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Neuroprotective effects of *Lepidium meyenii* (Maca)

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The neuroprotective activity of the plant *Lepidium meyenii* (Maca) was studied in two experimental models: *in vitro* and *in vivo*. Crayfish neurons were pretreated with vehicle or the pentane extract from Maca, subjected to H_2O_2, and their viability determined microscopically and chemically. A significant concentration-neuroprotective effect relationship was demonstrated. The pentane extract was then administered intravenously to rats prior to and following middle cerebral artery occlusion. While infarct volumes were decreased for the lower dose, higher doses increased infarct volumes compared to controls. These results suggest a potential application of Maca as a neuroprotectant.

Keywords: Maca; *Lepidium*; crayfish neurons; cerebral ischemia

Introduction

Much interest has focused on the prevention and treatment of neurodegenerative diseases and stroke,[1,2] but challenges relating to the complexity of the central nervous system (CNS) and neuronal death have been difficult to overcome.[3–9] One focus of the search for effective new drugs has been on complementary and alternative medicines. Medicinal plants have the great advantage that they have followed an indirect clinical trial during centuries of traditional use and usually many of the adverse effects and toxicities are known. In addition, they have been an important source of chemical compounds used as drugs or to model the synthesis of new drugs. Naturally occurring compounds also have the advantage of having been developed in living organisms during evolution to protect and improve the organism's survivability. Facing the complexity of neurodegeneration,[9,10] natural based drugs may be good candidates to target oxidative stress, stabilization of membranes, inactivation of enzymes, inhibition of apoptosis, or inhibition of inflammation so as to inhibit or decrease the cellular death and neuronal injury observed in focal ischemic stroke and many neurodegenerative diseases.

The Peruvian plant, *Lepidium meyenii* (Maca), was probably domesticated between the years 4000–1200 BC as indicated by primitive Maca cultivars that have been found in archeological sites in Peru.[11] Knowledge of this plant and its medicinal properties has been transferred from generation to generation and continues today.[12] The root (hypocotyls) of Maca can be baked or eaten after decoction, but typically is dried and stored for years before its consumption.[13] Thereafter, Maca has been traditionally used as a food and an essential folk medicine;[14] its tremendous value has led many Peruvians to regard Maca as a fundamental component of their diet and medicinal treatments.[15] Used to enhance sexual behavior in men and women, fertility, energy, and reduce stress and menopausal symptoms,[13] it is also recommended in traditional medicine as effective in cancer prevention and treatment, anemia, gastritis, high blood pressure, and depression.[12,16] While several studies have already been conducted to examine Maca as a potential treatment in various disorders, pharmacological studies are still needed to determine and better characterize the properties attributed to this plant.

Chemical studies have demonstrated that the hypocotyls of the plant contain various compounds: alkaloids (macaridine, lepidilin A, lepidilin B, lepidine, tetrahydro-β-carbolines, and others),[17–19] glucosinolates (glucotropaeolin, glucoalyssin, glucobrassicanapin, glucobrassicin, and others),[19–21] aromatic isothiocyanates (benzylisothiocyanate, 4-methoxybenzylisothiocyanate, and others),[19]

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steroids (brassicasterol, ergostadienol, and others),[22] benzylated amides (macamides),[18,23–25] and acyclic polyunsaturated oxoacids (macaenes).[19,23] Most likely other compounds will be discovered in the future. The aerial parts of the plant contain essential oils mainly constituted by phenylacetonitrile, benzaldehyde, and 3-methoxyphenylacetonitrile.[26]

The published pharmacological results of Maca investigations provide information about the effect of the whole hypocotyls or its crude extract administered orally, especially regarding the fertility enhancer and pro-aphrodisiac effects in mice,[27,28] rats,[29–37] and humans,[38–40] and the preventive effect on the UV-induced skin damage.[41] Other results demonstrate the antistress,[42] antiosteoporosis,[43] and prostate size reduction effects in mice and rats.[44,45] Recent studies have demonstrated the effect on learning and depression in ovariectomized mice,[46] on memory impairment induced by scopolamine[47] and ovariectomy in mice.[48] Clinical studies have also demonstrated the effect of the dried powdered hypocotyls on serotonin-selective reuptake inhibitor (SSRI)-induced sexual dysfunction[49] and on postmenopausal psychological signs.[50] *In vitro* studies have demonstrated antioxidant and antiapoptotic activity in macrophages,[51] absence of toxicity to rat hepatocytes[52] and also the lack of direct androgenic effects in prostatic cancer cells.[53] One study reported estrogenic effects as increasing proliferation of human breast cancer cells (MCF-7).[52] Most of the studies in animals did not report toxicity,[13] however preliminary toxicological assessments in humans have shown that 90 days consumption of 0.6 g/day of dried hypocotyl powder leads to significant increases in diastolic blood pressure and plasma aminotransferase (AST).[54] Overall, there still is a need for additional pharmacological studies to demonstrate other properties attributed to this plant and answer questions remaining about the efficacy, doses, mechanisms of action, responsible compounds, biopharmaceutical properties, safety, toxicity, adverse effects, and possible clinical applications of the plant, preparations, or its isolated compounds.

