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Article

Study of acute toxicity and investigation of the presence of β-*N*-methylamino-L-alanine in the *Gunnera manicata* L. a species native to Southern Brazil

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Gunnera (Gunneraceae) forms a complex association with the cyanobacterium *Nostoc puctiforme* L. *Gunnera-Nostoc* symbiosis is the only one reported involving a flowering plant, and results in the formation of the neurotoxic amino acid β-*N*-methylamino-L-alanine (BMAA). The species *Gunnera manicata L*., for which phytochemical, pharmacological and toxicological studies are lacking, is found in Southern Brazil. Therefore, acute toxicity and the presence of neurotoxic amino acid were investigated in aqueous extracts of G. *manicata*. The acute toxicity test was conducted by administering aqueous root extract of *G. manicata* at a concentration of 2000 mg/kg in a single dose orally to Wistar rats. Lethality was monitored daily for 14 days after treatment. The relative mass of organs was analyzed by one-way ANOVA and macroscopic changes were investigated. The analysis of BMAA, a procedure performed by GC/MS, involved a preliminary derivatization step. The ESI-MS/MS analysis was done by direct infusion. The present study demonstrated absence of neurotoxin in the samples of *G. manicata* analyzed and absence of acute toxicity in aqueous root extracts. These data confirm that extracts from the roots of *G. manicata* have a high margin of drug safety.

Uniterms: *Gunnera manicata* L./toxicity. β-*N*-methylamino-L-alanine/identification. Neurotoxins/identification. Acute toxicity.

Gunnera (Gunneraceae) forma uma complexa associação com a cianobactéria *Nostoc puctiforme* L. A simbiose *Gunnera-Nostoc* é a única relatada envolvendo uma angiosperma e, em decorrência desta, ocorre a formação da neurotoxina β-*N*-metilamino-L-alanina (BMAA). No sul do Brasil, encontra-se a espécie *G. manicata* L., da qual não constam, na literatura científica, estudos fitoquímicos, farmacológicos e toxicológicos. Assim, o presente estudo avaliou a toxicidade aguda e a presença da neurotoxina BMAA em extratos aquosos de *G. manicata*. O ensaio de toxicidade aguda foi realizado com extrato aquoso das raízes de *G. manicata* na concentração de 2000 mg/kg, administrado em dose única via oral em ratos Wistar. Letalidade foi observada diariamente durante 14 dias pós-tratamento. Após a eutanásia, a massa relativa dos órgãos foi analisada por ANOVA de uma via e investigou-se a presença de alterações macroscópicas. A análise do BMAA por CG/EM envolveu uma etapa preliminar de derivatização, já a análise por ESI-EM/EM foi realizada por infusão direta. O presente estudo demonstrou a ausência da neurotoxina nas amostras de *G. manicata* analisadas bem como a ausência de toxicidade aguda no extrato aquoso das raízes. Esses dados demonstram alta margem de segurança dos extratos testados.

Unitermos: *Gunnera manicata* L./toxicidade. β-*N*-metilamino-L-alanina/identificação. Neurotoxina/identificação. Toxicidade aguda.

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INTRODUCTION

Gunnera (Gunneraceae) comprises more than forty species occurring mainly in the Southern Hemisphere (Wanntorp, 2003). Some cyanobacteria are involved in symbioses with hosts such as fungi (lichens), bryophytes, cycads, mosses, ferns (*Azolla*) and *Gunnera*. The most common cyanobacteria found in symbioses with plants belong to the filamentous heterocystous genus *Nostoc* (Nilsson, Bergman, Rasmussen, 2000). *Gunnera-Nostoc* symbiosis is unique in being the only angiosperm genus involved in symbiotic associations with the nitrogen-fixing cyanobacterium *Nostoc puctiforme* L.. This symbiosis is possibly a relic of an earlier partnership which evolved during a period when there was a limited supply of inorganic forms of nitrogen (Osborne *et al.,*1992).

