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Research Article

Dimeric Phloroglucinol Derivatives but Not Benzopyrans from *Hypericum* Species Have No Toxicity on Cerebellar Primary Neurons

Betti A H[1,2*], Stolz E D[1], Barros F M C[1], Müller L G[1], Von Poser G L, Rego J-L[3], Rego J-C[4], Vaudry D[2], Rates S M K[1]

[1]*Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal do Rio Grande do Sul. Av. Ipiranga, 2752. Porto Alegre – RS. CEP 90610-000, Brazil.*
[2]*Equipe Facteurs Neurotrophiques et Différenciation Neuronale, INSERM U982 (Institut National de la Santé et de la Recherche Médicale, Unité 982), Université de Rouen, France*
[3]*Institut de Recherche et d'Innovation Biomédicale (IRIB), Université de Rouen, France*
[4]*Service Commun d'Analyse Comportementale (SCAC), Institut de Recherche et d'Innovation Biomédicale (IRIB), Faculté de Médecine et Pharmacie, Université de Rouen, France*

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ABSTRACT

Hypericum polyanthemum, H. caprifoliatum and *H. myrianthum,* are vegetal species native to southern Brazil and demonstrated antinociceptive and antidepressant-like effects. These species have a strong tendency to accumulate phenolic compounds with the phloroglucinol substitution pattern, such as benzopyrans (HP1, HP2, HP3) and dimeric phloroglucinol derivatives (uliginosin B, hyperbrasilol B, japonicin A). Pre-clinical studies suggest that dimeric phloroglucinols and benzopyrans might constitute promising molecular patterns to develop central nervous system drugs: uliginosin B and hyperbrasilol B displayed antinociceptive and antidepressant-like effects; japonicin A and HP1 showed antinociceptive effect only. However, limited information is available on their neuronal toxicity. The aim of this study was to investigate whether these compounds can be associated with neuronal toxicity by using cerebellum primary cell cultures. Cell cultures of primary cerebellar granule cells were prepared from 7 to 9 days rats. Growing concentrations of HP1, HP2, HP3, uliginosin B, hyperbrasilol B and japonicin A (10^{-10} to 10^{-6} M final concentration) were added directly to cell cultures and neuronal survival was assessed 72 h later. Exposure of cell cultures to HP1, uliginosin B, hyperbrasilol B and japonicin A (10^{-6} and 10^{-10} M) for up to 72hours, did not affect granule cell survival suggesting that these compounds are not neurotoxic, while HP2 (10^{-6} and 10^{-7} M) and HP3 (10^{-7} M) were. This study indicates that uliginosin B, hyperbrasilol B and japonicin A are devoid of neuronal toxicity, which encourages further studies on dimeric phloroglucinol derivatives, aiming at developing new antidepressant and analgesic drugs.

Keywords:

INTRODUCTION

Natural products play an important role in drug discovery, development process and influence the design of new molecules with biological activity, due to the chemical diversity[1,2] however, toxicity studies are generally scarce. The genus *Hypericum* was highlighted in this scenario from studies with *Hypericum perforatum* Linaeus, an European species used to treat mild to moderate depression[3,4] effect attributed to the presence of hypericin (naphthodianthrone) and hyperforin (monomeric phloroglucinol derivative)[3]. Pre-clinical studies also showed that this species had antinociceptive effect[5] as well as it was effective in neuropathic pain[6]. Brazilian *Hypericum* species occur primarily at southern regions. Species found in Rio Grande do Sul, belong to the sections Brathys and Trigynobrathys, the latter with a greater number of native species[7]. Regarding chemical composition, the classes of secondary metabolites that have been commonly isolated from *Hypericum* species of sections Brathys and Trigynobrathys are dimeric phloroglucinols. Differently from *H. perforatum*, which produces monomeric phloroglucinol derivatives such as hyperforin, the native species produce dimeric phloroglucinol derivatives. These compounds were proposed as chemotaxonomic markers for the southern Brazilian species[8]. Other secondary metabolites that have been found in the native species are benzopyrans[9], benzophenones, xanthones, flavonoids, phenolic acids, terpenoids[10-12] and essential oils[13]. Compounds of these classes have been shown to present antibacterial, antifungal, antiviral, antiproliferative, antinociceptive, monoamine oxidase inhibitory and antidepressant activities, among others[14]. The antidepressant-like and antinociceptive effects of *Hypericum* species native to Southern Brazil in rodents were previously demonstrated by our group[15-19]. Of note, Brazilian native species differ from *H. perforatum*, since they do not produce hypericin, and usually exhibit compounds with a dimeric