The goal of this study was to determine the possible neuroprotective properties of the lipophilic compounds from *Lepidium meyenii* tested *in vitro* in cell culture, and *in vivo* in rats subjected to focal ischemic stroke. The lipophilic nature of some of the constituents of Maca could facilitate their passage through the blood-brain barrier and act within the CNS. Some constituents present in Maca have reported activity in the CNS.[55–57] Standardized extracts could act along with endogenous compounds, or mimic endogenous compounds with demonstrated neuroprotective efficacy, or block neurodegenerative processes.[58] Maca could be a neuroprotective agent alone or act synergistically with other agents to prevent neurodegeneration and/or reduce cell death in stroke and other neurodegenerative diseases.

Materials and methods

Plant material and extract preparations

Dried hypocotyls of *Lepidium meyenii*, procured from Arequipa, Peru, were botanically identified at the faculty of Pharmacy and Biochemistry of the Catholic University of Arequipa, and then were chemically identified at the Massachusetts College of Pharmacy and Health Sciences (MCPHS) and the University of Mississippi. The dried hypocotyls (750 g) were comminuted into a powder and extracted with 3 L of methanol (HPLC grade, Pharmco-AAPER, Brookfield, CT) for a period of 48 h. The methanol extract was then filtered, concentrated in a rotary-evaporator to 1 L and mixed with an equal volume of distilled water, underwent a liquid–liquid continuous re-extraction with n-pentane 98% (Sigma-Aldrich, St. Louis, MO) for 24 h, after which time it was dried in a rotary-evaporator and the residue was kept under refrigeration. The yield of the pentane extract was 0.68%.

In all of the *in vitro* experiments, the pentane fraction was dissolved in 10% DMSO (Sigma-Aldrich) in phosphate-buffered saline (PBS, Mediatech, Manassas, VA). For the *in vivo* study, the pentane extract was redissolved in methanol with six times its weight of poly-vinyl-pirrolidone (PVP) 30 K (GAF Chemicals Corporation, Wayne, NJ), the solution was dried in a rotary evaporator until constant weight and the residue dissolved in sterile water before administration.

Cells and culture media

In this study we used *Orconectes limosus* (crayfish) neuronal cells, OLGA-PH-J/92 (ATCC, Manassas, VA). This cell line exhibits certain transformation

features, such as anchorage independence and loss of contact inhibition. Its stable growth characteristics and morphology from the primary culture throughout higher passages may suggest that the cells are neuronal stem cells (ATCC product description).

The crayfish neuronal cells were cultured in minimum essential medium (MEM, Sigma-Aldrich) supplemented with sodium bicarbonate (2.2 g/L, Sigma-Aldrich), HEPES (9.52 g/L, Sigma-Aldrich), sodium pyruvate (0.11 g/L, Sigma-Aldrich), and 10% fetal bovine serum (ATCC). The pH of the supplemented base media was then adjusted to 6.8.[59]

In vitro neuroprotection assay

The cells were cultured in T-flasks at 27° C until 80% confluent growth. The medium was removed from the flask, a scraper was used to detach the cells, and the cells were suspended in supplemented MEM. The cell suspension underwent total cell counting with trypan blue dye using a hemocytometer to determine the number of viable cells per μL to ensure a seeding density of 1×10^5 cells per well. MEM was subsequently added to yield a total volume of 180 μL per well. Twenty-four h after the cell seeding the wells were pretreated with 20 μL of the pentane extract solutions or vehicle. The final concentrations of pentane extract assayed were 0.1, 0.3, 1, 3, 10, and 30 μg/mL. Five replicates were performed for every assay. Three h later, 20 μL of 0.001 mol/L hydrogen peroxide (H_2O_2, Sigma-Aldrich), as a neurotoxic agent,[60] or vehicle, were added to the wells. Microscopic morphology of the cells was observed and photographed 18 h later.

