

***In vitro* ruminal digestion and micrographic analysis of the poisonous plant *Wedelia glauca* (Ort.) Hoffm. ex Hicken (Asteraceae)**

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Summary

Poisonous weeds are a serious threat to cattle in countries where animals are fed on natural as well as planted pastures. These may cause slight to severe lesions to different organs and, ultimately, death depending on the toxic principle and amount of ingested plant material. *Wedelia glauca* (Ort.) Hoffm. ex Hicken (Asteraceae) is a perennial plant from South America present in Southern Brazil, Central Argentina and Uruguay. It is considered a poisonous weed, and its toxicity is due to the presence of an hepatotoxic terpenoid known as atractyloside, which is a potent inhibitor of mitochondria respiration and synthesis of ATP. Although intoxications caused by poisonous plants are frequent in Argentina, diagnosis is sometimes difficult. Signs and macroscopic as well as microscopic lesions are not often clear, constituting a challenge to veterinarians. Some authors have stated that fragments of poisonous weeds - leaves, in particular - can be found in the ruminal content of dead animals. The purpose of this study is to present the results of the micrographic analysis of *W. glauca* leaves submitted to artificial ruminal digestion using the miniature artificial rumen. Despite the artificial digestion process, the epidermal structures that are considered important for botanical identification of the weed were easily found in all the samples. The micrographic analysis of the ruminal content of animals is a useful tool that, together with hematology, necropsy and histopathology findings, will help veterinarians to determine the responsible poisonous species.

Digestión ruminal *in vitro* y análisis micrográfico de la planta tóxica *Wedelia glauca* (Ort.) Hoffm. ex Hicken (Asteraceae)

Resumen

Las malezas tóxicas constituyen una amenaza en países donde el ganado es alimentado con pasturas naturales e implantadas. Esas plantas pueden causar de daños leves a graves en diferentes órganos y, en ciertos casos, finalmente provocar la muerte según el principio tóxico y la cantidad de material vegetal ingerido. *Wedelia glauca* (Ort.) Hoffm. ex Hicken (Asteraceae) es una planta perenne de América del Sur, presente en el sur de Brasil, la región central de la Argentina, y Uruguay. Es considerada tóxica debido a la presencia de un terpenoide hepatotóxico conocido como atractilósido, que es un potente inhibidor de la respiración mitocondrial y de la síntesis de ATP. A pesar de que las intoxicaciones por vegetales tóxicos son frecuentes

Key words: *Wedelia glauca* - toxicity - atractyloside - cattle - micrographic analysis.

Palabras clave: *Wedelia glauca* - toxicidad - atractilósido - ganado - análisis micrográfico.

en la Argentina, el diagnóstico a veces resulta dificultoso. Los signos y las lesiones macro y microscópicas no siempre son claras, y constituyen un desafío para los veterinarios. Algunos autores han establecido que los fragmentos de malezas tóxicas –particularmente las hojas– pueden hallarse en el contenido ruminal de animales muertos. El propósito de este trabajo es presentar los resultados del análisis micrográfico de las hojas de *W. glauca* sometidas a digestión ruminal *in vitro* utilizando el rumen artificial en microescala. A pesar del proceso de digestión artificial, las estructuras epidérmicas, que son consideradas importantes para la identificación botánica de la maleza, fueron fácilmente reconocidas en todas las muestras. El análisis micrográfico del contenido ruminal de los animales constituye una herramienta importante que, conjuntamente con los estudios hematológicos y los hallazgos de necropsias e histopatología, ayudarán al veterinario a determinar la especie tóxica responsable.

Introduction

Poisonous weeds are a serious threat to cattle in countries where animals are fed on natural as well as planted pastures. These may cause slight to severe lesions to different organs and, ultimately, death depending on the toxic principle and amount of ingested plant material (Tokarnia *et al.*, 1979). Although weeds generally have bad taste and odor they are consumed in situations of extreme hunger, particularly during winter time when forage is scarce due to the lack of rains (Zeinsteger *et al.*, 2009).

Wedelia glauca (Ort.) Hoffm. ex Hicken belongs to the Asteraceae family. It is a perennial plant from South America present in Southern Brazil, Central Argentina and Uruguay. It is 0.3-0.8 meters high, with opposite, simple lance-shaped leaves that usually have 2-3 basal teeth. Its yellow and daisy-like flowers, blossom during spring time (Figure 1). Fructification takes place during summer and early autumn. In Argentina this weed is also known as “yuyo sapo” or “sunchillo” (Gallo, 1987).