**Author for Correspondence*

phloroglucinol nucleus and, less frequently, benzopyrans. Among dimeric phloroglucinol derivatives, japonicin A, hyperbrasilol B and uliginosin B were already identified and isolated from *H. myrianthum* Cham. & Schltdl. (Hypericaceae), *H. caprifoliatum* Cham. & Schltdl. (Guttiferae) and *H. polyanthemum* Klotzsch ex Reichardt (Guttiferae)[8,20]. The main benzopyrans are HP1 (6-isobutyryl-5,7-dimethoxy-2,2-dimethyl-benzopyran), HP2 (7-hydroxy-6-isobutyryl-5-methoxy-2,2-dimethyl-benzopyran) and HP3 (5-hydroxy-6-isobutyryl-7-methoxy-2,2-dimethyl-benzopyran)[20-22] found in the aerial parts of *H. polyanthemum*. All chemical structures are represented in Figure 1. The benzopyran HP1 exhibited antinociceptive effect in the hot-plate test, which is mediated by the opioidergic system[15]. The uliginosin B showed antidepressant-like and antinociceptive effects in rodents that seem to be due to its ability to inhibit the neuronal monoamine uptake with consequent activation of the monoamine receptors and indirect stimulation of the opioid system[16,23]. The hyperbrasilol B showed antinociceptive effect in hot-plate test[24] and antidepressant-like effect on the forced swim test, which seems to be dependent of the intracellular sodium balance[25]. The japonicin A showed antinociceptive effect in the hot-plate test[21]. Altogether, these data suggest that dimeric phloroglucinols and benzopyrans might constitute promising molecular patterns to develop central nervous system drugs. However, other studies indicate that these molecules may affect cell survival and/or proliferation. In particular, HP2 and uliginosin B exhibited cytotoxicity against mammalian VERO cells[26]. HP1, HP2 and HP3 displayed a potent growth inhibitory activity against NCI-H460 (lung adenocarcinoma), HT-29 (colon adenocarcinoma) and U-373MG (glioblastoma) human cell lines[13,27]. Japonicin A and uliginosin B exerted antiproliferative effects against OVCAR-3(ovarian carcinoma) human cell line but not against HT-29 and U-251 (glioma)[28]. Nevertheless, a putative neurotoxicity of these drugs candidates has not been reported yet. Therefore, the aim of this study was to investigate whether HP1, HP2, HP3, uliginosin B, hyperbrasilol B and japonicin A exert any neuronal toxicity on an *in vitro* model using the well characterized cerebellar granule neurons primary cell culture.

MATERIALS AND METHODS

Plant material

The aerial parts of *H. polyanthemum, H. caprifoliatum* and *H. myrianthum* were collected in Caçapava do Sul (October and December, 2009), Porto Alegre (October and December, 2009), and Paraíso do Sul (October and December, 2009) Rio Grande do Sul, South Brazil, respectively. The species were identified by Sérgio Bordignon, Ph.D. (UNILASALLE-RS-BRAZIL) and vouchers were deposited in the Herbário do Departamento de Botânica (Instituto de Biociências, Porto Alegre, Brazil) - ICN *H. polyanthemum*: 175915; ICN *H. caprifoliatum*: Bordignon et al. 2287; ICN *H. myrianthum*: Bordignon et al. 3059. The plant collection was authorized by Conselho de Gestão do Patrimônio Genético and Instituto Brasileiro do Meio Ambiente - Number 003/2008, Protocol 02000.001717/2008 – 60.

