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Research article

Distribution of the cardiotoxin pavettamine in the coffee family (Rubiaceae) and its significance for gousiekte, a fatal poisoning of ruminants

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ABSTRACT

Gousiekte, a cardiac syndrome of ruminants in southern Africa, is caused by the ingestion of plants containing the polyamine pavettamine. All the six known gousiekte-causing plants are members of the Rubiaceae or coffee family and house endosymbiotic *Burkholderia* bacteria in their leaves. It was therefore hypothesized that these bacteria could be involved in the production of the toxin. The pavettamine level in the leaves of 82 taxa from 14 genera was determined. Included in the analyses were various nodulated and non-nodulated members of the Rubiaceae. This led to the discovery of other pavettamine producing Rubiaceae, namely *Psychotria kirkii* and *Psychotria viridiflora*. Our analysis showed that many plant species containing bacterial nodules in their leaves do not produce pavettamine. It is consequently unlikely that the endosymbiont alone can be accredited for the synthesis of the toxin. Until now the inconsistent toxicity of the gousiekte-causing plants have hindered studies that aimed at a better understanding of the disease. *In vitro* dedifferentiated plant cell cultures are a useful tool for the study of molecular processes. Plant callus cultures were obtained from pavettamine-positive species. Mass spectrometric analysis shows that these calli do not produce pavettamine but can produce common plant polyamines.

1. Introduction

South Africa has a rich and varied flora that includes some 600 poisonous plants [1–3]. Plant poisoning of livestock is responsible for considerable economic losses in southern Africa (that part of the African continent south of the Kunene, Okavango and Zambezi Rivers). One of the six most important plant toxicoses in this region is gousiekte, causing the death of about 7000 head of livestock, mainly sheep, goats and cattle, each year [1,4,5]. Gousiekte (Afrikaans for "quick disease") is a cardiac syndrome of domestic ruminants caused by the ingestion of certain poisonous plants. The disease is characterized by sudden death four to eight weeks after the initial intake of toxic plants, usually without obvious prodromal symptoms. At present six plant species, all belonging to the Rubiaceae, are known with certainty to cause the disease: *Vangueria pygmaea* (syn. *Pachystigma pygmaeum*) [6], *Vangueria thamnus* (syn. *Pachystigma thamnus*) [7], *Vangueria latifolia* (syn. *Pachystigma latifolium*), *Pavetta schumanniana* [8], *Pavetta harborii* [9] and *Fadogia homblei* (syn. *Fadogia monticola*) [4].

Research on gousiekte commenced in 1908 when Walker attempted to establish the cause of the disease [6]. After many earlier authors have failed in their attempts, Fourie and coworkers [10] succeeded in isolating the causal toxin. They demonstrated the presence of the gousiekte-inducing compound in *Pa. harborii*, *Pa. schumanniana*, *V. pygmaea* and *F. homblei*. The chemical structure of the toxin was published in 2010 [11]. It is a polyamine and was named pavettamine after the genus *Pavetta*, of which two species have been identified to cause the disease. It was hypothesized that endosymbiotic bacteria could be involved in the production of the toxin due to the fact that all six gousiekte-causing plants house

Abbreviations: UPLC™, ultra performance liquid chromatography; MS/MS, tandem mass spectrometry; IS, internal standard; BR, the National Botanic Garden of Belgium; PRU, the Manie van der Schijff Botanical Garden, University of Pretoria; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; TQD, triple quadrupole detector; BEH, ethylene bridged hybrid; MRM, Multiple reaction monitoring.

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bacteria of the genus *Burkholderia* in their leaves [12–14]. At present it is not known whether the endosymbiont plays any role in the production of the poisonous compound. Analysis of *in vitro* cultures of the *F. homblei* endosymbiont, however, did not reveal production of pavettamine [15].

In the past, studies aimed at proving the link between gousiekte and suspected plants met with considerable difficulties as a significant number of animal feeding experiments gave negative results [10,13,16]. The toxicity of the known gousiekte-causing plants is variable and diminishes during drying. Animals differ in their susceptibility to the toxin and the disease cannot be induced in small laboratory animals. Moreover, feeding experiments have to deal with a long latency period and the lack of premonitory signs [16]. An earlier experiment in which sheep were fed limited quantities of *F. homblei* gave negative results. It was assumed that the dose employed at that time was too low since subsequent studies proved this plant to cause gousiekte [4]. Therefore it was suggested that any rubiaceous plant could only be discounted as a possible cause of gousiekte if subjected to extensive feeding experiments [13].

Many plants closely related to the six known gousiekte-causing species occur in southern Africa. The Rubiaceae or coffee family is the fourth most species-rich flowering plant family with more than 13 000 species comprising about 600 genera [17]. The Rubiaceae is particularly well represented in humid tropical forests, with species diversity decreasing rapidly from the subtropics through the temperate regions to the poles [17,18]. In southern Africa alone there occur more than 30 species of *Pavetta* [19,20]. It would be helpful to determine if in any of these plants the toxic principle is present and in which order of magnitude. Other Rubiaceae, or even species from other plant families, might contain pavettamine, perhaps in a lower concentration, insufficient to cause gousiekte. Alternatively such plants may not be consumed in significant quantities by domestic ruminants. The isolation procedure for the toxin described by Fourie et al. [10] made it possible to chemically assay plants for their toxicity. However, this method does not quantify the concentration of pavettamine and, as the authors stated, the procedure is tedious. Recently, a mass spectrometry based method for the analysis of pavettamine was reported [15]. It allows detection and quantification of pavettamine in biological samples in a fast and sensitive manner without the need for large sample volumes. Hitherto, plants or plant fractions could only be assayed for toxicity by using ethically questionable biological trials [10].

