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Development of a selective HPLC-DAD/ELSD method for the qualitative and quantitative assessment of commercially available *Eurycoma longifolia* products and plant extracts

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ABSTRACT

Aqueous extracts of the roots of *Eurycoma longifolia* are traditionally used to improve sexual performance, to treat infertility and other sexual dysfunctions but also to increase muscle strength. Nowadays, many different products are commercially available which are promoted as *E. longifolia* extracts and claim to possess beneficial aphrodisiac effects. Since such herbal aphrodisiac preparations have been recently the target of fraudulent product counterfeiting and because eurycomanone, one of the main quassinoids of *E. longifolia*, is suspected to possess toxic effects at higher concentrations, a highly selective HPLC-DAD/ELSD method has been established to analyze commercially available products and extracts of plant material. The presented method was established by the use of a mixture of 27 reference compounds for qualitative issues and fully validated according to the ICH guidelines for the quantification of three quassinoides: laurycolactone A, longilactone, and eurycomanone. The calibration curves of these showed a linearity over a range of 0.05 to 1.0 mg/ml, with a regression coefficient not lower than $R^2 = 0.9969$. The inter-day and intra-day precision (indicated as relative standard deviation) of the developed method was < 2.9%. The recovery ranged from − 3.3% to + 6.0%. Eight randomly purchased products have been analyzed with this method, but only five of them contained *E. longifolia* compounds in detectable amounts. The concentration of eurycomanone in these products varied from 0.22 ± 0.002 mg eurycomanone per capsule to 1.84 ± 0.08 mg corresponding to a maximal recommended daily intake of 0.76 ± 0.02 to 31.90 ± 0.21 mg.

1. Introduction

Eurycoma longifolia, an evergreen, slender tree, part of the Simaroubaceae family, native to Indonesia and Malaysia, is widely used in traditional medicine of many Southeast Asian countries for a vast variety of indications. In particular aqueous extracts from the roots are historically used to increase sexual performance, for the treatment of infertility and other sexual dysfunctions [1]. Several studies have concluded that root extracts from *E. longifolia* could help improving the libido and could be useful for the treatment of sexual problems. Although most of the studies used a full plant extract, some studies claim that quassinoids [2], especially eurycomanone, might be responsible for the described effects. Despite the bioactive components have not been identified yet numerous products containing root extracts from *E. longifolia* are commercialized claiming beneficial aphrodisiac effects. Most of the products are freeze- or spray-dried water extracts of the roots of *E. longifolia*, which are sold as capsules, pills or liquid formulations. As a recent report of the Food and Drug Administration showed that dietary supplements or food marketed for enhancement of sexual performance might be contaminated by hidden drug ingredients [3], the general quality of such products must be critically challenged. Furthermore, toxic effects for *E. longifolia* extracts have been described which might be related to quassinoids. Especially eurycomanone at high dosages showed toxicity in a mice model and a brine shrimp model [4]. Nevertheless *E. longifolia* extracts are considered safe, as long as the daily intake of a consumer would not exceed the calculated acceptable daily intake (ADI) of up to 1.2 g/60 kg adult/day (with a safety factor of 100), equivalent to 12 times the recommended intake dosage for humans (100 mg/60 kg adult/day) [5]. For the individual compounds like eurycomanone and other quassinoids from *E. longifolia* no ADI has been determinate. Therefore, low quality *E. longifolia* products could create potential health risks in two ways; first because of excessive doses of eurycomanone and/or related quassinoids and secondly because of contamination with undisclosed substances.

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Table 1
List of used reference compounds of *Eurycoma longifolia*.

N°	Name of compound	Compound class	Retention time	MW	*m/z* in positive mode	*m/z* in negative mode$^-$
1	Eurycomanone	C_{20}-type quassinoids	22.77	408.4	391.1; 409.0; 839.1;	389.4; 407.0;
2	13,21-dehydroeurycomanone	C_{20}-type quassinoids	23.24	410.4	411.2;	409.1;
3	Isoaloeresine D	Chromone component	25.13	556.8	557.1; 612.7;	n.d.
4	Vanillic acid	Phenolic compound	25.45	168.1	169.2;	167.1;
5	Syringic acid	Phenolic compound	26.97	198.1	198.9; 220.9; 418.4;	197.2; 477.9;
6	Longilactone	C_{19}-type quassinoids	27.50	366.4	367.2; 755.2;	365.1; 411.1;
7	Vanillic aldehyde	Phenolic compound	28.33	152.1	125.0; 152.9;	150.9; 305.8;
8	9-Glucoside canthine-6-one	Canthin-6-one alkaloid	29.87	398.1	399.0;	443.2; 632.8;
9	14,15ß-Dihydroxyklaieanone	C_{20}-type quassinoids	30.44	396.4	397.1;	395.0; 441.0;
10	Scopoletin	Coumarin	32.01	192.1	193.3	n.d.
11	Eurycomalactone	C_{19}-type quassinoids	32.05	348.3	349.1; 719.2;	347.8;
12	1,1′-biphenyl-3,3′-dicarboxylic acid	Phenolic compound	32.22	274.2	274.8; 548.6;	273.1;
13	Fraxidin	Coumarin	32.97	222.1	222.9; 466.9;	221.2; 541.9;
14	1-Methoxycarbonyl-ß-carboline	ß-carboline alkaloid	33.89	226.2	227.0; 474.9;	n.d.
15	7α-Hydroxyeurycomalactone	C_{19}-type quassinoids	36.07	350.4	351.1; 732.2;	n.d.
16	11-Dehydroklaieanone	C_{20}-type quassinoids	36.55	362.4	363.3; 724.9;	361.4; 407.3; 732.2;
17	Eurycolactone E	C_{19}-type quassinoids	36.91	350.4	351.1; 647.1; 723.2;	349.2; 395.4
18	9-Hydroxycanthin-6-one	Canthin-6-one alkaloid	37.19	236.2	236.8;	235.0; 389.1
19	Laurycolactone A	C_{18}-type quassinoids	39.67	318.3	319.0; 637.1;	318.1; 363.2;
20	5,6-Dehydroeurycomalactone	C_{19}-type quassinoids	39.99	346.3	347.2;	345.4;
21	Laurycolactone B	C_{18}-type quassinoids	41.92	316.4	317.1; 655.2;	314.9; 421.1;
22	4-Hydroxy, 5-methoxycanthin-6-one	Canthin-6-one alkaloid	42.05	266.2	266.9; 554.9;	n.d.
23	9,10-Dimethoxycanthin-6-one	Canthin-6-one alkaloid	42.92	280.2	281.0; 582.9	n.d.
24	9-Methoxycanthin-6-one	Canthin-6-one alkaloid	47.33	250.2	250.9; 522.9;	249.3; 404.0; 538.7
25	Pedunculoside[a]	Cyclic triterpene	54.50	650.8	n.d.	695.6; 763.2
26	Heptamethoxyflavone	Flavonoid	55.31	432.4	433.1;	n.d.
27	Eurylene[a]	Triterpene/squalene	58.09	594.8	553.4; 594.7; 611.4	n.d.