Extraction and isolation

All organic solvents were analytical grade from F. Maia (Cotia, São Paulo, Brazil). Dried and powdered aerial parts of *H. polyanthemum, H. caprifoliatum* and *H. myrianthum* were extracted with *n*-hexane by maceration (3 × 24 h at 20°C). The extracts were evaporated to dryness under reduced pressure (Rotavapor 8020 Fisatom®, São Paulo, Brazil) and treated with acetone to remove waxes and insoluble impurities. The acetone soluble fractions were submitted to column chromatography on silica gel 60 (Merck®) (70 – 230 Mesh, Merck®, Darmstadt, Germany) using mixtures of *n*-hexane:dichloromethane or hexane:ethyl acetate gradient system as the mobile phase[8,20,29]. Japonicin A was obtained from *H. myrianthum* as a precipitate and, subsequently, purified by repeated washing with *n*-hexane and recrystallization with dichloromethane. Hyperbrasilol B was obtained from *H. caprifoliatum*, and uliginosin B, HP1 (6-isobutyryl-5,7-dimethoxy-2,2-dimethyl-benzopyran), HP2 (7-hydroxy-6-isobutyryl-5-methoxy-2,2-dimethyl-benzopyran) and HP3 (5-hydroxy-6-isobutyryl-7-methoxy-2,2-dimethyl-benzopyran) were obtained from *H. polyanthemum* and purified by preparative TLC performed on 20 cm × 20 cm glass-supported plates covered with 0.5 mm layers of silica gel GF_{254} (Merck®, Darmstadt, Germany) and with *n*-hexane:dichloromethane (1:1 v/v) as eluent. The identity of compounds were confirmed by HPLC and identified by ^{1}H-NMR (60 MHz) spectroscopy (Eft-60®, Anasazi Instruments), and compared with literature data[22]. The separation and purification of the dimeric phloroglucinol derivatives (japonicin A, hyperbrasilol B and uliginosin B) and the benzopyrans (HP1, HP2 and HP3) were carried out according to previous studies of our group[8,9,15,21,22,25,30].

Primary cell culture

The *in vitro* assays were performed in the Equipe Facteurs Neurotrophiques et Différenciation Neuronale, INSERM U982 (Institut National de la Santé et de la Recherche Médicale, Unité 982), Université de Rouen, France. The study was performed according to guidelines of The National Research Ethical Committee (published by National Heath Council - MS, 1998) and Brazilian law (Brazil, 2008), which are in compliance with the International Guiding Principles for Biomedical Research Involving Animals (CIOMS, 1985) and with the European Parliament and of the Council Directive of September 22, 2010 (2010/63/EU) on the protection of animals used for scientific purposes. Cerebellar granule cells were prepared from 7 to 9 days rats, as previously described[31, 32]. Briefly, cells were dissociated from the cerebellum by mechanical chopping and treatment with trypsin and DNase and plated in poly-D-lysine precoated 24-well plates, at a density of 1.2×10^6 cells/mL. Dissociated cells were cultured in basal medium (74% DMEM, 25% HAM's F12, 1% antibiotic, Gibco®), supplemented with 10% fetal bovine serum (Lonza®) and

