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First Report on Rotenoids as Neurotoxic Principles of Seeds from *Aeschynomene indica* (Leguminosae)

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Abstract: Toxic principles from seeds of *Aeschynomene indica* collected in Brazil were analyzed. Dalpanol, 12 α-hydroxydalpanol and 11-hydroxydalpanol were identified using ^{1}H NMR in *A. indica* for the first time. 11-hydroxydalpanol has not been previously reported in the existing literature. Furthermore these rotenoids are likely the toxic principles that cause neurological signs in mice.

Key words: Poisonous plants, neurotoxicants, dalpanol, 12 α-hydroxydalpanol, 11-hydroxydalpanol, mice

INTRODUCTION

The invasive plant known as *Aeschynomene indica* (Leguminosae Fabaceae) occurs in India, Malaysia, Australia, the Pacific Islands, Africa, the southern of United States and southern Brazil where it is prevalent in the state of Rio Grande do Sul in wetlands often as a weed within rice paddies. The seeds of this plant are harvested with the rice and during rice processing contaminate a by-product consisting of broken rice used for animal feeds including swine feed.

Two reports of spontaneous outbreaks of poisoning have been observed from ingestion of diets containing 13 and 40% of *A. indica* seeds contaminating the broken rice in swine rations in the state of Rio Grande do Sul, Brazil (Riet-Correa *et al*., 2003; Oliveira *et al*., 2004). Clinical signs in pigs included variable degrees of uncoordinated gait, sternal recumbence, difficulty in rising and lateral recumbence followed by death. Histopathological alterations were symmetric focal degeneration in the cerebellar and vestibular nuclei. Feeding seeds of *A. indica* to swine it was observed clinical signs similar to those observed in spontaneous cases (Riet-Correa *et al*., 2003; Oliveira *et al*., 2004). To the best of the knowledge, there are no studies in the literature relating toxic compounds in *A. indica* seeds to this disease (intoxication).

Bioassay guided fractionation of *A. indica* in swine and mice demonstrated that the ethanol extract and its ethyl acetate fraction of seeds of *A. indica* were lethal to swine and mice when administered orally (Haraguchi *et al*., 2003). Thus, the main objectives of this study were to identify the toxic substance(s) of the ethanol extract and its ethyl acetate fraction of *A. indica* seeds by continued bioassay guided studies in mice.

MATERIALS AND METHODS

Preparation of extracts from *A. indica* seeds: *A. indica* seeds were obtained from a rice processing company in Pelotas, Rio Grande do Sul, Brazil. A voucher specimen was identified as Brazil, Rio Grande do Sul, Claudio Timm, N° 17598 (PEL). The ground seeds were exhaustively extracted first with hexane and then followed by 96% ethanol. After extraction, the solvent was removed by rotary evaporation under reduced pressure yielding hexane and ethanol extracts. The ethanol extract when suspended in 10 mL of 80% ethanol yielded a white solid residue; this residue was filtrated and identified as starch by the Molish test. The filtrate after concentration under reduced pressure, yielded an ethanol extract free of white solid residue (EE). The EE was further fractionated by partitioning the EE fraction between water and ethyl acetate to obtain after evaporation of solvents an Ethyl Acetate Residue (EAR) and an aqueous residue.

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Phytochemical analysis: The EAR was applied to a silica gel 60 (70-230 mesh, Merck) chromatographic column and eluted sequentially under reduced pressure with ethyl acetate, a mixture of 5, 10 and 50% methanol/ethyl acetate and finally methanol to obtain 5 fractions. Each fraction was evaporated under reduced pressure until dryness. The 5% methanol/ethyl acetate fraction (5% MEAF) was applied to a silica gel 60 (70-230 mesh, Merck) chromatographic column and eluted with mixtures of methanol and ethyl acetate in increasing order of polarity. After evaporation, each fraction was monitored by Thin Layer Chromatography (TLC) employing a plastic plate impregnated with silica gel 60G F254 (Merck) and developed with ethyl acetate, methanol and water (100:13.5:10). The TLC spots were visualized by UV and by treatment of the plates with an alcohol solution of 10% iron chloride and 10% sulphuric acid on a hot plate for 10 min. Fractions that were similar were combined, yielding 8 subfractions. Further separation by reversed phase HPLC employing a semipreparative C-18 chromatographic column and an acetonitrile: water (1:1) mobile phase was made only in subfractions that showed increased residue on a TLC plate sprayed with 10% alcohol iron chloride. Detection was accomplished using a UV-vis detector at λ = 300 nm and the mobile phase flow rate was 7 mL min^{-1}. Structural identifications were accomplished using Nuclear Magnetic Resonance (NMR) and Infrared (IR) spectroscopic analyses.

