European Journal of Medicinal Plants
8(3): 128-141, 2015, Article no.EJMP.2015.098
ISSN: 2231-0894

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Berberis vulgaris/Picrorhiza kurroa: Extending the Treatment Window for Reperfusion Injury

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Authors' contributions

This work was carried out in collaboration between all authors. Authors TMS and BJC designed the study, performed the statistical analysis, wrote the protocol and author MCS wrote the first draft of the manuscript. Author MCS managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/EJMP/2015/18000
Editor(s):
(1) Marcello Iriti, Faculty of Plant Biology and Pathology, Department of Agricultural and Environmental Sciences, Milan State University, Italy.
Reviewers:
(1) Marina Quartu, Department of Biomedical Sciences, Section of Cytomorphology, University of Cagliari, Italy.
(2) Rajendra Nath, Pharmacology and Therapeutics, King George's Medical University, Lucknow (UP), India.
(3) Weiting Wang, Tianjin Institute of Pharmaceutical Research, Tianjin, China.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=1085&id=13&aid=9035

Original Research Article

Received 31st March 2015
Accepted 17th April 2015
Published 28th April 2015

ABSTRACT

Aims: The present study investigates the neuroprotective effects of *Berberis vulgaris* and *Picrorhiza kurroa* in a rodent model of cerebral ischemia – reperfusion.
Study Design: Rats were pretreated with *Berberis vulgaris, Picrorhiza kurroa* or vehicle 30 minutes prior to cerebral ischemia and reperfusion injury. Post-mortem infarct measurements of the cerebral cortex were used to assess neuroprotection for each treatment.
Place and Duration of Study: The study took place in the Department of Biomedical Sciences, Atlantic Veterinary College, University of Prince Edward Island between July 2014 and January 2015.
Methodology: The right middle cerebral artery (MCA) was occluded for 30 minutes followed by 5.5 hours of reperfusion in anaesthetized male Sprague-Dawley rats (250-350 g). *Berberis vulgaris* (0.001-0.1 mg/kg) and *Picrorhiza kurroa* (0.0001-1.0 mg/kg) were administered singly and in combination (0.001 mg/kg each) 30 minutes prior to MCA occlusion as well as at several intervals during the reperfusion period. Infarct volume in the affected hemisphere was measured to assess neuroprotection.

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Results: Occlusion of the right MCA for 30 minutes followed by 5.5 hours of reperfusion in anaesthetized male Sprague-Dawley rats produced a focal ischemic lesion localized to the prefrontal cortex. Intravenous administration of either *Berberis vulgaris* or *Picrorhiza kurroa* 30 minutes prior to MCA occlusion provided significant neuroprotection following ischemia-reperfusion. Furthermore, *Berberis vulgaris* (0.1 mg/kg, i.v.) or *Picrorhiza kurroa* (1.0 mg/kg, i.v.) could be administered during the ischemic period itself, as well as up to 90 minutes into the reperfusion period to provide neuroprotection. A combined injection of *Berberis vulgaris* with *Picrorhiza kurroa* using subthreshold doses of each (0.001 mg/kg) reduced infarct volume in the ischemic cortex when injected 30 minutes before MCA occlusion, 15 minutes into the ischemic period or up to 150 minutes following reperfusion. While *Berberis vulgaris* and *Picrorhiza kurroa* co-administration significantly reduced both baseline mean arterial pressure and heart rate in non-ischemic animals, suggestive of a central effect on autonomic tone, no significant effect was observed on baroreflex sensitivity in rats undergoing ischemia-reperfusion

Conclusion: Natural products such as *Berberis vulgaris* and *Picrorhiza kurroa* may hold the key to reducing morbidity and mortality associated with post-stroke complications due to reperfusion injury.

Keywords: Stroke; ischemia/reperfusion; middle cerebral artery occlusion; antioxidant therapy; neuroprotection.