Motile filaments of cyanobacteria, known as hormogonia, colonize *Gunnera* plants through cells in the plant's specialized stem glands. In an *in vitro* experiment, optimized conditions were determined for establishing *Nostoc*-*Gunnera* symbiosis by inoculating mature glands with hormogonia from *Nostoc punctiforme*. *Gunnera manicata* L. plants were grown in nitrogen-replete medium and nitrogen status was found to be the main determinant of gland development. In contrast to uninoculated plants, *G. manicata* plants colonized by *N. punctiforme* were able to continue growing in N-limited medium (Chiu *et al.,* 2005).

Beta-N-methylamino-L-alanine (BMAA), a non protein amino acid, was found to be produced by cyanobacterial root symbionts of the genus *Nostoc* (Cox *et al.,* 2005). The naturally occurring, non-essential amino acid BMAA has recently been found in high concentrations in brain tissue of patients with tauopathies such as the Amyotrophic Lateral Sclerosis-Parkinsonism-Dementia Complex (ALS/PDC) in the South Pacific island of Guam and in a small number of North American Caucasian patients with sporadic Alzheimer's disease. BMAA is produced by cyanobacteria present in all conceivable aquatic and/or terrestrial ecosystems and may be accumulated in living tissues in free and protein bound forms through the process of biomagnification (Papapetropoulos, 2006). Once ingested, BMAA can be bound by proteins within the body, resulting in a slow release of free BMAA over years as contaminated proteins are metabolized. The demonstration, by enzymatic cleavage, that BMAA is incorporated within the actual amino acid sequence of the protein would lend weight to this hypothesis. BMAA was discovered in the brain tissues of nine Canadian Alzheimer's patients (Cox *et al.* 2005) and in neurotoxic foods, including cycad flour, flying foxes and other feral or wild animals that are important components of the traditional Chamorro diet (Banack, Murch, Cox, 2006). In addition, it was also found in cyanobacterial-plant symbiosis such as *Gunnera kauaienisis* (Cox *et al.,* 2005). Nilsson and coworkers (2000) found 45 cyanobacteria isolated from 11 different *Gunnera* species originating from different geographical areas. The most common cyanobacteria found in symbioses with plants belong to the filamentous heterocystous genus *Nostoc* (Nilsson, Bergman, Rasmussen, 2000).

The *G. manicata* species, on which there is scant scientific data from phytochemical, pharmacological or toxicological studies, is found in Southern Brazil, mainly in coastal regions. Considering that *G. manicata* plants were able to be colonized by *N. punctiforme* and that the formation of the BMAA neurotoxin can result from this symbiosis, the present study investigated the presence of BMAA and acute toxicity in aqueous extracts of the genus *G. manicata*, native to Southern Brazil.

MATERIALS AND METHODS

Chemicals and reagents

BMAA (CAS 15920-93-1) was purchased from Sigma Chemical Co. (St. Louis, USA). Water was purified using a Milli-Q system (Millipore, Bedford, USA). Pyridine and acetonitrile (HPLC grade) were obtained from Merck (Darmstadt, Germany), ethanol from Proquimios (Rio de Janeiro, RJ, Brazil), dichloromethane from Cromato (Diadema, SP, Brazil) and ethyl-chloroformate from Sigma Chemical Co. (St. Louis, USA).

Plant material

Roots of *G. manicata* were obtained from the Aparados da Serra region, Southern Brazil, and identified by Rodrigo B. Singer. A voucher specimen (R.B. Singer s. n., Serra da Rocinha, January 15th, 2009) has been deposited at the ICN herbarium - Departamento de Botânica - UFRGS in Porto Alegre, Brazil.

Preparation of extracts

The aqueous extract was made from the dried roots or leaves extracted in a water bath at 50 °C for 3 hours. The extract obtained was collected, filtered and then concentrated to dryness under reduced pressure. The resulting residue was subsequently dissolved in water for analysis.