Animals: About 60 days male Swiss mice bred in the Department of Pathology at the Faculty of Veterinary Medicine and Animal Sciences. All experimental manipulations were approved by the animal care and use committee of the FMVZ-USP.

Phytotoxicity evaluation: Mice were randomly separated into seven groups including a control group with three mice per group. The groups were dosed the following fractions by single oral gavage: Ethanol Extract (EE), Ethyl Acetate Residue (EAR), Ethyl Acetate Fraction (EAF), 5% Methanol/Ethyl Acetate Fraction (MEAF), 10% MEAF, 4th subfraction from MEAF at the doses of 0.9, 0.45, 0.2, 0.15, 0.1 and 0.2 g kg^{-1}, respectively. All samples were suspended with Tween 80 and water. The control group received a mixture of Tween 80 and water as vehicle. After administration, the animals were observed at 1, 2, 4 and 8 until 30 h and the behavioral changes and lethality in comparison with control mice were recorded. After 30 h, the surviving mice were sacrificed by decapitation and the brain collected and fixed in neutral buffered formalin for histopathology. Histopathologic analyses were performed on sections (5 μm thick) that had been stained with hematoxylin and eosin.

RESULTS

Phytochemical analysis: The 4th subfraction showed increased concentrations of residue with three dark spots when visualized on a TLC plate sprayed with 10% alcohol iron chloride indicating constituent aromatics at R_f 0.43, 0.36 and 0.33. The spraying with 10% sulphur solutions followed by heating showed additional spots with minor intensity.

Therefore, the 4th subfraction was further separated by reversed phase HPLC employing a semipreparative C-18 chromatographic column and an acetonitrile: water (1:1) mobile phase. Detection was accomplished using a UV-vis detector at λ = 300 nm and the mobile phase flow rate was 7 mL min^{-1}. Three principal substances (1-3) (Fig. 1) were obtained.

Structural identifications were accomplished using NMR and IR spectroscopic analyses. Proton signals in 6.30-7.80 ppm region and carbon 13 signals in the 100-130 ppm region indicated aromatic groups. Proton signals in the 3.00-5.00 ppm region and carbon 13 signals in the 73.00-79.00 ppm region indicate carbonyl groups. Proton signals in the 3.60-3.80 ppm region and carbon 13 signals in the 55.00-57.00 region indicate methoxy groups. Proton signals in the and 1.20-1.40 ppm region and carbon 13 signals in the 20-27 ppm region indicate of methyl groups among other signals (Table 1 and 2). The infrared spectra showed an absorption band at 1676 cm^{-1} indicating the presence of carbonyl groups linked with aromatic group. In comparison with predicted spectral data the compounds were identified as rotenoids: dalpanol (1), 12 α-hydroxydalpanol (2) and 11-hydroxydalpanol (3) (Fig. 1). Compounds 1 and 2 were isolated previously from *Amorpha fructinosa* (Li *et al.*, 1993). However, compound 3 has not been previously reported in the existing literature.

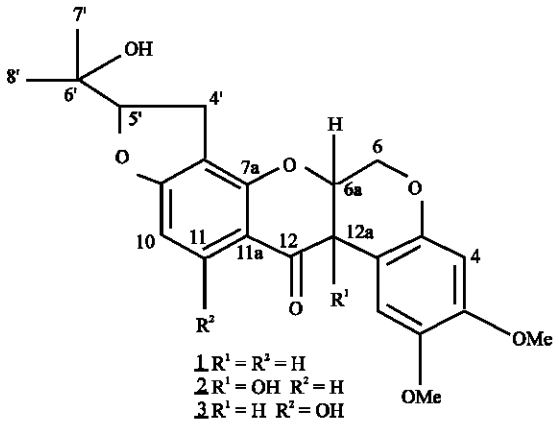

Fig. 1: Structures of the rotenoids from *A. Indica*

Table 1: ^{1}H NMR data for rotenoids from *Aeschynomene indica* seeds (ppm, $CDCl_3$, J in Hz, 500 MHz)