1. INTRODUCTION

According to the American Heart Association's 2015 Heart Disease and Stroke Statistics Update, cardiovascular disease continues to be the leading global cause of death [1]. Stroke follows in second place, accounting for 11.3% of total deaths worldwide. While statistics indicate that death from stroke has in fact decreased within the last 10 years, post-stroke deficits affecting quality of life continue to prove challenging to both treat and prevent. In part, these effects are attributed to a phenomenon known as reperfusion injury, or clinically as reperfusion syndrome [2]. Cerebral injury resulting from the return of blood flow to ischemic tissue evolves from several mechanisms including invasion of neutrophils which secrete inflammatory cytokines and cellular adhesion molecules, and increased production of reactive oxygen species (ROS) which contribute to lipid peroxidation as well as disruption of the blood brain barrier [3]. Treatment modalities have routinely targeted what are believed to be the most critical effectors, namely oxidative radicals and inflammatory mediators. Antioxidant therapy has proven to be only marginally successful with some therapies proving ineffective and, on occasion, toxic depending on the tissue involved. Antagonizing the inflammatory response of invading neutrophils following ischemia using antibodies to cellular adhesion molecules such as I-CAM [4] have proven more successful, however it continues to be a challenge to provide consistent treatment options in a timely fashion to patients suffering stroke.

With the growing interest in natural product pharmaceuticals, research aimed at finding neuroprotective agents derived from the plant kingdom has identified several promising compounds. *Berberis vulgaris* L. (barberry) is an isoquinoline alkaloid found in several plants, including *Hydrastis Canadensis* (goldenseal), *Coptis chinensis* (golden thread) and *Berberis aristata* (tree turmeric) [5]. *Berberis vulgaris* (*B. vulgaris*) possesses multiple biological activities including being a potent antioxidant [6] and anti-inflammatory agent [7]. The neuroprotective potential of *B. vulgaris* has been demonstrated in both mice and gerbil models of global cerebral ischemia [8-11], as well as in rats undergoing permanent middle cerebral artery occlusion to produce focal ischemic lesions localized to the insular region of the cortex [12].

Picrorhiza kurroa (*P. kurroa*) is a small, perennial herb, the leaves and roots of which are common constituents of several Ayurvedic preparations. Traditional uses of *P. kurroa* include treatment of liver and gastrointestinal disorders, allergies and skin ailments [13]. Extracts of the root produce several active compounds including apocynin which has been shown to exert potent neuroprotection in several animal models of stroke and reperfusion injury [14,15]. While less commonly used, extracts of *P. kurroa* leaves have been shown to protect hippocampal-derived HT22 cells against ischemia-reperfusion induced injury as well as hydrogen peroxide-induced oxidative stress in part through scavenging of nitric oxide [16]. In the current study, these

compounds are administered both individually and combined in a rat model of ischemia-reperfusion injury as a means of identifying lowest therapeutic dose, possible synergism or potentiation and lastly, potential extension of the therapeutic window for treatment following re-establishment of cerebral blood flow.

2. MATERIALS AND METHODS

2.1 Reagents and Animals

B. vulgaris (Berberine hydrochloride) was purchased from Sigma Aldrich (St. Louis, MO, USA) and diluted in physiological saline solution (0.9%). A single batch of *Picrorhiza kurroa* in powder form was purchased from Carmel Biosciences Inc., (Atlanta, GA, USA) and diluted in 0.3% DMSO to a concentration of 1 mg/mL with subsequent dilutions being made in 0.9% saline. Male Sprague-Dawley rats (250-350 g) were purchased from Charles River Laboratories (Montreal, PQ, Canada).

2.2 Surgical Procedures

Surgical procedures described herein were approved by the University of Prince Edward Island Animal Care Committee and were in compliance with the requirements of the Canadian Council on Animal Care (protocol #14-041). Our surgical protocol has been described previously [17]. Briefly, rats were anaesthetized with sodium thiobutabarbital (Inactin, Sigma Aldrich; 100 mg/kg, i.p.) and kept warm on a heating pad maintained at 37±1°C. Cannulae were inserted into the right femoral artery and vein and secured with fine suture. The arterial cannula was connected to a pressure transducer and tachograph (Gould, Cleveland, OH, USA) permitting acquisition of blood pressure and heart rate data which were displayed and analyzed using PolyviewPro/32 analysis software (Grass, Warwick, RI, USA). Drug treatments and supplemental anaesthesia, if required, were administered through the venous cannula. Rats were then carefully transferred to a stereotaxic frame where surgical access to the right middle cerebral artery (MCA) was achieved. Once exposed, a 3 point occlusion of the MCA was accomplished by inserting suture under the vessel and gently lifting. Blood flow was re-established by removal of the sutures. The MCA was occluded for 30 minutes followed by 5.5 hours of reperfusion (transient ischemia model, tMCAO).