Animals

Twelve male and twelve female Wistar rats weigh-

ing 191.16 ± 12.92 g and 222.5 ± 22.05 g, respectively and obtained from the State Foundation of Production and Research in Health (FEPPS) were used. The animals were housed in 47x34x18 cm polyethylene cages (6 animals per cage) under standard conditions of temperature (22 ± 2 ºC), controlled humidity and 12 h-light/dark cycle. All animals were fasted overnight but were provided with water *ad libitum*. Experimental procedures were performed during the light phase of the cycle. The experiments were performed after approval of the protocol by the FEPPS Research Ethics Committee (number CEP/FEPPS-04/2008) and were carried out in accordance with current guidelines for the care of laboratory animals (Olfert, Cross, McWilliam, 1998).

Acute toxicity

The experiments were conducted by dividing the rodents into groups of six rats each, all of which were treated orally (by gavage) with water (control) or 2000 mg/kg of *G. manicata* aqueous root extract. The 2000 mg/kg dose was chosen based on previous experiments by our research group, showing signs of low or non-toxicity. All treatments were administered at a constant volume of 10 mL/kg. The animals were observed after dosing for 1 min and at 5, 15, 30 min and 1, 2, 3, 4, 5, 6 h for the presence of respiratory, digestive and neurological alterations. The specific signs monitored were alterations in locomotor activity, stimulus reaction, piloerection, salivation, gasping, ptosis, tearing, tremors, writhing, and convulsions. The number of deaths and weight were noted every 24 h for 14 days. After the 14th day, surviving rats were euthanized, necropsied and analyzed for macroscopic alterations in heart, liver, kidneys, adrenals, lungs, brain and spleen. The weight of the organs was determined and expressed as relative weight (organ mass/ body weight x 100). In acute toxicological studies, 6 animals per group were used, and only at the high dose, in order to minimize the biological variability and to minimize the number of animals used in the study, considering the ethical aspects (Brasil, 2004; Cazarin, Corrêa, Zambrone, 2004).

Derivatization and extraction procedures

Standard of BMAA derivatives were prepared by treating of 250 µL of BMAA solution (10 ng/mL) with a mixture of 100 µL ethanol and 80 µL pyridine (65:25:10 by volume). A 15 µL aliquot of ethyl-chloroformate was added and the mixture was shaken vigorously for about 30 s until the evolution of CO_2 gas was complete. The derivatives were extracted with 100 µL of dichloromethane, with vigorous shaking. The mixture solution was centrifuged at 3500 rpm for 10 min. The extraction was performed twice. The organic layer was dried under N_2 gas and reconstituted by adding 100 µL of dichloromethane. A 1 µL aliquot of extract was injected into the GC injection port running in a solvent vent mode (Guo *et al.,* 2007). The same treatment was received for aqueous extracts of roots and leaves which were analyzed by gas chromatography–mass spectrometry (GC-MS). Both were analyzed with or without spiking standard BMAA.

Another extraction procedure was tested to investigate the presence of BMAA in roots and leaves. Acid hydrolysis was accomplished using 6 M HCl as the extraction medium, and by incubating for 17h at 110 ºC to release any protein-bound BMAA. Excess HCl was evaporated and the residue dissolved in water (600 µL). After centrifugation the extracts were passed through syringe filters. Ultrafiltration was used when necessary before injecting aliquots directly into the tandem mass spectrometry (ESI-MS/MS). These samples were also derivatized and analyzed by the GC-MS system. All of these samples were analyzed with or without spiking standard BMAA.

