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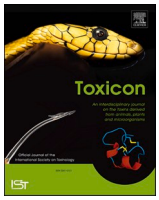

Indigofera cryptantha-induced pigmenturia in cattle in South Africa

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ABSTRACT

Two field cases of reddish-black pigmenturia occurred where cattle grazed on an established *Cenchrus ciliaris* (blue buffalo grass) pasture in South Africa. The pasture was noticeably invaded by *Indigofera cryptantha*, which was heavily grazed. Apart from the discolored urine, no other clinical abnormalities were detected. Urinalysis revealed hemoglobinuria, proteinuria and an alkaline pH. When the animals were immediately removed from the infested pasture, they made an uneventful recovery. However, a bull died when one of the herds could not be removed from the *I. cryptantha*-infested pasture. Macroscopically, the kidneys were dark red in color and the urinary bladder contained the dark pigmented urine. Microscopically, the renal tubules contained eosinophilic, granular pigment casts in the lumen. In addition, many renal tubular epithelial cells were attenuated with granular cytoplasm and were detached from the basement membranes. Chemical analysis was performed on dried, milled plant material and two urine samples collected during the field investigations. Qualitative UPLC-UV-qTOF/MS analysis revealed the presence of indican (indoxyl-β-glucoside) in the stems, leaves and pods of *I. cryptantha* and indoxyl sulfate was identified, and confirmed with an analytical standard, in the urine samples. It is proposed that following ingestion of *I. cryptantha*, indican will be hydrolysed in the liver to indoxyl and conjugated with sulfate. Indoxyl sulfate will then be excreted in relatively high concentrations in the urine. In the alkaline urine, two indoxyl molecules might dimerize to form leucoindigo with subsequent oxidation to indigo, thus, contributing to the dark pigmentation of the urine. It is also possible that indoxyl sulfate contributed to the renal failure and death of the bull. Although *I. suffruticosa*-induced hemoglobinuria has been described in Brazil, this is the first report of *I. cryptantha*-induced pigmenturia in cattle in South Africa.

1. Introduction

In South Africa pigmented, reddish-brown or coffee-colored urine in cattle is often associated with bovine babesiosis or redwater. Bovine babesiosis is a tick-borne disease where the intra-erythrocytic protozoan parasite *Babesia* induces intravascular hemolysis with subsequent icterus and hemoglobinuria (De Vos et al., 2004). Other causes of hemoglobinuria in cattle in South Africa, such as leptospirosis are less common (Hunter, 2004) and cold water- and post-parturient hemoglobinuria are rare in the country (Tarr, 1947). Bacillary hemoglobinuria, caused by *Clostridium novyi* type D, has not been diagnosed in South Africa (Kriek and Odendaal, 2004). Myoglobinuria is uncommon in cattle, but may arise in downer cows as well as with vitamin E and/or selenium deficiency (Constable et al., 2017).

Toxicological causes of plant-induced hemolytic anaemia and hemoglobinuria in cattle in South Africa, although infrequent, can occur following excessive ingestion of members of the Brassicaceae family and *Allium* species (e.g., onions and garlic) (Kellerman et al., 2005). Chronic copper poisoning in cattle, with associated hemoglobinuria, is even more sporadic (Gummow et al., 1991). Hematuria, as a result of a hemorrhagic tendency and bleeding tumours in the urinary bladder, following ingestion of *Pteridium aquilinum* (bracken fern) is seldom reported (Tustin et al., 1968). Although *Senna occidentalis* is an invasive species in South Africa, myopathy and subsequent myoglobinuria in

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cattle, as described in South America, have not been reported (Riet--Correa et al., 2009).

In north-eastern Brazil, *Indigofera suffruticosa*, has been confirmed as a cause of a usually, non-fatal hemoglobinuria in cattle (Barbosa Neto et al., 2001). This invasive weed is especially prevalent in relatively wet seasons (Riet-Correa et al., 2009). Salvador et al. (2010) reported that five days after 25 cows and a bull were introduced to a camp, severely invaded by *I. suffruticosa*, the cows voided reddish urine, but the animals remained in the camp for 10 days. Seven days after removal from the camp one animal was found dead, reportedly dying of acute hemolytic anaemia, but the other affected animals recovered uneventfully (Salvador et al., 2010). The authors suggested that the anaemia is caused by aniline contained in the plant (Salvador et al., 2011).

Historically, the natural dye, indigo, was extracted from the leaves of certain *Indigofera* species, such as *I. tinctoria* in Asia. Indigo has a distinctive blue colour, well known for staining denim jeans (Cordin et al., 2021). Production of indigo dye involves the processing of the leaves (Fig. 1). The leaves containing indican (indoxyl-β-glucoside) (1) are then hydrolysed to release β-D-glucose and indoxyl (2), the indoxyl then dimerises to form leucoindigo (3), which is then ultimately oxidised to indigo (4), also referred to as indigotin (Zou and Koh, 2007; Molino and Junio, 2021) (Fig. 1). However, nowadays indigo (4) is synthesized with aniline as a starting material (Cordin et al., 2021).