2.3 Measurement of Infarct Volume

Rats were sacrificed after 5.5 hours of reperfusion (tMCAO). Brains were removed and sliced into a series of 1 mm coronal sections through the area of infarct using a rat brain matrix (Harvard Apparatus; Holliston, MA, USA) and subsequently immersed in a 2% solution of 2,3,5-triphenol tetrazolium chloride (Sigma Aldrich, St. Louis, MO, USA) for 5 minutes. Both sides of each section were scanned and infarct volume was measured using imaging software (Image J; Scion Corporation, Frederick, MD, USA). Images were coded and sent over a secure server to a reviewer blinded to treatment groups for measurement of infarct volume. The average area for each section was multiplied by section thickness (1mm) to provide an infarct volume. The sum of volume measurements for all sections from each brain provided a total infarct volume for each animal. All data was then sent back to the experimenter for decoding.

2.4 Dose-Response Curves for *B. vulgaris* and *P. kurroa*

All animals were randomly assigned to each treatment or vehicle group on a daily basis using free online random number generator software (www.random.org). A dose-response curve for *B. vulgaris* was generated by administering doses of *B. vulgaris* (0.001 – 0.1 mg/kg; n=5 or 6 per group) intravenously (1 mL/kg) 30 minutes prior to occlusion of the MCA. Sutures were removed following 30 minutes of occlusion and reperfusion lasted 5.5 hours. Similarly, *P. kurroa* was administered intravenously (0.0001 – 1.0 mg/kg; n=6 or 7 per group) 30 minutes prior to MCA occlusion. Control groups received vehicle injections (0.9% saline or 0.3% DMSO, n=6 each) 30 minutes prior to MCA occlusion.

2.5 Testing the Treatment Window

The most potent doses of *B. vulgaris* (0.1mg/kg) and *P. kurroa* (1.0 mg/kg) determined above were subsequently administered at various points during the transient MCAO protocol. Specifically, intravenous drug injections (n=4 or 5 per group) were made either 15 minutes after MCA occlusion, immediately prior to reperfusion, or 30, 60, 90, 120 minutes into the reperfusion period. In all cases, experiments were terminated following 5.5 hours of reperfusion.

2.6 Combinatorial Drug Treatments

Non-neuroprotective doses of *B. vulgaris* (0.001 mg/kg) and *P. kurroa* (0.001 mg/kg) were determined from the dose-response curves and combined into a single solution that was administered intravenously 30 minutes prior to MCA occlusion or at various points throughout the tMCAO protocol, namely 15 minutes post-MCA occlusion, at the start of reperfusion, or 30, 60, 90, 120, 150, 180 minutes into the reperfusion period (n=5 or 6 per group).

2.7 Cardiovascular Effects of *B. vulgaris* and *P. kurroa*

In separate groups of anaesthetized animals instrumented to record mean arterial pressure and heart rate, the combined solution of *B. vulgaris* with *P. kurroa* (0.001 mg/kg each) was administered intravenously in the absence of ischemia/reperfusion (n=4). Measurements of MAP and HR were made 5 minutes prior to drug administration, as well as 5, 10, 15, 30, 45, 60, 90, 120, 150, 180 minutes following drug delivery. A similar experiment was conducted for vehicle administration (n=4).

Baroreflex sensitivity was used to assess autonomic tone before and during the tMCAO protocol with and without *B. vulgaris* + *P. kurroa* (0.001 mg/kg each) pretreatment. The baroreceptor reflex was evoked by administering 250 ng of phenylephrine hydrochloride (100 µl, i.v.). The ratio of the peak change in the magnitude of the reflex bradycardia to the magnitude of the phenylephrine-induced pressor response ($\Delta HR/\Delta MAP$) was calculated as a measure of baroreflex sensitivity (BRS).

2.8 Statistical Analysis

All data are presented as mean±S.E.M. Data were analyzed by a one-way ANOVA, followed by a Bonferroni post-hoc test. When comparing 2 groups only, a Student's t-test was used. In all cases, *P* values ≤ 0.05 were considered statistically significant.