Cell viability assay for concentration-effect correlation

Cell viability was measured using a colorimetric method for the determination of cells in proliferation, with tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium salt (MTS, Promega, Madison, WI), which is bioreduced by cells into a formazan blue product that is soluble in the cell culture medium. Ten μL of MTS reagent was added to wells 3 h after the H_2O_2 treatment and an absorbance measurement was performed on well plates using a 96-well plate reader at a wavelength of 490 nm, after an incubation period of 24 h. Estimation of the viable cells is possible as the absorbance of the blue color is directly proportional to the number of metabolically active cells. The neuroprotective effect is estimated as the percentage of absorbance increase compared with the absorbance in the wells treated only with H_2O_2 (maximal damage), 0% of neuroprotection. The absorbance in the wells treated only with vehicle (no damage) was considered as 100% of neuroprotection.

Animals

Thirty-six (36) adult male Sprague–Dawley rats (300–350 g) from Charles River Laboratories (Wilmington, MA) were housed under standard conditions of ambient temperature (74 ± 3°F), relative humidity (55 ± 15%), and 12:12 h light:dark cycles, five days before the study and were divided into four groups of nine animals each. Food and water were available *ad libitum*. All experimental animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

Development of focal infarction

Animals were anesthetized with i.p. chloral hydrate (Sigma-Aldrich) 400 mg/kg. The common carotid arteries were isolated via a ventral midline cervical incision and both arteries were eventually ligated for one h. Then, a 1.5 cm scalp incision was made at the midpoint between the right eye and the right ear. The temporalis muscle was separated and retracted to expose the zygoma and squamosal bone. A hole was made with a dental drill 1 mm rostal to the anterior junction of the zygoma and squamosal bone. Bone and dura mater were carefully pierced to expose the middle cerebral artery. The artery was occluded with a bipolar coagulator (Radionics, LabX, Riverview, FL). The craniotomy was cleaned and the temporalis muscle and overlying skin were allowed to fall back and were sutured separately. After one h the common carotid arteries were reperfused and the ventral incision cleaned and sutured.[61]

Treatment

The four groups of animals received a double tail vein injection, the first when anesthetized before surgery (30 min prior to stroke), and the second when the common carotid arteries were reperfused at the end of surgery (one h after stroke). One group

of animals (Control) was treated with aqueous PVP solution and the other three groups (Maca) were treated with the pentane extract at doses of 3, 10, and 30 mg/kg per injection.

Infarct volume evaluation

Twenty-four h after the treatment, the animals were deeply anesthetized with i.p. chloral hydrate and decapitated. The brains were extracted and kept in a cold saline solution under refrigeration for 15 min. Brains were sliced and treated with 2% triphenyltetrazolium-chloride (TTC, Sigma-Aldrich) solution for 30 min and kept in the dark. The stained slices were transferred to 10% formalin buffered solution (Sigma-Aldrich), for photographs after 24 h. Infarct volumes, measured using the Image J program, were expressed as percentages relatives to the total tissue in the brain hemisphere.

Statistical analysis

Data were analyzed for statistical significance and results are presented as mean ± SEM. Homogeneity of variances was assessed using the ANOVA. A P-value less than 0.05 was considered significant. If the P-value in the ANOVA test was significant, post hoc Bonferroni testing was performed when indicated. The concentration-effect relationship was analyzed by linear regression.

Results

Crayfish neurons neuroprotective assay

Following pretreatment of crayfish neurons with pentane extract solutions or vehicle, and treated with H_2O_2, differences in cell appearance could be microscopically detected as shown in Figures 1A to D. As compared to the dendritic shape and dense concentration of untreated cells, Figure 1A, and the spherical shape and sparse concentration of cells subjected to H_2O_2 (negative control), Figure 1B, cells treated with different concentrations of the pentane fraction exhibited varying cell shapes and densities. Cells treated with 30 μg/mL pentane extract displayed healthier dendritic cell shapes than cells treated with 3 μg/mL pentane extract as shown in Figures 1C and D. Linear regression analysis of the six concentrations of pentane extract demonstrated a significant concentration effect ($P \leq 0.03$ and $r = +0.85$). Figure 2 shows the percentages of neuroprotection as calculated from the absorbances of the MTS-treated wells after the experiments with the various pentane extract solutions (0.1 to 30 μg/mL) and H_2O_2 in the crayfish neurons. Results indicate that the effective concentration 50% (EC_{50}) to be approximately 2.8 μg/mL.