The aim of this study was to describe two field outbreaks of *Indigofera cryptantha*-induced pigmenturia in cattle in South Africa and to compare them to *Indigofera suffruticosa*-induced hemoglobinuria in cattle, reported from north-eastern Brazil. The specific objectives were to confirm the presence of indican in various plant parts and to ascertain if the suggested hemolytic agent, aniline, is present in the plant material.

2. Materials and methods

2.1. Field outbreaks

2.1.1. Case 1

A farmer in the Stella district (26.78573°S; 24.76675°E), North-West Province of South Africa, moved 42 Bonsmara heifers and a bull to a 9-ha *Cenchrus ciliaris* (blue buffalo grass) pasture on a Monday at the beginning of February 2023. By Friday (4 days after exposure), it was noticed that almost all cattle voided dark reddish-black urine.

The farmer consulted a general practitioner who confirmed the reddish-black urine, but on clinical examination could not detect any other abnormalities, *i.e.*, temperature, pulse, respiratory rate and rumen motility were within the normal range, no icteric mucous membranes, and blood smears were negative for blood parasites. Clinically, the cattle were behaving normally, were ruminating and did not appear sick. The herd was removed from the pasture and, when checked a week later, all previously affected animals had recovered with no visible discoloration of the urine. When the heifers were removed from the camp on the Friday, the farmer placed adult cows on the pasture, but after the weekend, by Monday (3 days later), three cows were seen with reddish-black urine. The cows were then also removed. No mortalities were reported.

On closer inspection of the pasture, it was noticed that the cattle also grazed on a small leguminous bush growing abundantly in the camp. The bush grew upright, and the pods were curved, with constrictions, and borne close to the stem below the leaves (Fig. 2). Fresh plants were randomly collected, while criss-crossing through the camp. The aerial parts of the pod-bearing plants were chopped-off and placed in five 50-kg hessian bags. Thereafter, the plants were spread out in a shed and allowed to dry for 10 days. The dried plants were collected and submitted for botanical identification and chemical analysis. A small volume of urine was also collected from a heifer for urinalysis.

2.1.2. Case 2

During April 2023, 40 cross-bred cows, with 2–3-month-old calves at foot, and a bull were moved to an established 20 ha *Cenchrus ciliaris* (blue buffalo grass) pasture in the same district (26.62613°S; 24.968618°E). A similar erect, bushy plant grew in the cultivated field. The bull, cows and calves were moved to the blue buffalo grass pasture on a Thursday and by the following Monday (4 days later) the majority of the cows and the bull voided dark, reddish urine. The calves were unaffected. The animals appeared clinically normal with no increase in body temperature, and the visible mucous membranes were not icteric. Blood smears were negative for parasites. The cattle could not be removed from this pasture. The bull died on the Wednesday, 6 days after being introduced to the established pasture.

A necropsy was performed 6–8 h following death, and a urine sample was collected for urinalysis and chemical analysis. Tissue samples (heart, lung, kidney, liver and spleen) were collected and placed in a 10% buffered formalin solution for microscopical examination.

2.2. Urinalysis

The urine was evaluated with a dipstick (Combur-Test® strip, Roche) and also submitted for further laboratory analysis at the Clinical Pathology Laboratory, Faculty of Veterinary Science.

2.3. Microscopy

Tissue samples were routinely processed, sectioned and stained with hematoxylin and eosin. To exclude myoglobinuria, kidney sections were also processed for immunohistochemical staining. An indirect immunoperoxidase technique, using rabbit polyclonal anti-myoglobin antibody (ab187506, Abcam) and VECTOR NovaREd as the chromogen, was performed by hand, following validated protocols. Kidney tissue from a previous case of capture myopathy in a rhinoceros was used as the positive control. For transmission electron microscopy (TEM), samples were processed following standard protocols and embedded in Agar 100 epoxy resin. Ultra-thin sections (80–90 nm thick) were cut and stained with uranyl acetate and counterstained with lead citrate. The sections were viewed with a JEOL JEM 1400-FLASH transmission electron microscope (Tokyo, Japan).

2.4. Chemical analysis

2.4.1. Solvents and purchased standards

For the extraction of the plant material, UnivAR®-grade dichloromethane (DCM) and methanol (MeOH) were purchased from Merck®, South Africa (Merck KGaA, Darmstadt, Germany). Sample preparation

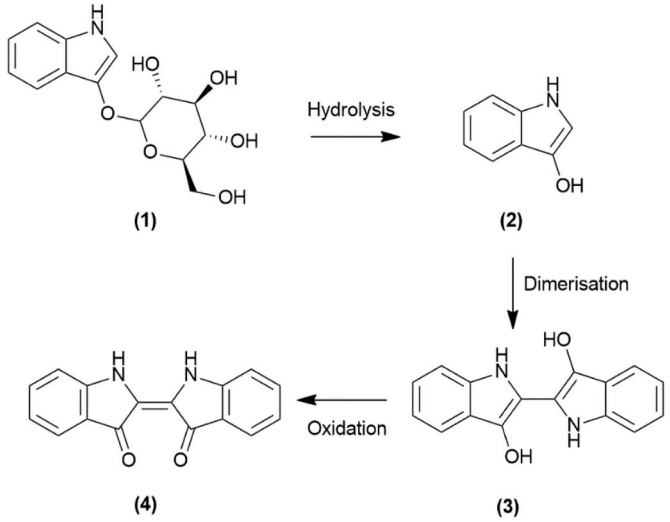

Fig. 1. Biosynthesis of indigotin (indigo dye) from *Indigofera* species.