3. RESULTS

3.1 *B. vulgaris* and *P. kurroa* protect against ischemia/reperfusion injury

Pre-treatment with either *B. vulgaris* or *P. kurroa* 30 minutes before MCA occlusion conferred neuroprotection as reflected by reduced infarct volumes measured following ischemia/reperfusion. Infarct volume was significantly lower in rats treated with 0.05 and 0.1 mg/kg *B. vulgaris* when compared to vehicle treated animals (Fig. 1). *P. kurroa* demonstrated significant neuroprotection in tMCAO at 0.1 and 1.0 mg/kg administered 30 minutes before occlusion of the MCA (Fig. 2). The most potent doses of each drug were used to study effects on treatment window. Non-neuroprotective doses of *B. vulgaris* and *P. kurroa* were determined from these graphs to be 0.001 mg/kg and thus, combined into a single injection to study effects on treatment window as well.

3.2 Combined injections of *B. vulgaris* and *P. kurroa* extend the Treatment Window for Neuroprotection against Ischemia/Reperfusion Injury

Intravenous administration of *B. vulgaris* (0.1 mg/kg) at points during the tMCAO protocol produced significant neuroprotection compared to vehicle-treated animals (Fig. 3A). Infarct volume was significantly reduced when *B. vulgaris* was injected during the 30 minute period of ischemia (t=-15 min), at the start of reperfusion (t=0 min), and at 30, 60, and 90 minutes into the reperfusion period (Fig. 3A). No neuroprotection was observed when *B. vulgaris* was administered 120 minutes after the start of reperfusion. Similar effects were seen with *P. kurroa* (1.0 mg/kg) with significant reductions in infarct volume being measured when *P. kurroa* was injected during the ischemic or reperfusion periods up until 90 minutes (Fig. 3B). Injection of *P. kurroa* 120 minutes into reperfusion did not provide neuroprotection.

Combining lower, non-neuroprotective doses of *B. vulgaris* and *P. kurroa* (0.001 mg/kg each) into a single treatment was significantly neuroprotective and contributed to a lengthened treatment window for neuroprotection (Fig. 3C). Infarct volume was significantly reduced in rats injected with both *B. vulgaris* and *P. kurroa* (0.001 mg/kg) 30 minutes prior to occlusion of the MCA (Fig. 4). Additionally, administering the combined drugs during the ischemic period or at several points within the reperfusion period also reduced infarct volume relative to vehicle-injected controls (Fig. 3C). The combination of B. vulgaris and *P. kurroa* at these lower doses was neuroprotective in our transient ischemia model when administered up until 150 minutes following the onset of reperfusion. At 180 minutes into the reperfusion period, injection of B. vulgaris + *P. kurroa* offered no neuroprotection (Fig. 3C).