The primary objective of the present study is to assess whether pavettamine is present in other plant species, including species that lack bacterial endosymbionts. To estimate the role of bacteria and plants in the production of the toxin, callus cultures of pavettamine-positive species were tested in their capacity to produce the toxin in the absence of bacteria.

2. Results and discussion

2.1. Pavettamine is present in other plants than the six known gousiekte-causing species

The potential presence and concentration of pavettamine were determined through detection by tandem mass spectrometry after derivatization with benzoyl chloride and separation by ultra-performance liquid chromatography (UPLC™) [15]. The selected plants, 82 taxa from 14 genera, are from the Rubiaceae since gousiekte has invariably been associated with plants of this family [4,16]. Given the presumed link between gousiekte-causing species and endosymbiotic bacteria, nodulated species were of particular interest. Plant taxa, in which pavettamine was detected, are listed in Table 1. Two additional species were found to be positive for the toxin, namely *Psychotria kirkii* and *Psychotria viridiflora*. The genus *Psychotria* was previously not linked to the aetiology of gousiekte. In fact, the six gousiekte-causing species all belong to the subfamily Ixoroideae, while the genus *Psychotria* is of the subfamily Rubioideae [14,21]. Two other accessions, one nodulated *Psychotria* and one nodulated *Pavetta* species also produce pavettamine. *Psychotria* is the world's third largest flowering plant genus and the largest in the Rubiaceae [17]. We were unable to detect pavettamine in any of the other genera tested. Considering the concentration of pavettamine detected in these plants, it appears that the *Psychotria* species produce pavettamine in higher amounts that the traditional gousiekte-causing plant species. However, it is known that the toxicity in these plants varies at different times of the year, as well as from year to year [4,6,8]. Toxicity apparently also varies according to locality, habitat and probably climatic conditions [6,8]. The conditions of the plants grown in the greenhouses of the National Botanic Garden of Belgium might not accurately correspond to in-field conditions. Furthermore, a threshold concentration of pavettamine in leaves has not been determined for causing the onset of the development of gousiekte.

Table 1
List of plant samples in which pavettamine was detected. Value in nmol/g fresh weight (* nmol/g dry weight) ± st error ($N = 5$).

Plant name	Accession	Pavettamine
Fadogia homblei[a,c]	Wild collected	296 ± 47*
Pavetta sp.[b]	BR-20060123-38	4135 ± 121
Pa. harborii[b]	Wild collected	1284 ± 68*
Pa. schumanniana[b]	BR-20041430-66	230 ± 18
Pa. schumanniana[b]	BR-20001942-57	1381 ± 79
Psychotria sp.[b]	BR-20001933-48	4084 ± 174
Ps. kirkii[b]	BR-19951273-22	3116 ± 101
Ps. kirkii[b]	BR-20010513-92	551 ± 21
Ps. kirkii[b]	BR-19761893	414 ± 31
Ps. kirkii[b]	BR-20070328-58	1352 ± 156
Ps. kirkii[b]	BR-19750521	3660 ± 151
Ps. kirkii[b]	BR-20021203-15	3330 ± 226
Ps. kirkii[b]	BR-20021526-47	1644 ± 118
Ps. kirkii[b]	BR-20070330-60	2095 ± 272
Ps. kirkii[b]	BR-20001946-61	2005 ± 79
Ps. kirkii var. *hirtella*[b]	BR-20001036-24	8674 ± 1124
Ps. kirkii var. *nairobiensis*[b]	BR-19981825-19	322 ± 26
Ps. kirkii var. *tarambassica*[b]	BR-19536779	1396 ± 219
Ps. viridiflora[b]	BR-20070138-62	1256 ± 49
Ps. cf. kirkii[b]	BR-20001943-58	5172 ± 513
Vangueria pygmaea[c]	Wild collected	374 ± 13*

[a] value adopted from Van Elst et al. [15].
[b] Species with leaf nodules.
[c] Species with non-nodulating bacterial endophytes.

The mass spectrometry method for the quantification of pavettamine as described by Van Elst et al. [15] allows the detection of several other important polyamines (diaminopropane, putrescine, cadaverine, spermidine, spermine and agmatine) alongside pavettamine in biological samples. Pavettamine certainly is an unusual polyamine and of the common plant polyamines most closely resembles cadaverine (see Fig. 1), both having a carbon chain of five carbon atoms. Cadaverine is formed by the decarboxylation of lysine [22]. We did not detect cadaverine in many of the selected plants. However, we observed that all plants able to produced pavettamine can also produce cadaverine (see Table A.1, Supplementary files). At the moment, it is not known how pavettamine is synthesised. Given their structural similarity, cadaverine might be involved in the biosynthesis of pavettamine. Further studies should elucidate the possible relation between cadaverine and pavettamine.

2.2. Gousiekte, a disease of southern Africa?

In 1923, the Director of Veterinary Services in South Africa, Arnold Theiler, claimed that 'Gousiekte is a disease of South Africa'