Fig. 2. *Indigofera cryptantha* var. *cryptantha* Benth. ex Harv. A. Compound leaves, with leaflets arranged in opposite pairs. B. Small, inconspicuous, pinkish-colored flowers. C. Curved pods, with constrictions between seeds and borne close to the stem below the leaves. (Courtesy of SAplants/Wikimedia Commons/CC BY-SA 4.0).

for UPLC-UV-qTOF/MS-analysis was done with ROMIL® SpS-grade methanol 215 (Microsep®, South Africa) and de-ionised water (sourced from an in-house Elga® Chorus II® Water Purification System – Labotec®). For solubilizing standards with poor aqueous solubility, dimethyl sulfoxide (DMSO, for molecular biology) was purchased from Merck®. Eluants for the UPLC were prepared using ThermoFisher formic acid for LC-MS (99%) and ROMIL® UpS grade methanol (ultra lc), and in-house de-ionised water.

Leucine enkephalin was sourced from Waters® via Microsep® (South Africa) as an internal calibrant for the monoisotopic mass analysis. Indican (indoxyl β-D-glucoside: I3750, Merck®), aniline (aniline: 51788, Merck®), and indoxyl sulfate potassium salt (urinary indican: I3875, Merck®) were purchased as standards for qualitative analysis of the extracts and urine samples.

2.4.2. Extraction of plant material

The plant material was air-dried, and the plant parts (stems, leaves and pods) were separated and milled (Ika Werke MF10 Microfine grinder, Staufen, Germany) with a 1 mm sieve. The plant material was extracted using a sonication-mediated extraction (SME) method. Of each plant sample, *ca.* 7.2 g was added to a separate in-house designed glass percolation vessel, together with 50 ml of a dichloromethane:methanol (DCM:MeOH [1:1]) solution and sonicated for 1 h. Thereafter, the solution was drained, and the plant material was re-extracted with 100% MeOH. The extract was drained from the plant material, combined with the DCM:MeOH solution, and dried to completion using an SP Scientific® Genevac® EZ-2.3 Plus centrifugal evaporator (Genevac Ltd., Ipswich, UK) (Thornburg et al., 2018).

2.4.3. Preparation of standards for UPLC-UV-qTOF/MS analysis

Standard solutions, of both indican (1) and aniline, were prepared in MeOH to, respectively, obtain 200 ppm working solutions. The potassium indoxyl sulfate (5a) (Fig. 8) standard was prepared in DMSO and diluted to 100 ppm.

2.4.4. UPLC-UV-qTOF/MS analysis of the plant extracts

Duplicate samples of the plant parts were analysed. A 5000 ppm solution of each plant extract was prepared in a MeOH:H_2O (1:1) solution before filtering through a 0.22 μm nylon filter to remove particulate matter. The samples were analysed on a Waters® Aquity® UPLC® system, fitted with Aquity® PDA and Xevo® G2 qTOF detectors.

Compound separation and analysis were conducted on an XBridge UPLC® C18 (2.1 × 150 mm, 1.8 μm) column (Waters® Inc., Milford, MA, USA). Compound separation was achieved using a linear gradient elution method employing H_2O (0.1% formic acid) as solvent A and MeOH (0.1% formic acid) as solvent B. The solvent method ran as follows: starting at 97% solvent A held for 0.1 min, a linear increase to 100% solvent B at 14 min, and a 2 min column wash hold (14–16 min) before returning to starting conditions to equilibrate the column (16.5 20 min). Both the column temperature and flow rate were kept constant throughout the run at 50 °C and 0.3 ml/min, respectively. The injection volume was set at 5 μl. All data analyses were performed using MassLynx v4.2.

For detection of the eluting analytes by UV, the Aquity® PDA was set to a scan range of 220–700 nm, with a scan time of 0.05 s and a full width at half maximum (FWHM) resolution of 4.8 nm. Monoisotopic mass analysis was performed in both the ESI(+)- and ESI(−)-ionisation modes. For the ESI(+)- and ESI(−)-ionisation the capillary voltage was set at 2.80 kV and 2.0 kV, respectively. For all analyses, the mass scan range was set to 50–1200 Da, and the mass resolution (FWHM) was 22 000. The source was kept at 120 °C and the desolvation gas at 350 °C. To account for any drift in mass accuracy, a lock mass internal standard (leucine enkephalin (*m/z* 555.2693)) was infused intermittently in-source at a fixed flow rate of 3 μl/min every 10 s.

For tentative identification, a mass error of less than 5 ppm in the quasi-molecular ion was required. The compounds aniline, indican (1), and indoxyl sulfate (5) (Fig. 8) were reported as 'found' if their retention time (R_t) values were within 0.1 min of the peaks in the purchased standards.