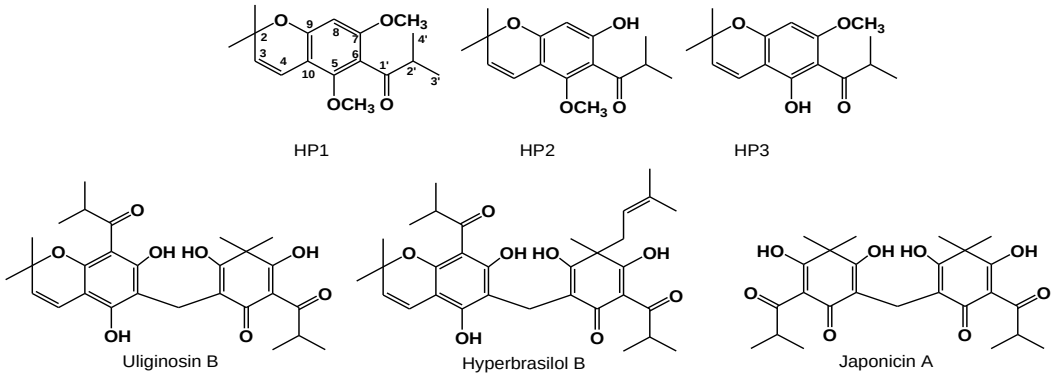

Figure 1: HP1 (A), HP2 (B), HP3 (C), uliginosin B (D), hyperbrasilol B (E) and japonicin A (F) chemical structures.

25 mM KCl. After 24 hours of culture, cytosine arabinoside (10 µM) was added to arrest the growth of non-neuronal cells. The cells were maintained in an incubator at 37ºC and 5% CO_2 for 5 days *in vitro* (DIV) before treatment. It has been well established that these cultures consist of more than 90% neurons after 8 days of culture.

Treatment and evaluation of granule cell survival

For neurotoxicity assay, growing concentrations of HP1, HP2, HP3, uliginosin B, hyperbrasilol B and japonicin A (10^{-10} to 10^{-6} M final concentration) were added directly to DIV cells and neuronal survival was assessed 72 hours later by measuring fluorescein diacetate uptake by living neurons, using a FlexStation3 microplate fluorometer[32]. The putative toxic effect of the tested compounds was also confirmed by visual observations of the cells by fluorescence and transmitted light microscopy. All the compounds were diluted in culture medium from a stock solution at 10 mM preparedin, 100% DMSO. The DMSO maximum final concentration in the culture medium was 0.01%. Each treatment was performed in quadruplicate and repeated 3 times in independent cultures. The plates were treated by direct addition of HP1, HP2, HP3, uliginosin B, hyperbrasilol B and japonicin A dilutions (20 µL) to the medium culture plates.

Statistical Analysis

One-way ANOVA was performed at Sigma Stat 2.03 software (Jandel Scientific Corporation). Differences were considered statistically significant at $P<0.05$.

RESULTS AND DISCUSSION

Hypericum perforatum is widely used to treat mild to moderate depression. Our group has been studying *Hypericum* genus species native to Southern Brazil, different extracts and secondary metabolites, for more than ten years aiming to characterize their *in vivo* antidepressant and antinociceptive properties. However, there is not much information regarding the putative toxicity of species of the genus *Hypericum* as well as their main secondary metabolites: phenolic compounds with the phloroglucinol substitution pattern, such as benzopyrans (HP1, HP2, HP3) and dimeric phloroglucinol derivatives (uliginosin B, hyperbrasilol B, japonicin A). Few studies evaluated the toxicity of the extract. Viana and coworkers[19] demonstrated that *H. caprifoliatum* petroleum ether extract is toxic to rats when acutely administered by intraperitoneal route (270 mg/kg); however, no signs of gross neurotoxicity (piloerection, hypothermia, apnea and prostration) nor neuromuscular impairment were found with petroleum ether and cyclohexane extracts when rats or mice were treated orally (50 - 2000 mg/kg). Betti and coworkers[33] demonstrated that a 28 days of treatment with lipophilic extract of *H. polyathemum* was not toxic to mice at doses used in pre-clinical trials; only doses five (450 mg/kg) or ten-times (900 m/kg) above the dose active *in vivo* showed biochemical and histological changes in the liver. In addition, the safety of the metabolites responsible for the antidepressant and antinociceptive properties[15,16,21,23-25] is still unknown; there is not much information in the literature about the toxicity of these metabolites. Since we have demonstrated an activity over the central nervous system of these compounds, it is crucial to show that they do not have any neuronal toxicity. The study of cell death has become a central issue in biology during the last years, in particular considering the Central Nervous System, where neuronal death is equally important as a physiological mechanism of developmental neuronal elimination[34]. In the present study, we evaluated the effect of several compounds isolated from species of the genus *Hypericum* natives to Southern Brazil on cultured cerebellar primary neurons. The primary cultures of cerebellar granule cells are a well-established and popular model to study almost every aspect of developmental, functional and pathological neurobiology[34]. In particular, these cells have been used *in vitro* to study the neurotoxicity of various compounds. The cerebellum is a prominent structure at the lack of the brain, with more than 90% of its neurons being granule neurons, constituting the largest homogeneous neuronal population in the mammalian brain[34]. In addition, the cerebellar granule cells can be cultured *in vitro* to differentiate into mature neurons, which *in vivo* exhibit an extensive neuritic network[35]. The benzopyran HP1 [$F(5,17)=1.883$, $P=0.1712$] and the dimeric phloroglucinols uliginosin B [$F(5,17)=1.807$, $P=0.1860$], hyperbrasilol B [$F(5,17)=1.619$, $P=0.2288$] and japonicin A [$F(5,17)=1.234$, $P=0.3523$] did not affect cell viability after 72 hours treatment (Fig. 2), when added directly to the culture, indicating that these compounds are not associated with neuronal toxicity under our experimental