H	1	2	3
1	6.71, s	6.48, s	6.78, s
4	6.39, s	6.43, s	6.38, s
6ax	4.12, d, 12.0	4.41, d, 12.0	4.11, d, 12.0
6eq	4.55, dd, 3.0, 12.0	4.51, dd, 2.0,12.0	4.55, dd, 3.0, 12.0
6a	4.86, dd, 3.0, 4.2	4.52, d, 2.0	4.80, dd, 3.0, 4.0
10	6.43, d, 8.5	6.38, d, 8.5	5.92, s
11	7.76, d, 8.5	7.74, d, 8.5	
12a	3.78, d, 4.2		3.78, d, 4.0
OH		4.40, s	
OMe-2	3.70, s	3.65, s	3.71, s
OMe-3	3.75, s	3.70, s	3.74, s
4'	3.06, d, 9.0	3.02, d, 9.5	2.90, d, 10.0
5'	4.61, t, 9.0	4.60, t, 9.5	4.57, t, 10.0
7'	1.29, s	1.26, s	1.26, s
8'	1.16, s	1.14, s	1.14, s

Table 2: ^{13}C NMR data for rotenoids from *Aeschynomene indica* seeds (ppm, $CDCl_3$, 75.4 MHz)

C	1	2	3
1	110.6	109.5	110.4
1a	104.8	105.0	105.0
2	144.0	144.0	144.8
3	149.6	151.2	150.0
4	101.0	101.1	101.1
4a	147.5	148.5	147.0
6	66.3	63.6	66.0
6a	72.3	76.0	71.7
7a	157.9	157.5	
8	113.6	114.3	101.1
9	167.2		166.0
10	104.8	105.2	91.8
11	129.8	129.9	
11a	113.5	112.0	
12	189.0		190.0
12a	44.7	67.6	43.7
2-OMe	56.4	56.5	56.4
3-OMe	55.9	55.9	55.9
4'	27.4	27.3	26.7
5'	91.5	91.6	91.0
6'	71.7	71.7	71.7
7'	26.2	26.1	26.1
8'	24.0	24.1	24.0

Rotenoids 1, 2 and 3 described for the first time in *Aeschynomene indica* in this study are likely the toxic principles that cause neurological signs in mice.

Phytotocicity evaluation: The toxicities of the extracts were determined using mouse bioassay. The treatment with Ethanol Extract (EE) at 0.9 g kg^{-1} in a single dose caused sternal recumbence, uncoordinated gait and hypothermia in all treated mice 1 h after administration. At 2 h after administration one mouse presented convulsion followed by death whereas other one died 3 h after treatment without signs of convulsion. Finally, the last mouse treated with EE was sacrificed 30 h after treatment.

The administration of Ethyl Acetate Residue (EAR) induced similar signs to those caused by EE. However, EAR caused death of only one mouse 5 h after its administration without signs of convulsion. Among the fractions, the groups that received Ethyl Acetate Fraction (EAF), 5% Methanol/Ethyl Acetate Fraction (MEAF) and 10% MEAF showed that these fractions were toxic to mice but did not induce death however. On the other hand, the 4th subfraction obtained from 5% MEAF as previously described when administered by gavage, provoked clinical signs such as hypothermia and uncoordinated gait in all treated mice at 3 and 30 h after its administration. Moreover, this subfraction induced death of two mice without signs of convulsion at 9 and 29 h after treatment. Finally, the last mouse treated with 4th subfraction from MEAF was sacrificed at 30 h of onset treatment. Histological evaluations of samples of brain recovered from treated mice revealed no differences from the samples obtained from control mice (data not shown).

DISCUSSION

After isolation, the primary compounds in 4th subfraction mentioned above were identified as rotenoids 1, 2 and 3. Rotenone, a substance isolated from plants, is an insecticide being toxic to fish (Cheng and Farrell, 2007). Although, it is safe for most mammals, rotenone is toxic to swine. In fact, previous studies administering this substance to pigs revealed that these animals presented neurological signs such as incoordination which progresses from staggering to paralysis of all limbs, respiratory depression and coma with rapid death and absence of pathological alterations (Oliver and Roe, 1957; Manahan, 2003). These signs were similar to those observed in Brazil in pigs fed with broken rice contaminated with *A. indica* seeds (Oliveira *et al*., 2005), further indicating that the toxicity of the seeds is due to rotenoids. Therefore, it is reasonable to hypothesize that dalpanol, 12 α-hydroxydalpanol and 11-hydroxydalpanol found in seeds from *A. indica* in the present study are the toxic principles responsible for the neurotoxic effects observed in mice and pigs.

CONCLUSION

In this study, these results showed for the first time that the neurotoxic effects of *A. indica* seeds in mice are caused by rotenoids.

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