2.4.5. Urine

The urine samples were prepared for UPLC-UV-qTOF/MS analysis by conditioning a 6 ml C8 SPE cartridge (Sigma® SupelcleanTM Envi18TM) with 18 ml MeOH. The cartridge was equilibrated with 18 ml de-ionised water (pH ~ 4.5). The urine (500 μl) was diluted with 4.5 ml acetate buffer (0.1 M, pH ~ 4.5) and loaded onto the cartridge. The cartridge was washed with 5 ml of de-ionised water, and all the eluting solution was collected. The retained sample on the cartridge was then eluted with 5 ml MeOH and collected. The eluting solutions were combined and evaporated to dryness under reduced pressure (Büchi® Rotavapor® RII, Switzerland) before being reconstituted in 1 ml of 1:1 MeOH:H_2O. The resulting sample was filtered through a nylon syringe

filter (0.22 μm) to remove residual particulate matter. The sample was then dried using an SP Scientific® Genevac® HT-6 Series 3i centrifugal evaporator, whereafter MeOH (50 μl) was added to each sample for UPLC-UV/qTOF/MS analysis.

3. Results

3.1. Plant identification

The leguminous plant collected during the outbreaks was identified as *Indigofera cryptantha* var. *cryptantha* Benth. ex Harv. (Fig. 2). A voucher specimen (PRU 130922) was lodged at the H.G.W.J. Schweickerdt Herbarium, University of Pretoria. *Indigofera cryptantha* is a woody, dwarf shrub, up to 0.8 m tall and grows in sandy bush and thornveld, along roadsides and infest old cultivated fields in South Africa (Fig. 3) (SANBI).

3.2. Urinalysis

The urine dipstick findings were markedly affected by the dark urine color, and all other parameters could not be assessed due to the marked pigmenturia. The cloudy, reddish-black urine collected from a heifer during the first outbreak had a pH = 9, protein 2+, hemoglobin/blood 4+. Microscopic examination of the urine sediment revealed 4–10 granular casts under a low-power field (LPF). The urine of the bull that died had an opaque appearance and was black in color. Urine pH = 9; protein 2+, hemoglobin/blood 4+.

3.3. Necropsy and microscopy

On macroscopical examination the urinary bladder was filled with very dark, pigmented urine and the kidneys were swollen and dark red in color. Only a few remnants of *Indigofera cryptantha* stems and pods could be discerned in the rumen contents. On histopathology, no specific lesions were noticed in the heart, and in the liver, only mild leukostasis was observed in multiple sinusoids. In the spleen, there was mild diffuse red pulp congestion and the white pulp follicles were active and moderately cellular. In the lung, multifocal alveoli contained fibrillar edematous fluid. The most striking lesions were noticed in the kidney. The cortical tubules contained eosinophilic, granular pigment casts in the lumen. Some of the affected tubules were mildly dilated with attenuated lining epithelial cells. Many renal tubular epithelial cells had granular cytoplasm, karyopyknosis and were detached from the basement membranes (Fig. 4A and B).

Immunohistochemical evaluation revealed only non-specific staining for myoglobin when compared to the positive control (Fig. 5A and B). Besides the renal tubular lesions observed with light microscopy, transmission electron microscopy of the glomeruli revealed structural changes to capillary endothelial cells and podocytes, characterized by swelling, vesiculation, and discontinuity and rupture of the cytoplasmic membrane. Destruction of cytoplasmic organelles was also observed (Fig. 5C).

3.4. Chemical analysis

3.4.1. Plant material and bovine urine samples

The data from the UPLC-UV/qTOF/MS analysis of each sample was analysed for confirmation of the presence of the following analytes: indican (1) ($C_{14}H_{17}NO_6$, monoisotopic mass: 295.1056 Da), indigotin (4) ($C_{16}H_{10}N_2O_2$, monoisotopic mass: 262.0742 Da), aniline (C_6H_7N, monoisotopic mass: 93.0578 Da) and indoxyl sulfate (5) ($C_8H_7NO_4S$, monoisotopic mass: 213.0096 Da).

Comparison of the detected monoisotopic masses, fragmentation patterns, and UV absorbance bands, to that of the reference standard, confirmed the presence of indican (1) in all the *I. cryptantha* samples analysed (Fig. 6, Table 1), when assuming the premise that the material is from plants of the genus *Indigofera*. Both the fragmentation pattern and UV absorbance bands of indican (1) match those described by Mohn

