent Technologies; Palo Alto, CA, USA); injector temperature, 250 °C; oven temperature programmed, 50 °C to 300 °C at 2 °C/min and held at 300 °C for 15 min; carrier gas, helium at 0.8 mL/min; injection volume, 1 μL (TBME solution); split ratio, 1/69.

The GC-MS analyses were performed on a TRACE GC 2000 series gas chromatograph interfaced to a Trace MS mass spectrometer (Thermo Quest; Milan, Italy) operating in the electron-impact mode (70 eV). The same operating conditions were used as for the GC-FID analyses.

The NIST/EPA/NIH mass spectral library (1998, version 1.6) of the GC-MS system, published linear retention indices (LRI) [19], and the mass spectra and LRI from authentic samples were used for the identification of the volatile constituents. The LRI were determined relative to the retention times of a series of *n*-alkanes (C_8 – C_{30}). The relative proportions of the essential oil constituents were obtained by electronic integration of the FID peak area data without the use of response factor correction (normalization method). The oil components as well as their percentage are presented in Table **1**.

Cell culture: *Trypanosoma brucei brucei* (strain 427) bloodstream forms and J774 cells (non-cancer murine macrophage-like cell line) were grown *in vitro*, respectively, in HMI 9 medium [20] and RPMI 1640 with L-glutamine medium. The media were supplemented with 10% heat-inactivated fetal calf serum. All cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Antitrypanosomal activity and cytotoxicity assays: The effect of the leaf essential oil of *S. spinosa* and of some of its components on the viability of *T. b. brucei* bloodstream forms and mammalian cells was evaluated using the Alamar Blue™ assay as previously reported [6], [21]. Suramin (Sigma), as well as colchicine (Sigma), were used as positive controls. All experiments were performed at least in triplicate.

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