In vivo neuroprotective assay

The effect of the pentane extract was also evaluated in rats subjected to focal ischemic stroke. The results show statistically significant differences for the three doses (3, 10, 30 mg/kg) as shown in Figure 3. The control group showed an overall percentage of ischemic tissue of 23 ± 1.5 and the dose of 3 mg/kg was the only dose showing significant neuroprotective effects (13.5 ± 2.2%; $P \leq 0.002$). Doses of 10 and 30 mg/kg actually showed a significant increase in brain damage. The neuroprotective effect of the 3 mg/kg dose on the brain of a representative animal compared to a control brain is shown in Figures 4A and B. The colorless area represents the ischemic or dead tissue.

Discussion

Several studies have found Maca extracts to produce different pharmacological effects in animals and humans. Lipophilic extracts, which concentrate most of the active constituents found in the hypocotyls of *Lepidium meyenii*, have demonstrated to be the most pharmacologically interesting.[31] Because traditional claims and preliminary studies have supported the suggestion that the pentane extract of the Maca root could act as a neuroprotective agent, this study was developed to demonstrate the possible neuroprotective properties of the pentane extract from Maca by both *in vitro* and *in vivo* models, attempting to demonstrate and associate cellular and organic protection.[62]

The *in vitro* model using crayfish neurons subjected to oxidative stress with H_2O_2 allowed for the evaluation of the protective effect of the pentane extract.[60] Oxidative stress, defined as a disturbance in pro-oxidant-antioxidant balance, has been implicated in several neurodegenerative diseases characterized by cell death. Although reactive oxygen species (ROS), such as H_2O_2, are produced by normal metabolic processes, overproduction may result in oxidative-stress injuries that damage the integrity of cell function causing subsequent death by either necrosis or apoptosis.[10] Under optimal conditions in cell culture, without oxidative stressors,