[a] Compound not detectable by DAD.

Thus, in the course of this project a highly selective high-performance liquid chromatography diode array/evaporative light scattering detector (HPLC-DAD-ELSD) method for qualitative and quantitative evaluation of products containing *E. longifolia* extracts was established. The application of this methodology was necessary since most of the *E. longifolia* products on the market are mixtures of multiple components, involving also extracts from different plants, as for example *Panax ginseng* and horny goat weed (*Epimedium* spp.), fungal material, like *Ganoderma lucidum* and synthetic additives. An additional aspect was to develop a method, which might be also used for HPLC-MS application in case of the detection of unexpected alterations/additives. Since the described quassinoids from *E. longifolia* could be the bioactive compounds but also responsible for toxic effects, calibration curves for the quantification of representatives of C_{18}-, C_{19}- and C_{20}–type quassinoids were established.

2. Material and methods

2.1. Commercial products

Eight commercially available products, containing root extracts of *E. longifolia* were randomly purchased. Three of them derived from companies registered in Europe (Switzerland and Netherlands), four from Vietnam and one from the United States of America.

2.2. Plant material

Plant material was collected on the Chua Chan Mountain (Dong Nai/Vietnam) in August 2010 (PT1) and in the Binh Phuoc province (Vietnam) in May 2015 (PT2). Identification of the plant material was carried out by Prof. Tran Hung (Department of Pharmacognosy/Faculty of Pharmacy, University of Medicine and Pharmacy of Ho Chi Minh City). Voucher specimens (DN107, BP145) are stored at the Department of Pharmacognosy/Faculty of Pharmacy, University of Medicine and Pharmacy of Ho Chi Minh City, Vietnam.

2.3. Reagents

All solvents needed were provided by VWR International (Darmstadt, Germany). Ultrapure water was produced onsite by a Sartorious Arium 611 UV water purification system (Sartorius AG, Göttingen, Germany).

2.4. HPLC-DAD-ELSD conditions

HPLC-diode array detection analyses was performed using an Agilent 1200 series (Agilent, Waldbronn, Germany) instrument equipped with photo diode array, autosampler, column thermostat and on-line degasser, coupled with a LT-ELSD Sedex 85 evaporative light scattering detector (Sedere, Alfotville, France). As stationary phase a Kinetex EVO C18 100 Å (150 × 4.6 mm; 3 µm particle size) column was used. Water containing 0.02% of trifluoroacetic acid (A) and acetonitrile (B) served as mobile phases. The solvent composition was set to: t = 0.0 min B: 0%; t = 32.0 min B: 20%; t = 42.0 min B: 25%; min t = 52.0 min B: 30%; t = 54.0 min.0 B: 98%; t = 65.0 min B: 98%. A post-time of 10 min was applied. The flow rate was t = 0.0 min 0.2 ml/min; t = 32.0 min 0.6 ml/min; t = 42.0 min 1.0 ml/min and temperature was set to 30 °C. The injection volume was 10.0 µl and chromatograms were recorded at 254 nm. For ELS detection, drift tube temperature was set to 70 °C, nitrogen gas flow to 3.5 l/min, and the gain was set to level 12.

2.5. HPLC-DAD-MS conditions

For purity and identity control of constituents of the analyzed products an Agilent 1100 series system, (Agilent, Waldbronn, Germany) equipped with autosampler, column thermostat, on-line degasser, quaternary pump and DAD, coupled with Esquire 3000 Plus (Bruker Daltonics, Bremen, Germany) was used. HPLC settings and gradient elution were selected as mentioned before except the composition of solvent A which consisted of water with 0.9% formic acid, 0.1% acetic acid (all v/v).The eluate was introduced into ESI-MS via a split (1:5) using alternating ionization mode with following settings: spray voltage