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### Original article

## *Aspidosperma subincanum* I. characterisation, extraction of an uleine-enriched fraction and potential health hazard due to the contaminant ellipticine

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#### A B S T R A C T

The bark of the Brazilian tree *Aspidosperma subincanum* Mart. ex A. DC., Apocynaceae, has been characterised, and its constituents concentrated to obtain an uleine-enriched extract with the aim to produce food supplements. The concentration of the contaminant alkaloid ellipticine was assessed, and its potential to elicit toxic effects on consumers evaluated. It was found that this alkaloid posed no danger.

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### Introduction

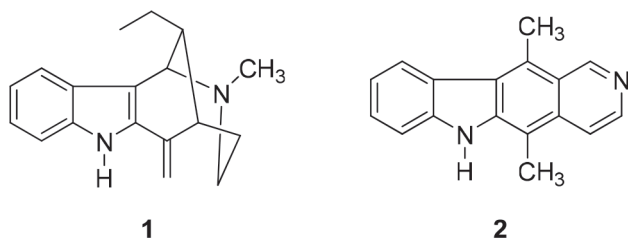
*Aspidosperma subincanum* Mart. ex A. DC., Apocynaceae, is a Brazilian tree commonly known as “*guatambu*”. The bitter tonic of its bark is known by the indigenous population to stimulate genitourinary, circulatory and respiratory functions, reduce fever and attenuate spasms. These physiological activities are caused by its principal alkaloid, uleine (1). Since 2004, Brazilian researchers have reported a broad spectrum of *in vitro* antimicrobial activity of an uleine-rich plant extract against pathogens, as well as having gastro-protective effects (Baggio et al., 2005), elicits alterations of vascular and non-vascular smooth muscle

responsiveness (Rattmann et al., 2005), produces nitric oxide (Souza et al., 2007), interferes with the inflammatory response (Nardin et al., 2008), regulates the immune system (Nardin et al., 2010) and has anticholinesterase activity (Seidl et al., 2010). All effects are attributed to the alkaloid uleine.

A research group from the Federal University of Paraná extracted the alkaloid uleine from *Himatanthus lancifolius*, and our laboratory endeavoured to purify uleine from the bark of *Aspidosperma subincanum* with the aim of concentrating uleine to be used as food supplement. For this purpose, it was imperative to verify the toxicity generated by the contaminant ellipticine (2).

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Plant material providers have a strong tendency to fail to distinguish between plants that possess similar medicinal effects and sell one for the other, this first publication exposes the characterisation of three different plants, namely *Geissospermum laeve* (Vell.) Miens, Apocynaceae; *Tabebuia impetiginosa* (Mart. ex DC.) Standl., Bignoniaceae; and *Aspidosperma subincanum*, three species easily confounded; and the potential toxic effect of ellipticine (2) present in trace amounts in the bark of *A. subincanum*.

## Materials and methods

### Plant material

Barks of *Geissospermum laeve*, (Vell.) Miens, Apocynaceae, *Tabebuia impetiginosa* (Mart. ex DC.) Standl., Bignoniaceae, and *Aspidosperma subincanum* Mart. ex A. DC., Apocynaceae, were commercially acquired in São Paulo (Brazil). The identification and authentication of the bark of *A. subincanum* was performed at the Laboratory of Pharmacognosy and Galenics of the University of Antwerp (Belgium) (Vlietinck, 2000).

Routinely, a sample is ordered and analysed in our laboratory (Arolab) by high-pressure liquid chromatography (HPLC) for conformity with the specifications and stored dry until processing. Samples of each lot are kept for reference at the laboratory.

### Methods

The methods used in this study, thin layer chromatography (TLC), high pressure liquid chromatography (HPLC) and mass spectrum analysis, are all well-known methods by the investigators active in these fields and do not need an exhaustive description. Specific details will be given in the results section.

The barks of the three species were extracted and the alkaloids content verified by HPLC.

### Extraction

The barks were mixed with alcohol at 70° in a ratio 1:10 under agitation, and heated with microwaves until a temperature of 50°C was reached. The extraction was done using 1000 mg of microgranules, refluxed for 45 min at 90°C in ethanol 70°, acidified with 2% H<sub>3</sub>PO<sub>4</sub>, and the yield was filtered.