Determination of BMAA by GC-MS

The samples were analyzed by GC/MS using a quadrupole MS system (Shimadzu CLASS17A-QP5000) operating at 70 eV. GC analysis was performed using a fused silica capillary column (30 cm x 0.25 x /0.25 mm, coated with DB-5). Injector and detector temperatures were set at 270 °C; the oven temperature was kept at 60 °C for 3 min and then ramped to 280 °C at a rate of 20 °C/min. The final temperature of 280 °C was reached in 5 min. Helium was employed as the carrier gas (1 m:/min). This methodology was adapted from Guo and co-workers (2007). Under these conditions, the retention time of the BMAA derivative was 12.45 min, as depicted by the chromatogram in Figure 1.

Determination of BMAA by ESI-MS/MS - Mass spectrometric condition

The analysis was done by direct infusion into a Sciex API 5000 triple stage quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA). The solvent used in the infusion syringe was a mixed of 0.1% formic acid and acetonitrile (50:50 v/v). The flow rate of the syringe was 10 µL min^{-1}. The ions were monitored full scan in positive mode, where nitrogen was used for both nebulizer and collision gas. The fragmentation profile was obtained from selection of a pattern ion and monitored in MS2 mode. Fragments were observed by tuning MS1 for m/z 119 and then scanning MS2 for product ions. The optimal collision

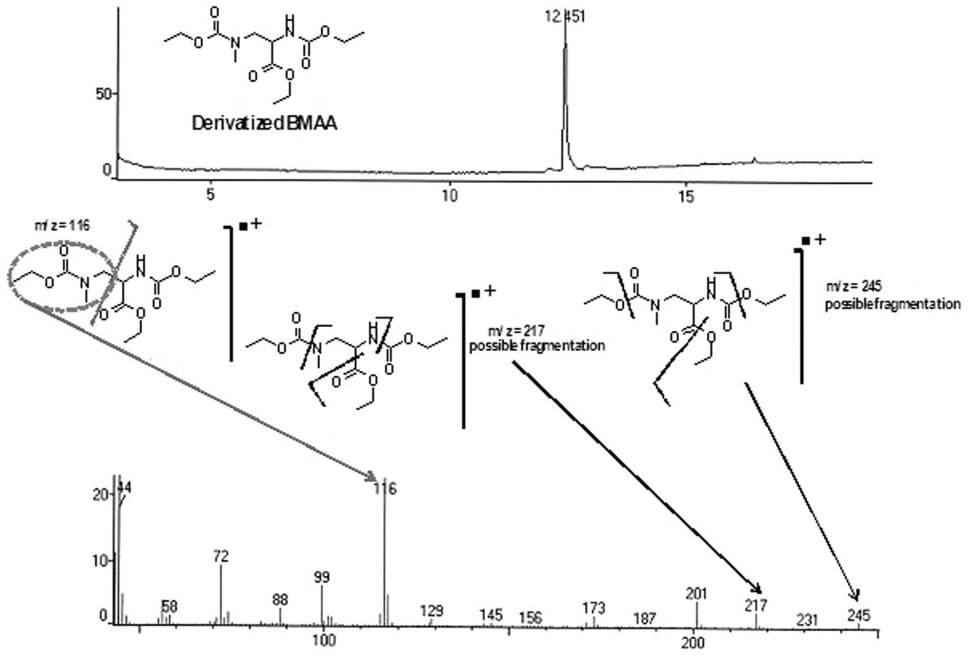

FIGURE 1 - a) Chromatogram. b) EI spectra of ethyl chloroformate derivatization of BMAA.

energy was 10 eV. For reliable identification, five specific product ions (m/z 102, 88, 76, 73 and 44), all derived from a precursor ion of m/z 119 and originating from different parts of the molecule, were detected (Rosén, Hellenäs, 2008).

Statistical analysis

All the results were expressed as mean ± SEM (standard error of mean). Statistical analysis for body weight gain was evaluated by repeated measures analysis of variance (ANOVA) and relative organ weight was analysed by one-way ANOVA using SPSS 10.0 software. The significance level was set at $p < 0.05$.