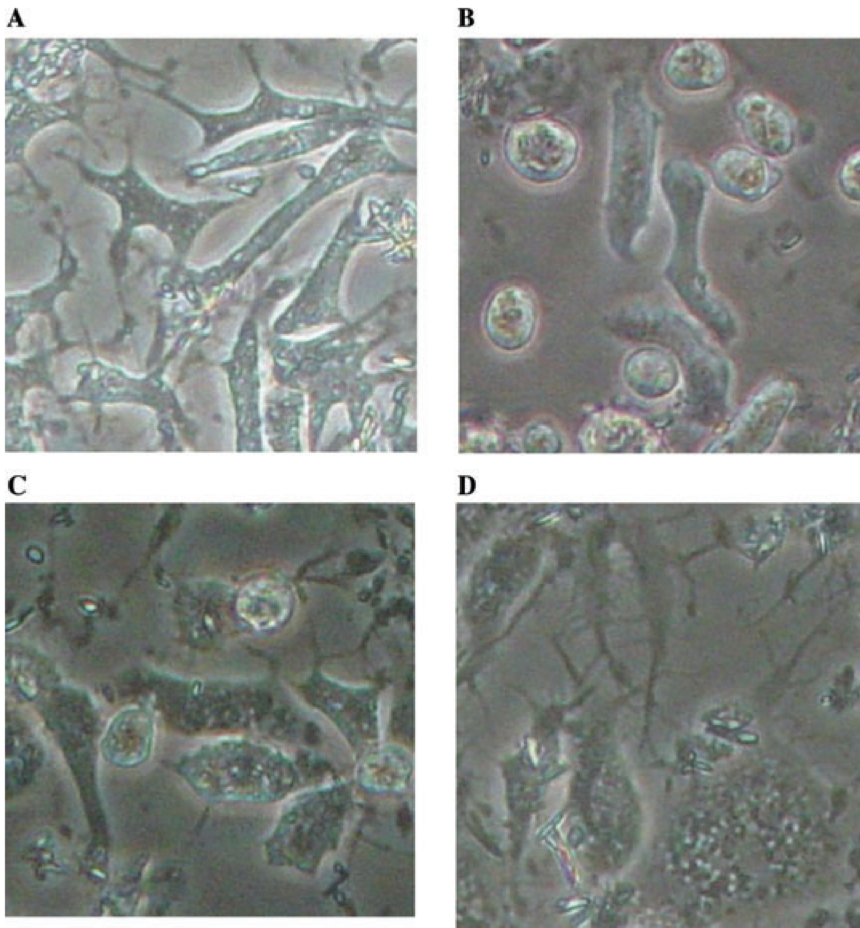

Figure 1. **(A)** ***In vitro* model of neuroprotection with crayfish neurons. Control crayfish neurons without treatment. (B) Crayfish neurons treated with H_2O_2 0.001 mol/L. (C) Crayfish neurons pretreated with 3 μg/mL of pentane extract of *Lepidium meyenii* (Maca) and treated 3 h later with H_2O_2 (0.001 mol/L). (D) Crayfish neurons pretreated with 30 μg/mL of pentane extract of *Lepidium meyenii* (Maca) and treated 3 h later with H_2O_2 (0.001 mol/L).**

the crayfish neurons assume appropriate dendritic shapes, extending and forming synapses with their surrounding neurons.[59,63] When oxidative stressors are applied these neurons lose their dendrites and transform into spherical shapes, suggesting a reversion to an undifferentiated state. Microscopic evaluations and cellular viability assays revealed a reduction in H_2O_2-induced stress in cells treated with the pentane extract. Cells treated with 30 μg/mL of the pentane fraction displayed more appropriate dendritic cell shapes and greater cell density compared to those treated with 3 μg/mL. Moreover, the cell viability assay demonstrated a significant linear correlation between the concentration of the pentane extract and the percentage of neuroprotection, ranging from 6.7% for the 0.1 μg/mL to 87.9% for the 30 μg/mL pentane extract.

The reduction of H_2O_2-induced cell oxidative stress *in vitro* by the pentane extract of *Lepidium meyenii* demonstrated in this preliminary study on crayfish neurons support the suggestion that this natural product has potential neuroprotective properties. Several possible mechanisms including the direct scavenging of ROS, stabilizing cell membranes to prevent lipid peroxidation, alleviating the damage by increasing the intracellular defensive mechanisms, or the inhibition or blockade of the biochemical cascades leading to cell death could be operating. Future studies are required to demonstrate the mechanisms involved and the

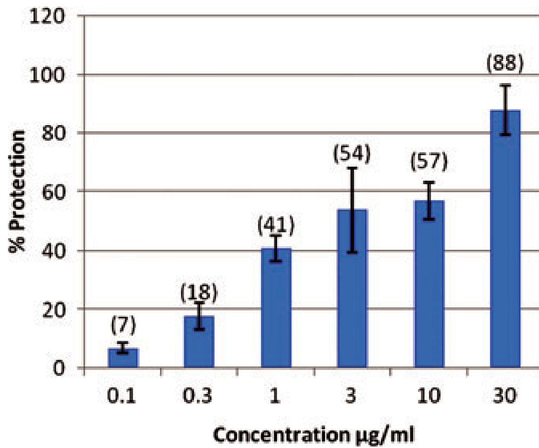

Figure 2. Concentration-effect of pentane extract of *Lepidium meyenii* (Maca) on % neuroprotection in crayfish neurons subjected to oxidative damage. Neurons were pretreated with the pentane extract and 3 h later 0.001 mol/L H_2O_2 was added. Bars represent the mean ± SEM, values in parentheses represent the mean.

participation of specific chemical entities. The techniques and comprehensive method developed to test neuroprotection on cells sets a foundation for future studies involving these and other neural cells and procedures.[64]

The effect of the pentane extract *in vivo* was then assessed in rats subjected to focal ischemic stroke. Occlusion of the middle cerebral artery (MCAO) in the rat is technically feasible and its histological consequences are consistent under controlled experimental conditions.[64] Improvement of the method includes 1 h transient occlusion of the common carotid arteries. Drugs demonstrating reduction of the infarct size in experimental stroke are considered potential neuroprotective candidates for the treatment of stroke and neurodegenerative diseases. In this study, 3 mg/kg of the pentane extract administered intravenously 30 min prior and 1 h after the onset of stroke showed a reduction from 23% to 13.5% in the percentage of infracted tissue resulting from MCAO. Contrary to this result, doses of 10 and 30 mg/kg produced a significant increase to 31.7% and 29.4%, respectively, of the infarct size demonstrating a neurodamaging effect under these stroke conditions.