### HPLC analysis

#### Conditions

An ODS C18 column 150 × 4.6; gradient: t<sub>0</sub> 10% ACN/H<sub>2</sub>O, 1.34 g KH<sub>2</sub>PO<sub>4</sub>, 220 mg 1-heptane sulfonic acid, 0.7 ml triethylamine qsp

1000 cc, pH 2.8 by H<sub>3</sub>PO<sub>4</sub>; t<sub>30</sub> 40% ACN; at t<sub>35</sub>, the concentration of ACN was reduced from 40% to 10 % and, at t<sub>40</sub>, the run ended. The peaks are integrated except the peak of injection; calibration was done with codeine as control (0.5 mg/10)

### Sample preparation

The liquid concentrate (1 g) was mixed with ammonia at 30 % (3 ml) and ethyl acetate (20-25 ml) under agitation for 15 min. The supernatant was recuperated and transferred to a vial; the rest is exhausted three times with ethyl acetate and added to the decantation vial. The organic phase is extracted three times with water (150 ml) and sulphuric acid (2%), and the aqueous phases recuperated. The aqueous phase was alkalinized with ammonia (30%) to reach pH 9 (9-12 ml); extracted thrice with 50 ml dichloromethane and washed three times with 70 ml water. The dichloromethane phase is evaporated to dryness, and further dissolved in 20 ml water with 2% phosphoric acid.

Uleine (1) and ellipticine (2) were characterised by mass spectrum analysis of the previously isolated molecules

## Results

### Drug identification

Barks of *Geissospermum laeve* (Vell.) Miens, Apocynaceae, *Tabebuia impetiginosa* (Mart. ex DC.) Standl., Bignoniaceae, and *Aspidosperma subincanum* Mart. ex A. DC., Apocynaceae, were chosen for comparison because they are all three endowed with similar desirable medicinal properties and are commonly interchanged by vendors of these prime material.

The HPLC chromatograms of their extracts differ substantially and make them easily recognisable (Figs. 1, 2 and 3) and allow the rejection of any material that does not conform to the specifications.

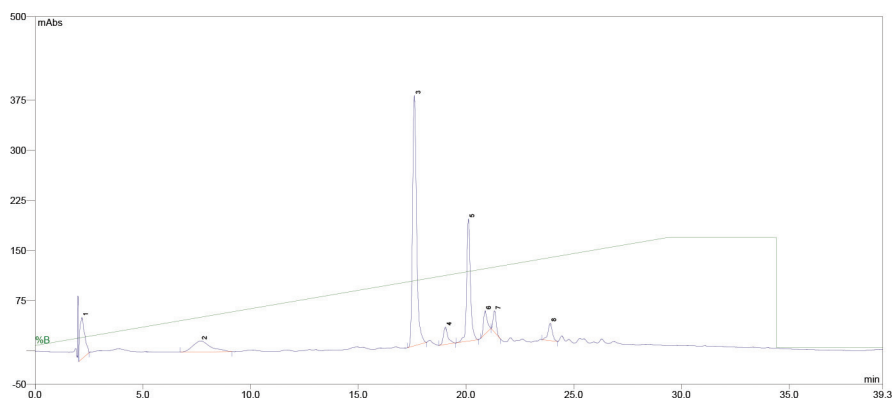
### Molecule characterisation

Is the molecule eluting last in the HPLC of *Aspidosperma subincanum* extract uleine?

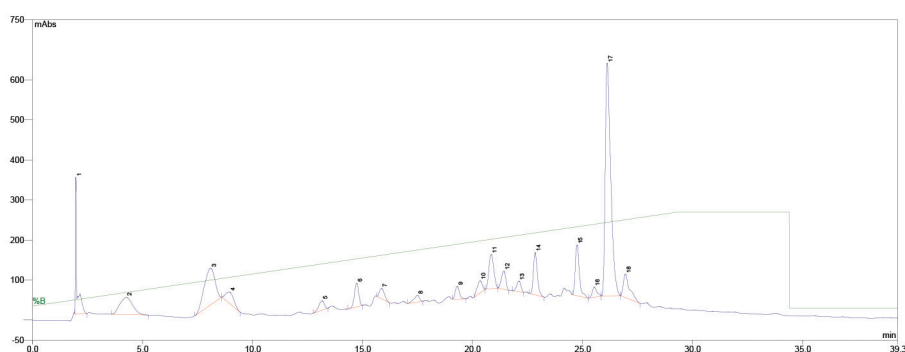
To confirm the chemical identity, the material isolated by HPLC was submitted to a mass spectrum analysis, which is a gas-chromatographic method of analysis of the atoms composing a molecule. The mass spectra of uleine (1) showed its molecular mass at *m/z* 266. Additional characteristic signals were detected at *m/z* 237, 223, 209, 194, 180 and 167. These are explained according to two simultaneously occurring schemes.