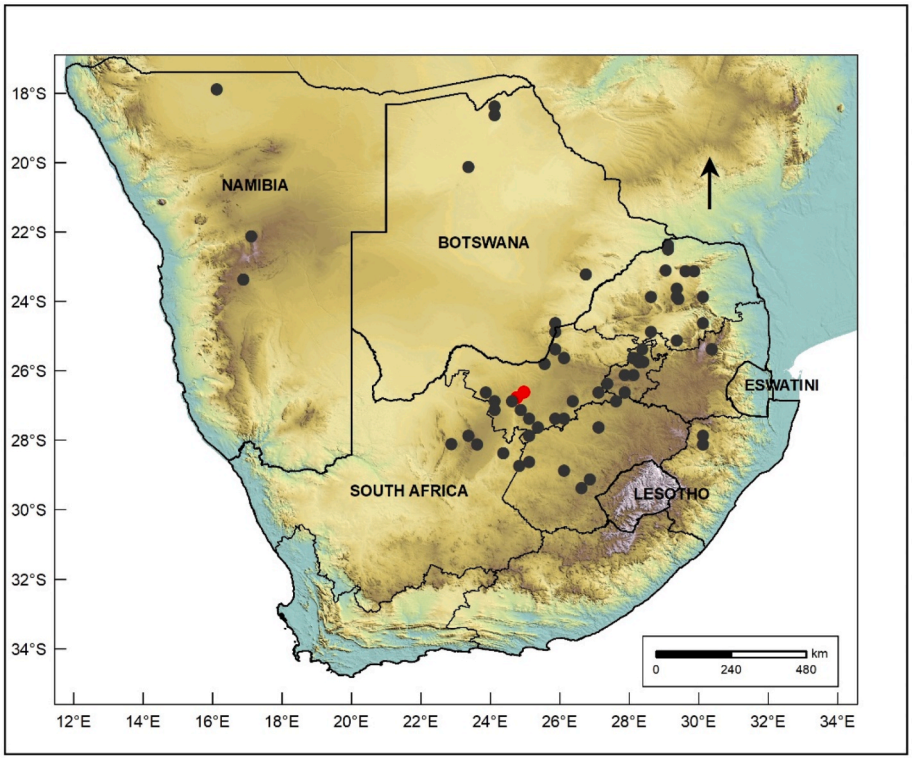

Fig. 3. Distribution of *Indigofera cryptantha* var. *cryptantha* (back dots). The black arrow indicates that this species also occurs to the north of the Flora of the southern Africa region (FSA). The red dots indicate the two farms where the field outbreaks occurred (Courtesy of H.M. Steyn, SANBI). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

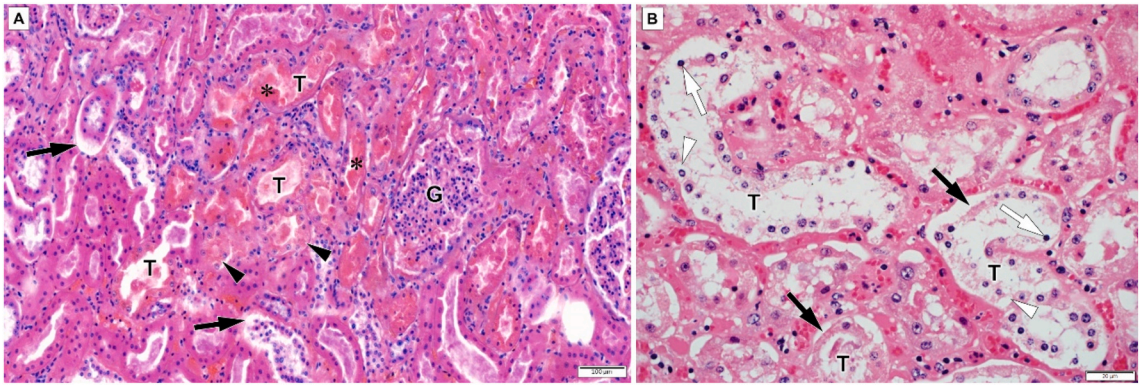

Fig. 4. Kidney of a bull that died following ingestion of *Indigofera cryptantha*. G = glomerulus, T = tubules. A and B. Severe damage to the renal tubular epithelial cells, including detachment from the basement membrane (black arrows). A. Eosinophilic pigment casts in the tubular lumen (black asterisks) and granular cytoplasm of renal tubular epithelial cells (black arrowheads); hematoxylin and eosin; bar = 100 μm. B. Cytoplasmic vacuoles (white arrowheads) and pyknotic nuclei (white arrows) in the epithelial cells of the tubules; hematoxylin and eosin; bar = 20 μm.