The toxicity of the plant is due to the presence of an hepatotoxic terpenoid known as atractyloside, which is a potent inhibitor of mitochondria respiration and synthesis of ATP (Lemaster and Sowers, 1979). In particular, atractyloside inhibits the ADP/ATP carrier through the organelle membrane altering oxidative phosphorylation by blocking translocation of adenine dinucleotide. As a consequence, there is an initial alteration of the intrahepatic blood circulation with necrosis of hepatocytes and periacinar hemorrhage. Such effects are characteristic of acute hepatotoxic compounds (Santos *et al.*, 2008).

Cattle, sheep and horses are susceptible to “sunchillo” intoxication which is generally acute and develops within 2-46 hours. Signs include depression, muscular fasciculations, and increment of respiratory and cardiac frequencies, opistotonous, sternal and lateral recumbency, tonic-clonic seizures and terminal paddling movements (Collazo and Riet-Correa, 1996). Animals might be either depressed or aggressive as a result of the encephalopathy due to the severe liver damage. Increments in serum AST (Aspartate aminotransferase), LDH (Lactate dehydrogenase) and GGT (Gamma-glutamyl transpeptidase) levels can be detected early.

Macroscopically the liver is swollen and dark reddish, and the wall of the gall bladder edematous. A yellowish fluid is usually found in thoracic as well as abdominal cavities. Petechiae and echymoses are observable on serous membranes. Hemorrhage of

Figure 1.- Flowers and leaves of *Wedelia glauca* (Ort.) Hoffm. ex Hicken, Asteraceae



the duodenum is a common find. Characteristic microscopic lesions in the liver constitute periacinar hemorrhagic necrosis (Collazo and Riet-Correa, 1996).

Although intoxications by poisonous plants are frequent in Argentina, diagnosis is sometimes difficult. Signs and macroscopic as well as microscopic lesions are not often clear, constituting a challenge to veterinarians. Some authors have stated that fragments of poisonous weeds - leaves, in particular - can be found in the ruminal content of dead animals (Yagueddu *et al.*, 1998). Despite the mechanic and enzymatic activities of the ruminal fluid, no important modifications are observable on the dermal structures such as trichomes and stomata as well as crystals of calcium oxalate for some species (Zeinsteger *et al.*, 2004; Zeinsteger *et al.*, 2009). The latter allows the identification of these structures by light microscopy, a method also known as micrographic analysis.

The purpose of this study is to present the results of the micrographic analysis of *Wedelia glauca* leaves submitted to artificial ruminal digestion using the miniature artificial rumen. Data will contribute to the identification of fragments of the poisonous plant in the ruminal content of animals suspected to die due to its ingestion. The use of micrographic analysis as a complementary diagnostic aid in Veterinary Medicine is emphasized.

Materials and methods

Plant material

W. glauca (Ort.) Hoffm. ex Hicken leaves were collected from Corrientes (Northeastern Argentina) and Buenos Aires (Argentina) during the flowering period. Voucher specimens were deposited after botanical identification at "Museo de Farmacobotánica Juan A. Dominguez, Facultad de Farmacia y Bioquímica", Universidad de Buenos Aires, Argentina. Zeinsteger, s.n.; Corrientes, Pcia. Corrientes (BAF 20251); Gurni, s.n., C.A. Buenos Aires (BA 20052); Bodenbender, s.n. Córdoba (BAF 3328), Caro 260, Córdoba.

In vitro ruminal digestion

Miniature artificial rumen technique was used, previously described by Huhtanen *et al.*, 1954, with modifications. It consisted essentially of a 10 cm dialysis membrane (Spectra/Por, Spectrum Medical Industries, Inc., California) tied at one end with a firm knot to form a tubular sac to hold 10 ml of rumen fluid (from a fistulized animal) and 2 g of plant material, and suspended in a screw-cap jar containing 100 ml of a solution similar in mineral composition to sheep saliva (McDougall's artificial saliva). The sac was held in place by screwing the cap onto the jar, and secured by catching the open end between the rim of the jar and the lid in such a way that about 1 cm of the sac remained outside of the jar after the lid was screwed in place (Figure 2 A).