The fragment M<sup>+</sup>-C<sub>2</sub>H<sub>5</sub> could be explained by the location of the ion bond C-14/15, which is activated by the allyl position, and the breaking of the connection and loss of the ethyl radical, with subsequent formation of fragment *m/z* 237. Alternatively, the loss of the C-21 substituent with a hydrogen transfer with N-methyl formation, could form the fragment *m/z* 181; the subsequent loss of the benzyl hydrogen of *m/z* 181 or the loss of the amine side chain will form the fully conjugated species *m/z* 180, in accordance to previous studies (Joule and Djerassi, 1964; Manske and Rodrigo, 1965).

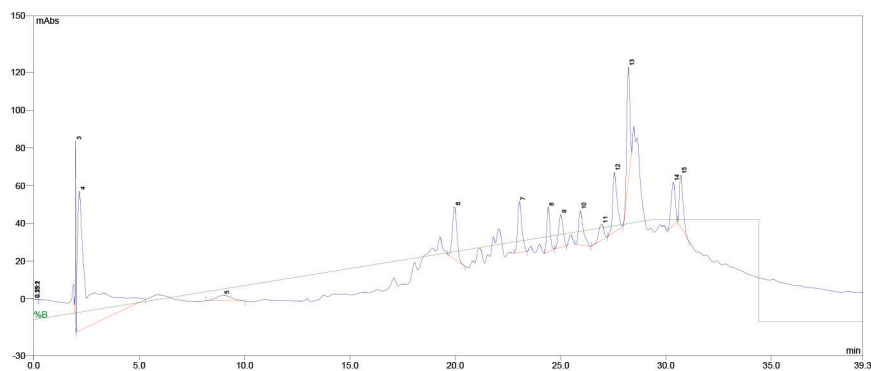
The ion *m/z* 209 can be formed by rearrangement of hydrogen through the six membered ring intermediary,



**Figure 1** – HPLC profile of *Geissospermum laevis* extract. The marker that elutes first, after two minutes, is codeine.



**Figure 2** – HPLC profile of *Aspidosperma subincanum* extract. The marker that elutes first is codeine. The major peak, which elute last, is uleine.



**Figure 3** – HPLC profile of *Tabebuia impetiginosa* extract. The marker that elutes in front is codeine.

breaking the benzylic additional bond with the loss of a methyl radical ion, which causes the formation of ion  $m/z$  194. Other fragments are present at  $m/z$  180, 151, 127, 112, 98, 77, 58 e 32 (Joule and Djerassi, 1964).

## Discussion

### Ellipticine

Ellipticine (2) is a toxic alkaloid present in minute amounts in the bark of *Aspidosperma*. The French Agency for Food

Safety (AFSSA) allows the presence of contaminants in food supplements inferior to 1% if their traditional use has been proven innocuous satisfactorily (Bertha et al., 2003). Two considerations must be taken into account for the evaluation of the deleterious impact of ellipticine on consumers: how much is there of this substance in the purified encapsulated uleine food supplement and how toxic is this amount absorbed by mouth?

### Concentration of ellipticine

A concentration of 0.063% of ellipticine and derivates was detected in a food supplement made from the bark of

*Aspidosperma subincanum* (Table 1). Accordingly, 0.26 mg of ellipticine and isomers were present in a 420 mg capsule (Angenot, 2004). Although the recommendation is a daily intake of three capsules, the investigator assumed an intake of six capsules, which accounts to a daily intake of 1.6 mg of ellipticine and derivate. This increased, amount has been deemed toxic for the consumer in the long run, and especially during pregnancy. To consolidate this argument, the abandonment of the use of ellipticine as an anticancer agent was encouraged due to its crippling collateral toxic activities. The conclusions of this investigation stated that mutagenic and teratogenic effects *in vivo* only led to the rejection of this food supplement by the Belgian Ministry of Health (Demotte, 2007) and by the French Ministry of Economy (Constans, 2008).

The total concentration of ellipticine reported (0.063%) in the capsules is well under the lower limit of tolerance of contaminants of food supplements defined by the AFSSA, which is 1%. Below this concentration, the contaminant is ignored.

**Table 1**  
Ellipticine (2) and derivates contents present in the bark of *Aspidosperma subincanum*.

Bark	Content of ellipticine (2) and derivates (%)
Methanol extract	0.012
Ethyl acetate extract	0.024
Ethanol 50% extract	0.018
Capsules	
Ethyl acetate	0.043
Ethyl acetate + NH <sub>4</sub> OH	0.020
Total	0.063

### Toxicity of ellipticine.

The toxicity of ellipticine varies considerably according to its evaluation *in vitro* and *in vivo*, and to route of administration. Food supplements are supposed to be absorbed exclusively by mouth. Studies *in vitro* showed promise for the use of ellipticine as an anticancer agent but *in vivo* assays failed to confirm it.