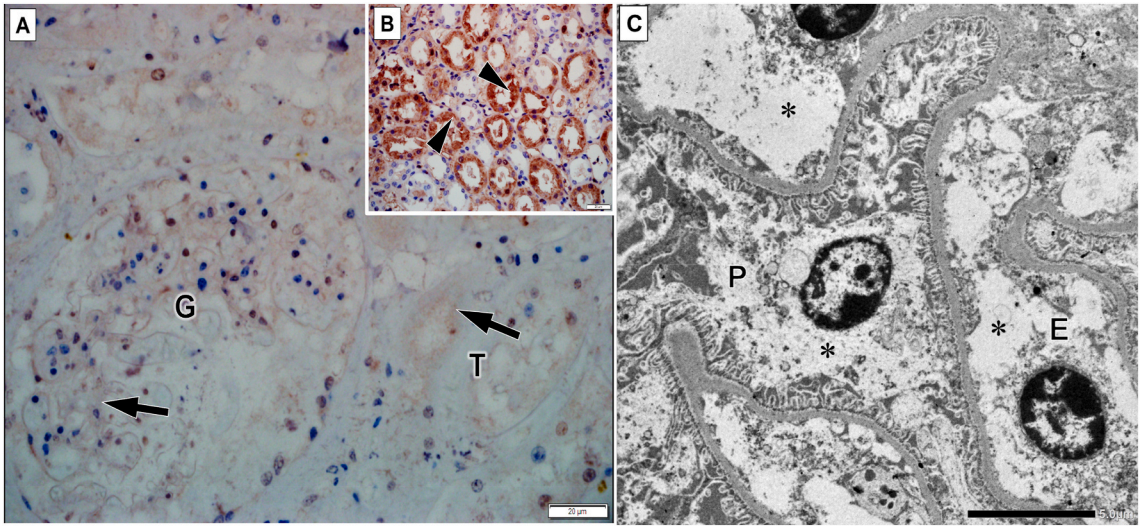

Fig. 5. Immunohistochemical myoglobin labeling (rabbit polyclonal anti-myoglobin antibody [ab187506], Abcam). A. Kidney of a bull that died following ingestion of *Indigofera cryptantha*. Weak, non-specific myoglobin labeling (black arrows); bar = 50 μm. B. Rhinoceros kidney (pathologic-anatomical diagnosis of capture myopathy - positive control). Myoglobinuric nephrosis with intense labeling of myoglobin (black arrowheads); bar = 20 μm. C. Micrograph of a glomerulus. Swollen capillary endothelial cell (E) and podocyte (P) with few or no cytoplasmic organelles (asterisks); bar = 5 μm.

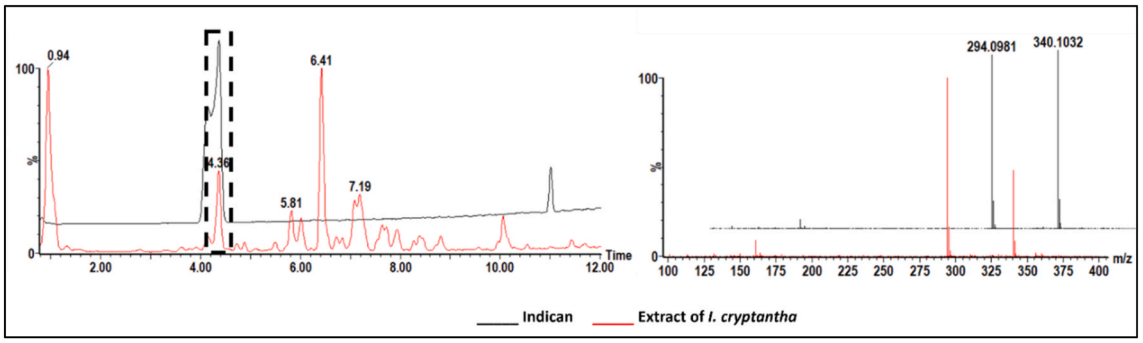

Fig. 6. Chromatograms (L) and mass spectra (R) overlays of indican analytical standard (black) and *Indigofera cryptantha* leaf extract (red). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

et al. (2009).

In the stems and leaves, the peak tentatively identified as indigotin (Table 1) was found to be small relative to the peak detected for indican (1), and the relevant peak was not detected in the extract from the plant pods.

Evaluation of the detected monoisotopic masses, fragmentation patterns, and UV absorbance bands confirm the presence of indoxyl sulfate (5) in the bovine urine samples analysed (Fig. 7, Table 1).

Table 1
Summarised results from the qualitative UPLC analysis of samples of interest.

Compounds	Molecular formula	R_t[a] (min)	λ_{max}[a] (nm)	Observed *m/z*[a] (mass error [ppm])	Observed Quasi-molecular ion	Observed fragments	Samples			
							S	L	P	U
Indican[a]	$C_{14}H_{17}NO_6$	4.4	224, 280	340.1031 (−0.1)	$[M\text{-}H + FA]^-$	131.0368, 161.0448, 294.0977	+	+	+	−
Indigotin[b]	$C_{16}H_{10}N_2O_2$	11.4	285, 560	263.0815 (−2.3)	$[M+H]^+$	199.0858, 219.0899	+	+	−	−
Aniline	C_6H_7N	1.5	234, 280	94.0663 (6.4)	$[M+H]^+$	65.0407, 77.0400	−	−	−	−
Indoxyl sulfate[c]	$C_8H_7NO_4S$	3.7	277	212.0009 (−4.2)	$[M\text{-}H]^-$	132.0439, 79.9554, 77.0378	−	−	−	+

S: stems, L: leaves, P: pods, and U: urine; + detected in the sample, - not detected in the sample.
Indican, aniline and indoxyl sulfate were confirmed through comparison to an analytical standard (tolerance in mass difference = 5 ppm).
[a] R_t: Retention time, λ_{max}: UV absorbance, *m/z*: mass-to-charge ratio.
References: [a]Mohn et al., 2009, [b]Sadler, 1956, [c]Yagil, 1967; Ebbel et al., 2010