RESULTS

Rats administered *G. manicata* roots extract developed no clinical signs of toxicity either immediately or during the post-treatment period with 2000 mg/kg. In the same observation period, no deaths occurred. Similarly, no macroscopic changes in organs and no statistically significant changes were observed in the assessment of the relative mass of organs of necropsied animals.

The analytical investigation of the aqueous root and leaf extracts, as well as extracts made by acid hydrolysis, showed no chromatographic profile or mass spectrum characteristic of the standard of ethyl chloroformate derivatization of BMAA. Similarly, on ESI-MS/MS analysis in both extraction procedures, no neurotoxin was observed. After several analyses of different samples, neurotoxin was considered absent in *G. manicata*.

DISCUSSION

G. manicata is a popular ornamental plant native to Southern Brazil. Despite its ornamental use, no toxicity studies were found in scientific literature. After an extensive review we observed that this species can be infected by *Nostoc puctiforme* L. (Chiu *et al.,* 2005) and as a result of this symbiosis the beta-*N*-methylamino-L-alanine (BMAA) can be produced. This is a non protein amino acid recently associated with Amyotrophic Lateral Sclerosis-Parkinsonism-Dementia Complex (ALS/PDC) in the South Pacific Island of Guam. Therefore, the aim of this study was to elucidate acute toxicity and investigate the presence of BMAA. The present investigation shows that the aqueous extract of the roots of *G. manicata* is non-toxic by the oral route in rats, at least up to maximum doses of 2000 mg/kg body weight.

For the chemical analysis by GC/MS, chloroformates derivatizing with simpler alkyls were used, with ethyl known for years as one of the favorable reagents for treating amino groups in gas chromatography. They have been revealed randomly as exceptionally rapid esterification agents (Husek, 1998). The chemical pretreatment of the sample was aimed at removing the active hydrogen atoms from all, or at least most, of the reactive groups. It is desirable that it be carried out as quickly as possible with

a minimum number of reagents and reaction steps. Since BMAA is a non-protein aminoacid, ethyl chloroformate was chosen for derivation procedures for GC/MS analysis.

To eliminate any doubt regarding the presence or absence of BMAA, it was confirmed by ESI-MS/MS analysis, focusing on the absence of this non-protein amino acid in the samples investigated.

It should also be stressed that *Nostoc* symbiosis is widespread amongst *Gunnera* species (Wanntorp, Wanntorp, 2003). *Nostoc* aggregates are clearly visible in transversal sections of the G. *manicata* stem, as greenish dots or areas (R.B. Singer, pers. obs). Even the tiny *G. herteri* (a small, annual species which is apparently the sister-group of all remaining *Gunnera* spp.) (Rutishauer, Wanntrop, Pfeifer, 2004) displays the anatomic stem channels that allow the *Nostoc* "infection". The absence of toxic compounds in *G. manicata* strongly suggests that the presence of symbiotic *Nostoc* colonies does not per se enable the secretion of the aforementioned toxic compounds. This situation suggests that other factors (e.g. ecological factors, phylogenetic biases etc.) may be involved. Sampling *G. manicata* stems in different regions may help to elucidate whether the presence/absence of toxic compounds is ecologically biased or not. On the other hand, a dense sampling within the *Gunnera* species may allow elucidation of whether or not the presence of toxic compounds is phylogenetically biased (e.g. toxic compounds are found or lacking in groups of phylogenetically-related species).

In conclusion, the present study demonstrated the absence of the neurotoxin BMAA in samples of *G. manicata* native to Southern Brazil. The absence of acute toxicity in aqueous root extracts of *G. manicata* under the conditions used in the experiment suggests that *G. manicata* may be safer for pharmacological use because samples without BMAA do not induce neurological or systemic injuries. These results demonstrate that the extracts of the roots of *G. manicata* have a high margin of drug safety. Further phytochemical and biological studies are underway in order to prospect bioactive compounds or plant extracts.