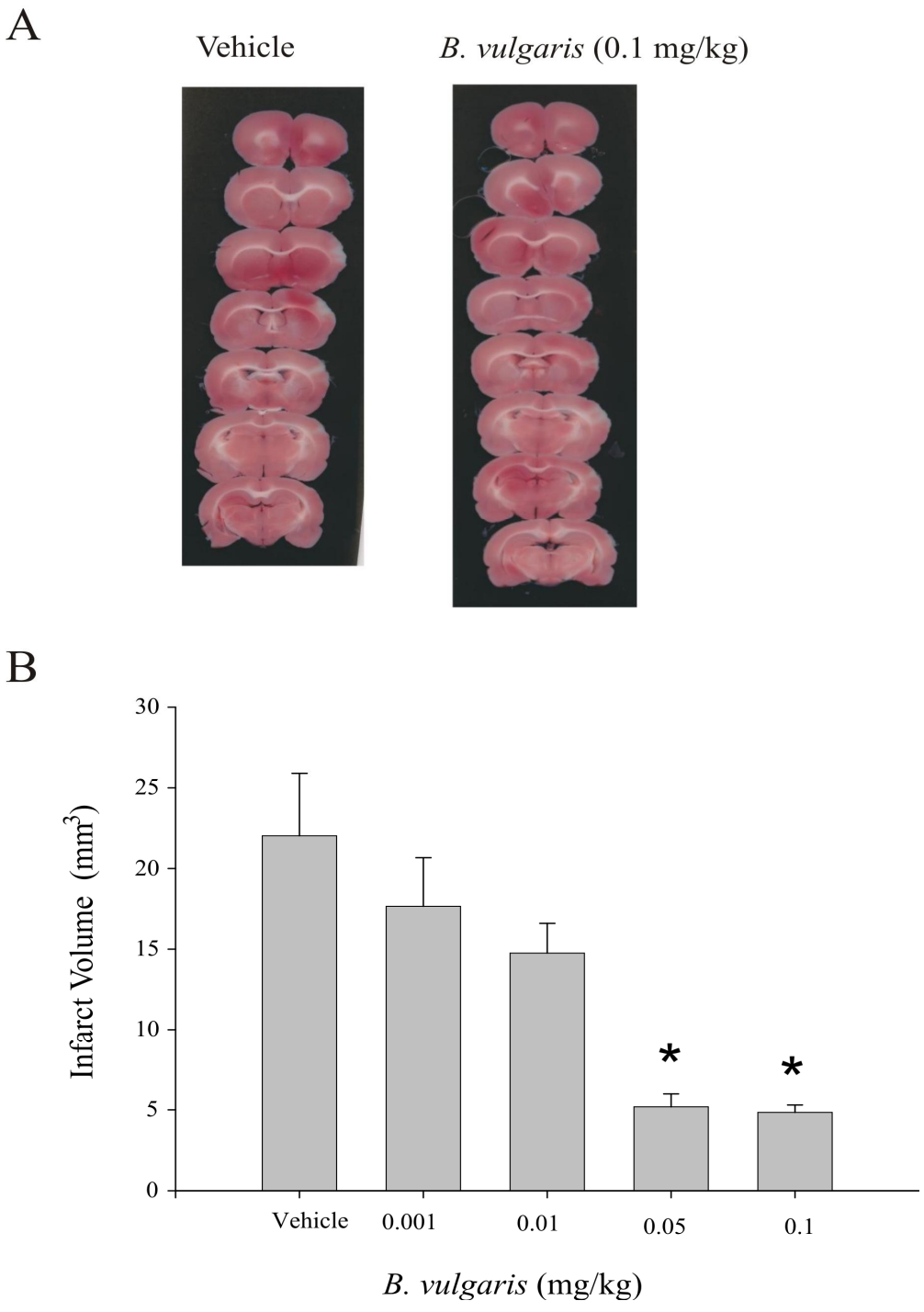

Fig. 1. Dose-dependent effect of *B. vulgaris* on infarct volume in tMCAO

A) Representative photomicrographs of TTC-stained, 1mm thick coronal slices illustrating the extent of infarct within the prefrontal cortex following 30 minutes of ischemia followed by 5.5 hours of reperfusion. Animals were either pretreated with 0.9% saline (Vehicle) or 0.1 mg/kg B. vulgaris (i.v.) 30 minutes prior to tMCAO. B) Bar graph summarizing the dose-response relationship between increasing doses of B. vulgaris and infarct volume calculated from TTC-stained brain slices following tMCAO. Each bar represents the mean±S.E.M. (n=5-6/group). Asterisk (*) indicates significance (p≤0.05) relative to vehicle-treated control group