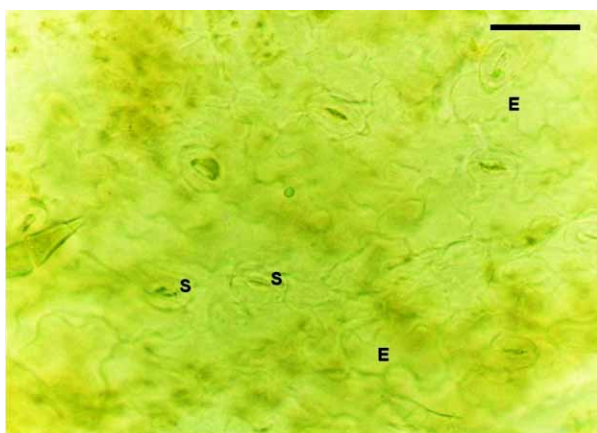
Prior to artificial digestion, CO₂ was bubbled vigorously into the artificial saliva for about 10 minutes, or until pH was 6.6 to 7.0. The whole artificial rumen was then placed at 38 °C in an incubator (Dubnoff Metabolic Shaking Incubator, Precision Lab) for 24 h. After 3 hours incubation, gas pressure was released by unscrewing the lid of the jar. The sac was once again placed between the lid and the top of the jar, the cap screwed on firmly, and the whole apparatus was shaken to remix the inoculums and substrate (Figure 2 B). After fermentation, the sac was removed from the miniature artificial rumen and digested plant material was washed with distilled water and then filtered. All the assays were performed 3 times for both controls and digested samples.

Figure 2.- *In vitro* ruminal digestion device



A: prior to digestion. B: after digestion. Gas product of fermentation is observable at the top of the tubular sac.

Figure 3.- Epidermal cells of *Wedelia glauca* control



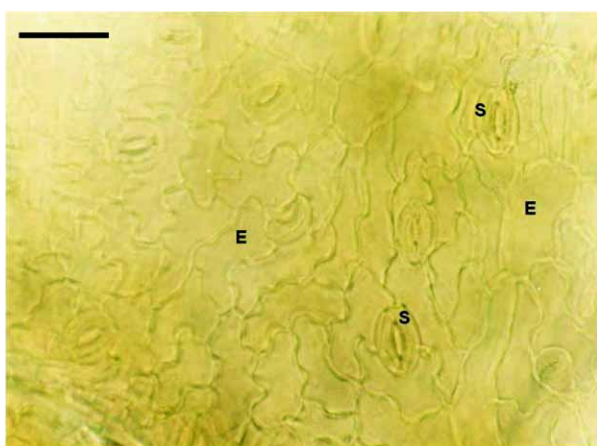
Epidermal cells (E) and stomata (S) of *W. glauca*. Control. Treatment with NaOH 5% at 100 °C. Scale: 50 µm.

Figure 5.- Tector trichome of *Wedelia glauca* control



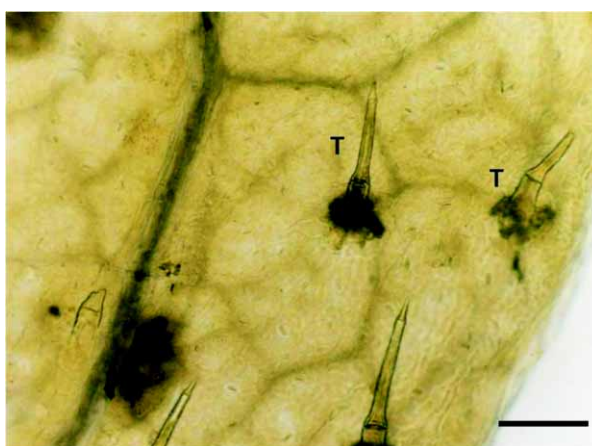
Treatment with NaOH 5% at 100 °C. Scale: 50 µm.

Figure 4.- Epidermal cells of *Wedelia glauca*



Epidermal cells (E) and stomata (S) of digested leaves of *W. glauca*. Treatment with NaOH 5% at 100 °C. Scale: 50 µm.

Figure 6.- Tector trichomes of *Wedelia galuca*



Tector trichomes (T). *In vitro* ruminal digestion. Treatment with NaOH 5% at 100°C. Scale: 200 µm.

Micrographic analysis

Digested plant material was placed in a 30 ml glass beaker and 10 ml 5% NaOH solution was added and heated for about 5 minutes. The sample was filtered and washed 4-6 times with distilled water and later mounted on a slide with coverslip. Pictures were obtained using a Carl Zeiss Axiolab MC 80DX microscope (Norma IRAM N° 37500, 1993).