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REFERENCES

BANACK, S. A.; MURCH, S. J.; COX, P. A. Neurotoxic flying foxes as dietary items for the Chamorro people, Marianas Island. *J. Ethnopharmacol.*, v.106, n.1, p.97-104, 2006.

BRASIL. Ministério da Saúde. Agência Nacional de Vigilância Sanitária. RE nº90. Guia para a realização de estudos de toxicidade pré-clínica de fitoterápicos. *Diário Oficial da União*, n.53, Brasília, DF, 18 de março de 2004. Seção1, p.34.

CAZARIN, K. C. C.; CORRÊA. C. L.; ZAMBRONE, F. A. D. Redução, refinamento e substituição do uso de animais em estudos toxicológicos: uma abordagem atual. *Rev. Bras. Ciênc. Farmac*., v.40, n.3, p.289-299, 2004.

CHIU, W. L., PETERS, G. A.; LEVIEILLE, G.; STILL, P. C.; COUSINS, S.; OSBORNE, B.; ELHAI, J. Nitrogen Deprivation Stimulates Symbiotic Gland Development in *Gunnera manicata*. *Plant Physiol.*, v.139, n.1, p.224-230, 2005.

COX, P. A.; BANACK, S. A.; MURCH, S. J.;RASMUSSEN, U.; TIEN, G.; BIDIGARE, R. R.; METCALF, J. S.; MORRISON, L. F.; CODD, G. A.; BERGMAN, B. Diverse taxa of cyanobacteria produce β-N-methylamino-L-alanine, a neurotoxic amino acid. *Proc. Nat. Acad. Sci. USA.*, v.102, n.14, p.5074-5078, 2005.

GUO, T.; GEIS, S.; HEDMAN, C.; ARNDT, M.; KRICK, W.; SONZOGNI, W. Characterization of ethyl chloroformate derivative of beta-methylamino-l-alanine. *J. Am. Soc. Mass Spectrom.*, v.18, n.5, p.817-825, 2007.

HUSEK, P. Chloroformates in gas chromatography as general purpose derivatizing agents. *J. Chromatogr. B*, v.717, n.1-2, p.57-91, 1998.

NILSSON, M.; BERGMAN, B.; RASMUSSEN, U. Cyanobacterial diversity in geographically related and distant host plants of the genus *Gunnera*. *Arch. Microbiol.,* v.173, n.2, p.97-102, 2000.

OLFERT, E.D., CROSS, B.M., MCWILLIAM, A. *Manual sobre el cuidado y uso de los animales de experimentación.* 2.ed. Ontario: Canadian Council on Animal Care, 1998. v.1, p.211.

OSBORNE, B. A.; CULLEN, A.; JONES, P. W.; CAMPBELL, G. J. Use of nitrogen by the Nostoc-Gunnera tinctoria (Molina) Mirbel symbiosis. *New Phytol.*, v.120, n.4, p.481-487, 1992.

PAPAPETROPOULOS, S. Is there a role for naturally occurring cyanobacterial toxins in neurodegeneration? The beta-N-methylamino-L-alanine (BMAA) paradigm. *Neurochem. Int.*, v.50, n.7-8, p.998-1003, 2007.

ROSÉN, J.; HELLENÄS, K. E. Determination of the neurotoxin BMAA (b-N-methylamino-L-alanine) in cycad seed and cyanobacteria by LC-MS/MS (liquid chromatography tandem mass spectrometry), *Analyst*., v.133, n.12, p.1785-1789, 2008.

RUTISHAUSER, R.; WANNTORP, L.; PFEIFER, E. Gunnera herteri – developmental morphology of a dwarf from Uruguay and Brazil (Gunneraceae). *Plant Syst. Evol.*, v.248, n.1-4, p.219-241, 2004.

WANNTORP, L.; WANNTORP, H. E. The biogeography of *Gunnera* L.: vicariance and dispersal. *J. Biogeogr.*, v.30, n.7, p. 979-987, 2003.

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