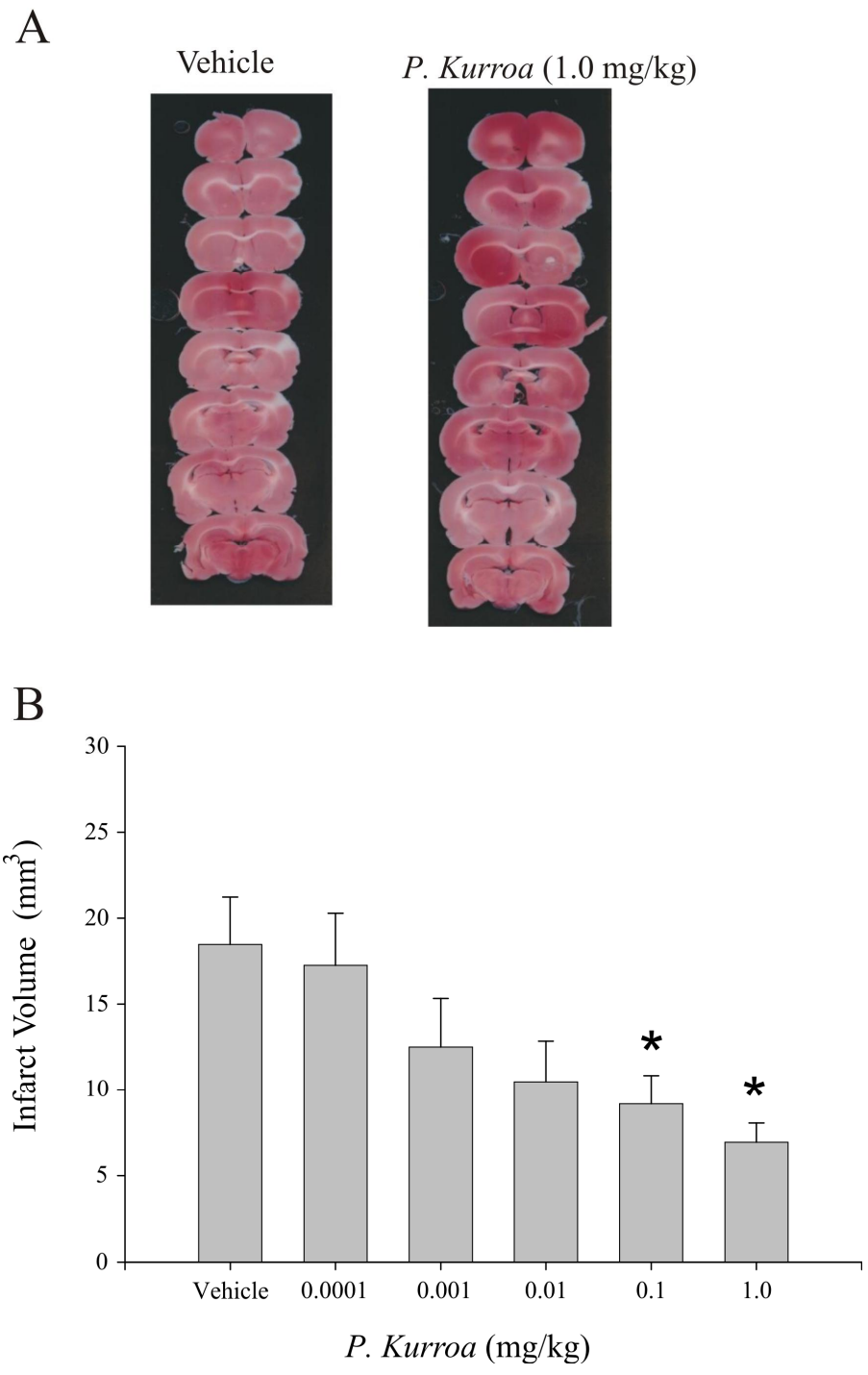

Fig. 2. Dose-dependent effect of *P. kurroa* on infarct volume in tMCAO

A) Representative photomicrographs of TTC-stained, 1mm thick coronal slices demonstrating the extent of cortical damage following ischemia-reperfusion. Rats were pretreated with either Vehicle (0.3% DMSO) or P. kurroa (1.0 mg/kg) 30 minutes prior to tMCAO. B) Bar graph summarizing the dose-dependent relationship between increasing doses of P. kurroa and infarct volume calculated from TTC-stained coronal sections of rat brain following tMCAO. Each bar represents the mean ± S.E.M. (n=6-7/group). Asterisk (*) denotes significance (p≤0.05) when compared to vehicle-treated group