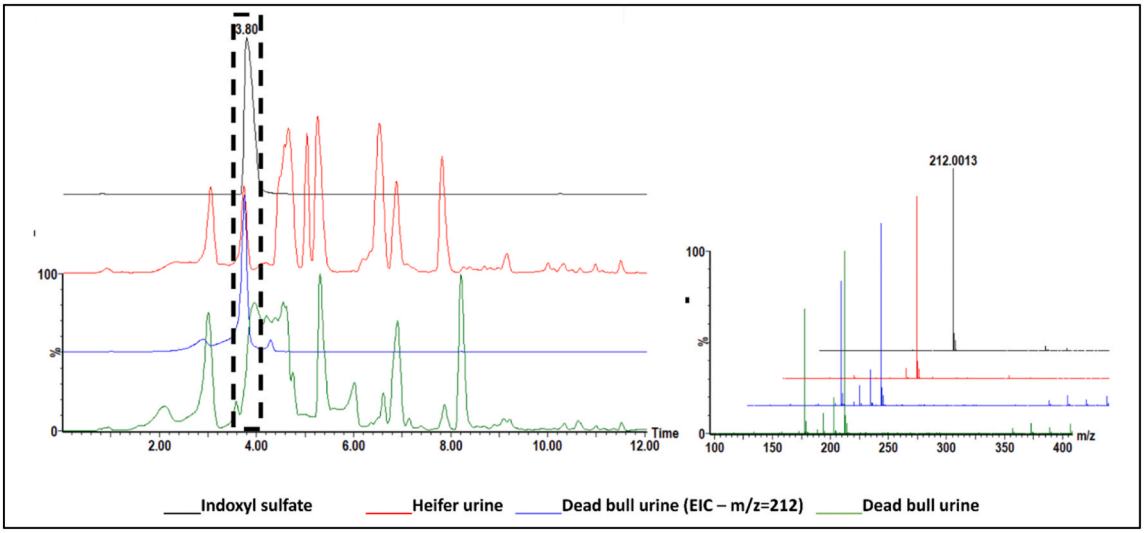

Fig. 7. Chromatograms (L) and mass spectra (R) overlays of indoxyl sulfate analytical standard (black) and urine samples from affected cattle (green, blue, and red). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Aniline was not detected in either the plant samples, nor in the bovine urine samples.

4. Discussion

Blood parasites, as well as other differential diagnoses causing hemoglobinuria in South Africa, were ruled out, and myoglobin as the cause of the pigmenturia was excluded with immunohistochemical staining of the kidney (Fig. 5A and B). Further investigation was prompted by the evidence that *I. cryptantha* was heavily grazed by the cattle during both outbreaks and taking the Brazilian reports of *I. suffruticosa* poisoning in cattle (Barbosa Neto et al., 2001; Salvador et al., 2010) into consideration. Good rainfall was reported during the summer months, and *I. cryptantha* grew abundantly. The outbreaks occurred in late summer and early autumn, and *Cenchrus ciliaris* (blue buffalo grass) is known to become more fibrous and less palatable late in its growing season (Van Oudtshoorn, 1991), which might have encouraged higher intake of the leguminous *I. cryptantha* by the cattle.

Qualitative UPLC-UV/qTOF/MS analysis of the plant parts (stems, leaves and pods) confirmed the presence of indican (1) (Fig. 6, Table 1), through a comparison of retention time, UV absorbance and mono-isotopic mass with an analysed analytical standard of indican. Indigotin (4) was tentatively identified in the stems and leaves, but not in the pods (Table 1). A search on the Dictionary of Natural Products (CHEMnetBASE, 2022), for compounds in *Indigofera* spp. that fall within the monoisotopic mass range around the experimental monoisotopic masses detected for indigotin (4), indicated the detected analyte may be one of three indigo isomers. These are indigotin (4), isoindigotin, and indirubin. A more in-depth analysis of the extract will be required to positively identify the analyte as one of the three compounds. Although not conclusively confirmed with an analytical standard, a comparison of the UV absorbance to a study conducted by Sadler (1956), does provide compelling evidence of its identity.

Salvador et al. (2011) surmised that aniline might be the cause of the intravascular hemolysis. In light of this suggestion, the extracts from *I. cryptantha* were scrutinised for the presence of aniline. However, no peaks conforming to the characteristics of the analysed aniline standard, were detected in any of the extract samples (Table 1). This could be attributed to the stability of indigo and the relatively harsh conditions required to convert it to aniline (Cordin et al., 2021). Thus, the cause of the intravascular hemolysis with resultant hemoglobinuria remains unclear.

Particularly noticeable in the current outbreaks was the intense dark red, to almost black urine which suggested, besides hemoglobin, the presence of another pigment in the urine. Barbosa Neto et al. (2001) reported that during experimental reproduction of *I. suffruticosa* poisoning in 6 cattle, the urine of the cattle initially had a bluish-green color, before the hemoglobinuria was noticed. In an attempt to reproduce the hemolytic anaemia and hemoglobinuria observed in cattle poisoned with *I. suffruticosa*, Salvador et al. (2011) dosed groups of guinea-pigs daily, for 2–15 days, with 10 g fresh plant material per kg body weight. Anaemia occurred, but without hemoglobinuria. Interestingly, 8–10 h after urination, they noticed that the urine was discolored and had a turquoise-blue tinge.