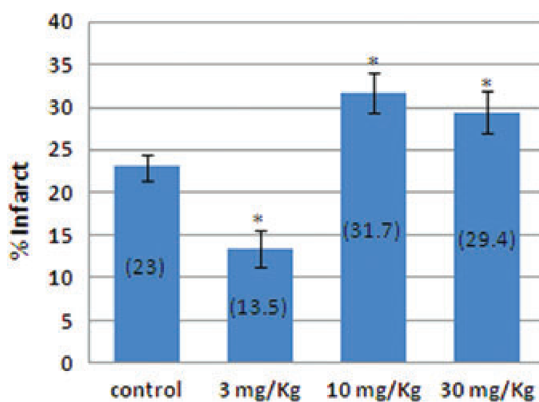

Figure 3. Effect of pentane extract from *Lepidium meyenii* (Maca) on % infarct in rats subjected to focal ischemic stroke. Values in parentheses represent the mean of the % infarct. Bars represent mean ± SEM. *Significantly $P < 0.05$ different from control.

This is a preliminary study of the effect of a pentane-extract of *Lepidium meyenii* administered intravenously in rats subjected to focal ischemic stroke. Previous pharmacological studies of the plant were performed with aqueous, hydroalcoholic or hexane extracts administered orally at equivalent doses without showing toxic effects. The pentane extract of Maca obtained by the method described contains mostly the lipo-soluble constituent alkaloids, benzylated amides (macamides), polyunsaturated oxoacids (macaenes), and benzylisothiocyanates. These compounds administered intravenously can likely reach the brain at higher concentration than other routes of administration. The results suggest that there are two different kinds of activity at the brain level in stroke conditions in rats, and possibly they depend on the different interaction of constituents with different cellular targets. One activity, at low doses, decreases the infarct volume indicating an inhibitory, antioxidative, or antiapoptotic mechanism that prevents cell death or protects neurons from ischemic insult. Another activity increasing the infarct volume, indicating that some excitatory, apoptotic, or neurotoxic processes are possibly activated at high doses. For instance, glucosinolates and isothiocyanates have reported pro-apoptotic and antiproliferative effects.[65,66]

The significant differences between the *in vitro* experiments that have not shown toxicity in cells at high concentrations, and the *in vivo* results showing deleterious effects at high doses may suggest new directions regarding potential mechanisms of action and toxicity that should be considered in

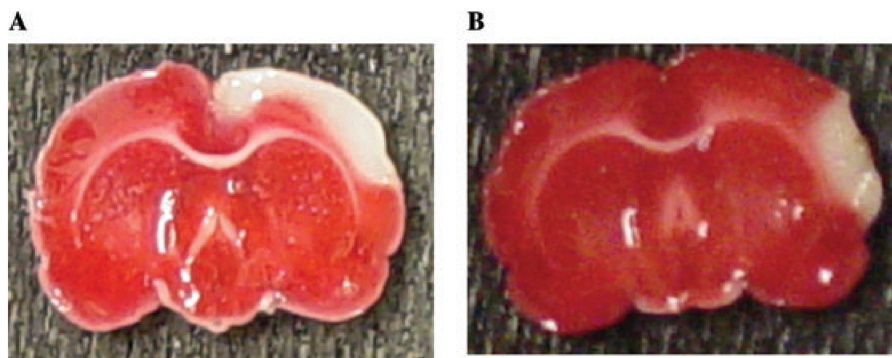

Figure 4. **(A) Brain section of a rat subjected to focal ischemic stroke and treated with vehicle (control). (B) Brain section of a rat subjected to focal ischemic stroke and treated with pentane extract from *Lepidium meyenii* (Maca) at 3 mg/kg.**

the future research on Maca. Other doses, different fractions of pentane extract, or isolated compounds need to be systematically evaluated for the neuroprotective mechanisms and toxicity to characterize the properties of *Lepidium meyenii.* The isolation and synthesis of derivatives may lead to the development of agents that can possibly be used to prevent or treat neurodegenerative diseases,[60] such as post-stroke, Alzheimer's disease,[67] and Parkinson's disease.[1]

Conflicts of interest

The authors declare no conflicts of interest.

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