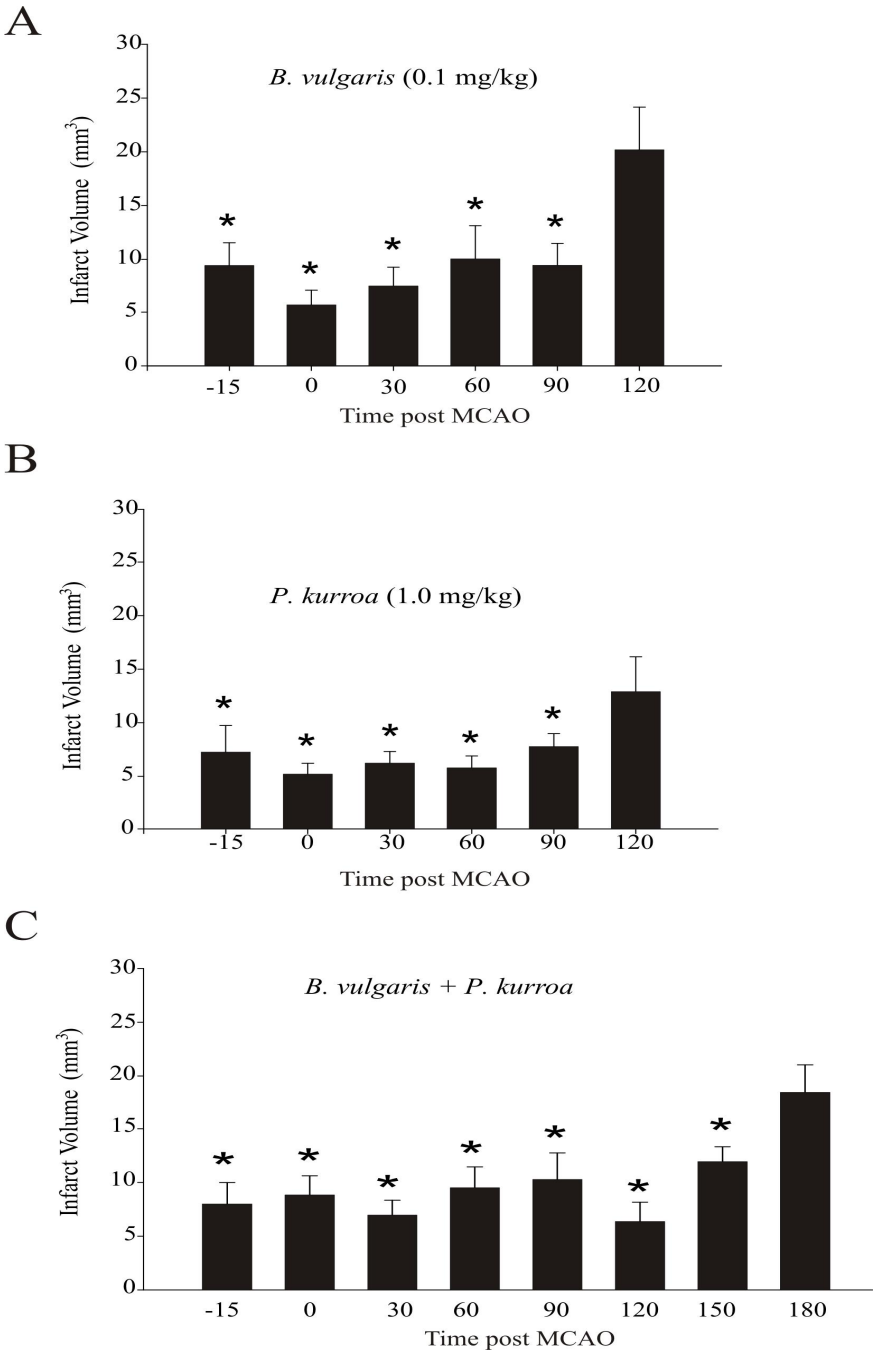

Fig. 3. Effect of *B. vulgaris* or *P. kurroa* on infarct volume following administration post-MCAO

A) Effect of B. vulgaris (0.1 mg/kg, i.v.) on infarct volume following tMCAO when administered 15 minutes into the ischemic period (-15min), at the time of suture removal (0 min) or 30, 60, 90 and 120 minutes into the reperfusion period (n=5/group). B) Effect of P. kurroa (1.0 mg/kg; i.v.) on infarct volume following tMCAO when administered during the ischemic period (-15min), upon suture removal (0 min) or during the reperfusion period (30, 60, 90, 120 min; n=4-5/group). C) Measurements of infarct volume following tMCAO in rats pretreated with both B. vulgaris and P. kurroa (0.001 mg/kg each; i.v.) either during the ischemic period (-15 min), upon suture removal (0 min) or during the reperfusion period (30, 60, 90, 120, 150, 180 min; n=5-6/group). In all cases, bars represent mean±S.E.M. with asterisk (*) indicating significant difference (p≤0.05) from vehicle-treated group injected 30 minutes prior to MCAO