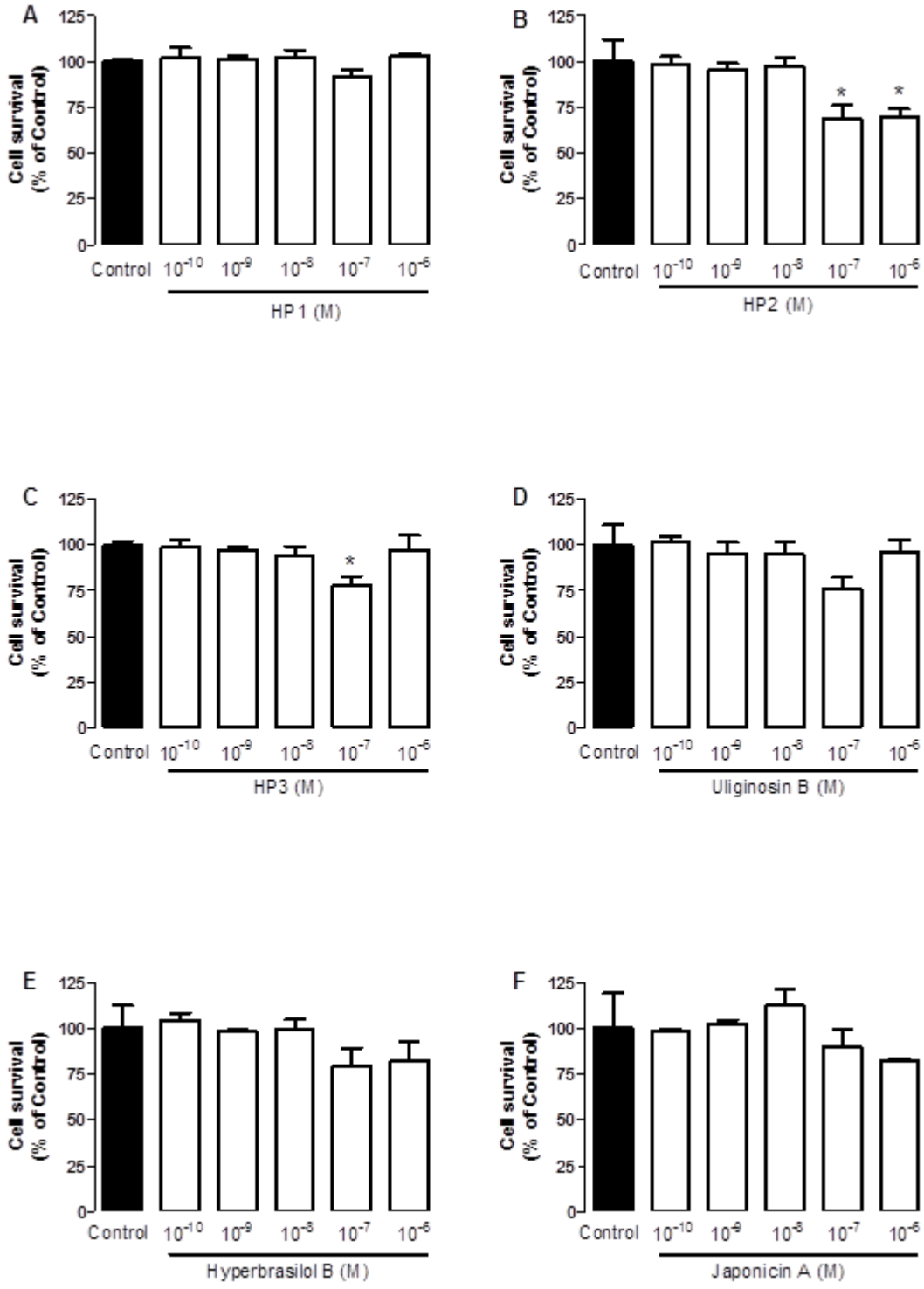

Figure 2: Effect of HP1 (A), HP2 (B), HP3 (C), uliginosin B (D), hyperbrasilol B (E) and japonicin A (F) (10^{-10} to 10^{-6} M) on cerebellar granule cells survival (3 plates, quadruplicate) after 72 h treatment. Data are expressed as mean ± S.E.M. ANOVA *post hoc* Dunnet. Difference from Control **P*<0.05.

conditions. These results are in line with those obtained with hyperforin[36-39], a monomeric phloroglucinol derivative found in *H. perforatum*, showing that in hippocampal cells hyperforin treatment did not induce neuronal toxicity *in vitro* at concentrations up to 10^{-6} M[36,37]. These results suggest that the phloroglucinol moiety is devoid of neurotoxic. Moreover, hyperforin and analogs prevented neurotoxicity induced by amyloid *β*-peptide in an Alzheimer's disease model[36]. The capacity of hyperforin to disaggregate amyloid deposits, decrease aggregation and amyloid formation could explain this effect, as well as the control of increased reactive oxidative species associated with amyloid toxicity[36-39]. It is noteworthy that acylphloroglucinols and other compounds structurally related to benzopyrans and dimeric phloroglucinols present antioxidant activity[40,41].