Results

Despite the artificial digestion process, the epidermal structures that are considered important for botanical identification of *W. glauca*, were easily found in all the samples. Epidermal cells are polyedric, elongated with rounded edges in controls (Figure 3) and digested (Figure 4). In number of 3 to 4, there are surrounding stomata which are anomocytic and

Figure 7.- Epidermal cells in a rosette of *Wedelia glauca*

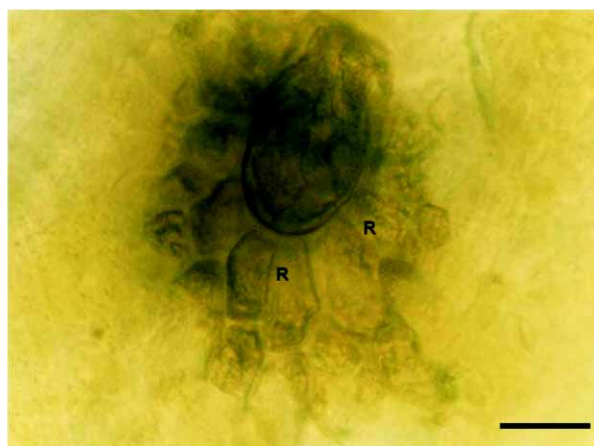
Epidermal cells (R) in a rosette. Control. Treatment with NaOH 5% at 100 °C. Scale: 50 µm.

anisocytic in controls (Figure 3) and in vitro digestion (Figure 4). One type of tector hair is predominating, it has a large body cell and ends in a small triangular one (Figure 5, controls; Figure 6, digested). At the base, a rosette-like structure appears (Figure 7, controls; Figure 8, digested). Some varieties of *W. glauca* may have ornamented tector trichomes, but this characteristic was not observed in this study.

Discussion

Ruminal digestion consists of mechanic as well as enzymatic processes used by animals to break down plant materials to obtain metabolically compounds, such as volatile fatty acids used for energetic purposes (Cronjé, 2000). Biological association between animal and microorganisms in the rumen is a symbiosis: ruminant gives bacteria and protozoa, a fermentation vat with favorable conditions where microorganisms degrade vegetal carbohydrates, lipids and proteins that will ultimately form part of the analogues in the ruminant (Ortiz-Rubio *et al.*, 2006).

Both natural and planted pastures are scarce during winter due to the lack of rains. On the other hand, some poisonous species may remain green throughout this season. Weeds usually have bad taste and odor, or they can be astringent, coriaceous or hirsute, these being defense mechanisms against herbivores (Bruneton, 2001). Despite these botanical

Figure 8.- Epidermal cells in a rosette of *Wedelia glauca*

Epidermal cells (R), rosette. In vitro ruminal digestion. Treatment with NaOH 5% at 100°C. Scale: 50 µm.

characteristics, extreme hunger makes animals feed on them with different consequences to their health.

As many countries with mild to sub-tropical climate, Argentina has good environmental conditions for the extensive beef production system. This is particularly important for the central area of the country, known as “The Pampas”, as well as for the Northeastern region. Almost 45 million heads are grazed in both regions (PNUD, 2009). Poisonous weeds are common there as well, and considering the geographical extension their presence is a constant concern for farmers. Epidemiology of intoxications for the country is scarce, although fatal cases are reported every year.

In general, ingestion of poisonous weeds may affect quite a high number of animals. Occasionally, only one animal is intoxicated, this being an accidental consequence of the presence of a poisonous species contaminating the alfalfa bale, for example. In many cases, signs are not always clear enough to diagnose poisonous plant ingestion and even the complementary diagnostic methods available to veterinarians may not give reliable results.

Micrographic analysis is an easy method used for many purposes such as botanic identification of plant material, and regarding animal biology for the determination of nutritional behavior in wild and domesticated animals (Alipayo *et al.*, 1992; Morrison *et al.*, 2008). For the latter, feces are analyzed under the microscope for the observation of plant microscopic structures in order to establish

identity of species (Van Lieverloo *et al.*, 2009). It is remarkable to mention that the digestive process does not modify the characteristics of trichomes, stomata and even epidermal cells. Regarding plants that have oxalate crystals, the pH of the rumen does not dissolve them (Zeinsteger, 2010).

The artificial digestion of *W. glauca* leaves did not modify the microscopic structures useful for its identification. Tector as well as glandular hairs are the most important structures that were observed without complications. Stomata and epidermal cells are not confirmative structures by themselves but they corroborate the botanical identification when first trichomes are considered. Regarding the reliability of the *in vitro* assay when compared to natural digestion, it has to be mentioned that a previous work has demonstrated that *Baccharis coridifolia* DC leaves digested *in vivo* were also identifiable without important modifications of their histological structures (Zeinsteger *et al.*, 2004).

The best diagnosis is the one performed by means of clinical examination with the implementation of as many complementary diagnostic methods as possible. The micrographic analysis of the ruminal content of animals is a useful tool that, together with hematology, necropsy and histopathology findings will help veterinarians to determine the responsible poisonous species.

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