Vanholder and de Smet (1999) reported that in humans, indole (derived from intestinal tryptophan) is metabolized in the liver to indoxyl sulfate (5) (Fig. 8) and is excreted in urine. Qualitative UPLC analysis confirmed the presence of indoxyl sulfate (5) in the urine samples of the affected cattle (Fig. 7), and importantly, was not detected in the plant samples (Table 1). In addition to the fragmentation pattern and UV absorbances matching that described by Yagil (1967) and Ebbel et al. (2010), the use of the analytical standard provided conclusive evidence for its presence in bovine urine, while not being detected in the plant samples. Indigotin (4), itself, could, however, not be detected in any of the urine samples. A possible explanation for the dark reddish-black urine observed in the cattle is that following ingestion of the *I. cryptantha* plant material, indican (indoxyl-β-glucoside) (1) will be hydrolysed in the liver to indoxyl (2) and conjugated with sulfate. Indoxyl sulfate (5) will then be excreted in relatively high concentrations in the urine. In the alkaline urine, two indoxyl molecules might dimerize to form leucoindigo (3) with further oxidation to indigotin (indigo) (4), thus, contributing to the dark pigmentation of the urine. In elderly, hospitalized human patients with long-term indwelling urinary catheters, a so-called 'purple urine bag syndrome' (PUBS), although rare, is reported (Abe et al., 2022). However, it was concluded that certain bacterial and/or fungal enzymes oxidize indoxyl sulfate (5) to blue indigo and red indirubin pigments, producing purple crystals on the surface of the urine collection bag (Abe et al., 2022). Thus, it is also possible that bacterial breakdown of indoxyl sulfate (5) to indigo (4) in the urine occurred, which could also explain the delayed turquoise-blue discoloration of the guinea-pig urine that Salvador et al. (2011) reported.

In the field outbreak in Brazil, only one cow died and the rest recovered uneventfully (Salvador et al., 2010). In the current outbreak, only one animal died as well. In both cases, where mortality was recorded, the cattle remained on the *Indigofera*-infested pasture and were not removed immediately.

Histopathological evaluation of the tissues of one animal that was sacrificed, after being force-fed *I. suffruticosa* collected in north-eastern Brazil, revealed coagulative necrosis of hepatocytes and nephrosis. Considerable filtrate and/or hemoglobin were noticed in the Bowman spaces, in the tubular lumens and also in the cytoplasm of the renal epithelial cells (Barbosa Neto et al., 2001). Salvador et al. (2010) also reported midzonal and occasional centrilobular necrosis of hepatocytes and tubular necrosis with hemoglobin deposits in the renal epithelial cells as well as hemoglobin casts in the tubules in a cow that died of *I. suffruticosa* poisoning. In the bull that died during the current outbreak, liver lesions, save for mild sinusoidal leukostasis, were absent. However, the kidney lesions were more conspicuous, with severe pigment nephrosis. The cortical tubules contained highly eosinophilic granular pigment casts in the lumen and many epithelial cells contained granular cytoplasm (Fig. 4A and B).

Hemoglobin will be filtered by the glomerulus, but renal excretion of indoxyl sulfate (5) is via tubular secretion mediated by organic anionic transporters (OAT), located at the basolateral membrane, in rats specifically rOAT3 (Deguchi et al., 2002). In human patients with chronic renal failure, indoxyl sulfate markedly accumulates in the tubular cells having hOAT1 and/or hOAT3 (Taki et al., 2006). Indoxyl sulfate plays a significant role in the progression of nephrotoxicity and chronic renal failure in humans (Niwa et al., 1999; Deguchi et al., 2002). The accumulated indoxyl sulfate in the renal tubular cells can induce renal damage by generating free radicals (Taki et al., 2006), phenotypic epithelial-to-mesenchymal transition (EMT) and/or apoptosis (Kim et al., 2012). Hence, it is possible that indoxyl sulfate contributed to the renal failure and death of the bull.

5. Conclusion

In conclusion, the available literature on *Indigofera*-induced hemoglobinuria in Brazil, as well as the qualitative analysis confirming the presence of indican (1) in the plant parts and indoxyl sulfate (5) in the urine of affected cattle, as well as the microscopical evaluation of the kidney, confirming severe pigment nephrosis, support a diagnosis of *Indigofera cryptantha*-induced pigmenturia in cattle in South Africa. This is the first report of *Indigofera*-induced pigmenturia in cattle in South Africa.

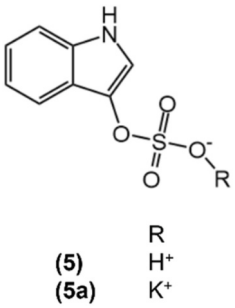

Fig. 8. Structures of indoxyl sulfate (5) and its potassium salt (5a).

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Ethical statement

The manuscript entitled "***Indigofera cryptantha*-induced pigmenturia in cattle in South Africa**" did not require approval from research and/or animal ethics committees, as the samples were collected during routine field investigations for diagnostic purposes. All the authors have approved the manuscript and agreed that it should be submitted to **Toxicon**. My co-authors and I declare no conflicts of interest. The authors also give the assurance that no part of this work is being considered for publication by another journal.

I declare that the present study was performed according to the international, national, and institutional rules considering biodiversity rights.

CRediT authorship contribution statement

Christo J. Botha: Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Magdaleen Vosser:** Writing – review & editing, Investigation. **Mohammed I.A. Ibrahim:** Writing – review & editing, Visualization, Methodology, Formal analysis. **Elizabeth du Plessis:** Writing – review & editing, Visualization, Methodology, Investigation. **Antoinette V. Lensink:** Writing – review & editing, Visualization, Methodology, Investigation. **Wiehan J. Rudolph:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation. **Luke Invernizzi:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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