A study performed *in vitro* demonstrated that ellipticine attaches to topoisomerase II, even in the absence of DNA (Froelich-Ammon et al., 1995). Furthermore, the topoisomerase-ellipticine complex attaches exclusively to abnormal portions of DNA (René et al., 2007). The claim that ellipticine is mutagenic because it modifies DNA is questioned because this molecule does not modify normal double-stranded DNA. It was shown *in vitro* that ellipticine induces the "natural" death of cancer cells by apoptosis (Kuo et al., 2005). This study suggested ellipticine to hold anticancer properties, instead of its alleged toxicity for normal diploid cells. Several *in vitro* studies proposed ellipticine to induce aberrations (i.e. clastogenesis) in human lymphocytes (Sakamoto-Hojo et al., 1988). It has been documented that ellipticine is mutagenic essentially for tumoral cells and have little mutagenic effects

on diploid cells, although these may suffer from clastogenesis (Moore et al., 1987). However, no corroboration of these results could be found in studies performed *in vivo*, where the drug may be administered via various routes, which impact significantly on drug assimilation.

*In vivo*, ellipticine administered intravenously in rats induces chromosomal aberrations in bone-marrow cells (Sakamoto-Hojo et al., 1988). However, to have an effect, ellipticine must first be metabolised by oxidation of 13-hydroxyellipticine and ellipticine N<sub>2</sub>-oxyde via cytochrome P450 3A4 (Stiborová et al., 2004). In the introduction of their publication, the authors mentioned that ellipticine showed little undesirable side effects when used as an anticancer agent. This statement contradicts the claim made by Angenot.

### Why is ellipticine a poor anticancer drug and why has it little side effects *in vivo*?

Ellipticine solubilises poorly in salty water. Human blood is salty and precipitates ellipticine. Ellipticine injected intravenously is not excreted via the kidneys but 84% of it is found in the faeces. To overcome the poor solubility of the genuine drug in media and use it as an anticancer agent in humans, it was solubilised in glucose by coupling it to acetate. The elliptitium acetate was injected three successive weeks at massive doses of 80 mg/m<sup>2</sup>/day, three days a week, with little response not due to intolerable side effects. It was abandoned because the ellipticine acetate induced the formation of antibodies. As a food supplement, the ellipticine is present at the dose of 0.063% per capsule, well below the lower-limit of 1% admitted for contaminants of food supplements, and is administered orally. In the stomach, high acidity precipitates it. In addition, the enzyme cytochrome P450 3A4 needed to metabolize the ellipticine into an active anticancer agent is not present in the stomach.

Traditional uses of extracts of *Aspidosperma subincanum* confirm these experimental observations: taken orally, extracts of *Aspidosperma subincanum* are non-toxic and can be used as food supplements. The plant was introduced in Europe in 1878. No report of toxicity was ever registered. The Belgian Ministry of Health admitted the use of extracts of *Aspidosperma subincanum* until 2007, and this food supplement was not reported to be toxic between 1878 and 2007. Exhaustive toxicity studies performed on mice (2000 mg/kg, 28 days, six days/week), rats (2000 mg/kg, single dose) and rabbits (500 mg/kg, 28 days, six days/week) confirmed that the food supplement is harmless when administered per os during a month at doses 60 times the average recommended daily dose for humans (SGS Lab Simon S.A. 1999 and 2000). Teratogenic and mutagenic effects were not detected.

### Conclusion

The study of the properties of uleine has been actively pursued for the last two decades by the Laboratory of Pharmacognosy of the University of Paraná, Arolab; aiming to fully characterise the *Aspidosperma* plant and Parabolic Biologicals established the potential toxic activity of the alkaloid ellipticine that this plant contains. It was concluded that ellipticine posits no danger

for the use of this plant as a food supplement, regardless the dose ingested.

### Author's contributions

JDF isolated and characterised the plant material. DM designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. RM wrote the manuscript. All authors have read and approved the paper for submission.

### Conflicts of interest

The authors declare no conflicts of interest.

### Acknowledgments

The company IFFRA Institut Français de Recherche Analytique, 38, rue de l'Industrie BP 192, 67 405 Illkirch Cedex) performed the gas-chromatographic method of analysis of the atoms composing the uleine molecule.

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