On the other hand, the results showed in Figure 2 demonstrated that HP2 [$F(5,17)=4.756$, $P=0.0126$] and HP3 [$F(5,17)=3.004$, $P<0.05$] were neurotoxic at the highest tested concentrations (10^{-7} and 10^{-6} M). This cytotoxic effect of HP2 and HP3 was also seen in glial tumoral cell lines[13,27] in addition to the antiproliferative activity observed in other cell lines[26,28]. The different profile of HP2 and HP3 in relation to HP1 could be, at least in part, due to the presence of a hydroxyl group at C7 and C5, respectively (Fig. 1). These hydroxyl groups can form intramolecular hydrogen bonds with the oxygen atom of the acyl moiety, closing a six-member ring[22,42]. Consequently, this suggests a possible conformational change of HP2 and HP3 in comparison to HP1, whose methoxyl groups do not act as hydrogen donators. The present study tested for the first time *Hypericum* isolated compounds over neuronal cells. Since *Hypericum* species have demonstrated Central Nervous System activity, it is important to evaluate the toxicity over neurons, and not just over different cell lines, as can be found in the literature. In conclusion, the data suggest that the dimeric phloroglucinol derivatives uliginosin B, hyperbrasilol B, japonicin A and the benzopyran HP1 present a low neurotoxicity, and encourage the further investigation of these effects *in vivo*, in animal models of acute and chronic neurological diseases.

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