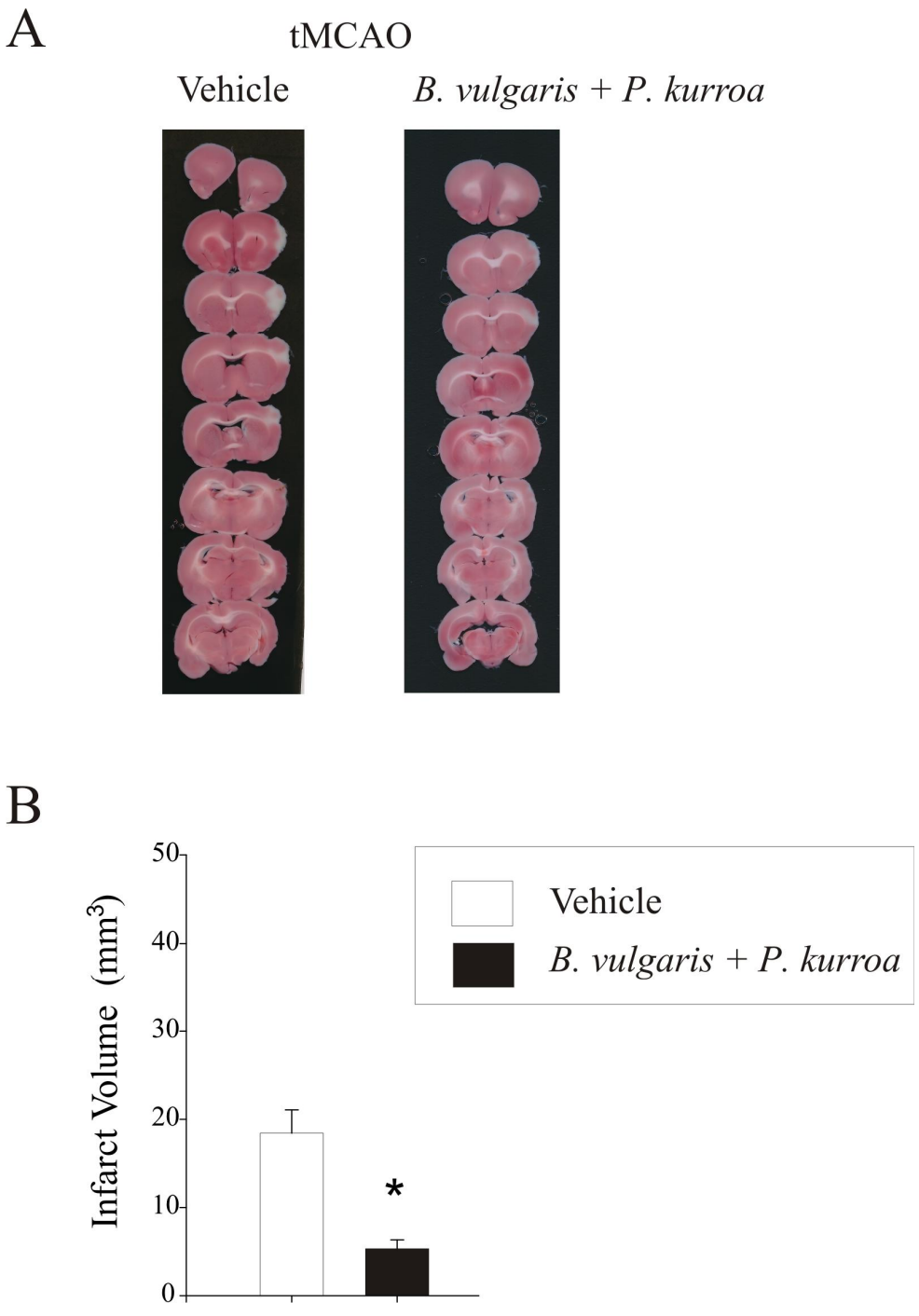

Fig. 4. Effect of combining *B. vulgaris* and *P. kurroa* pre-treatments on infarct volume following tMCAO

*A) Representative photomicrographs of TTC-stained, 1mm thick coronal sections illustrating the extent of infarct within the prefrontal cortex of rats pretreated with either 0.15% DMSO (Vehicle) or a combination of B. vulgaris and P. kurroa (0.001 mg/kg each) 30 minutes prior to tMCAO. B) Effect on infarct volume calculated from TTC-stained rat brain sections following tMCAO of pretreating with either 0.15% DMSO (Vehicle) or B. vulgaris + P. kurroa (0.001 mg/kg each). Each bar represents mean±S.E.M. and * indicates significantly different from Vehicle group (p≤0.05; n=5-6/group)*

3.3 Hemodynamic and ANS effects of *B. vulgaris* and *P. kurroa*

In a separate group of rats instrumented for the recording of mean arterial pressure (MAP) and heart rate (HR), baseline MAP was 110±9 mm/Hg while resting HR was 385±16 bpm (Fig. 5). Combined intravenous injection of *B. vulgaris* and *P. kurroa* (0.001 mg/kg each) produced a time-dependent decrease in MAP over a 2 hour period of observation, dropping to 77±12 mmHg. MAP was significantly decreased relative to vehicle-injected control animals by as early as 45 minutes post-injection (Fig. 5). Changes in mean HR were less dramatic but nonetheless significant by 2 hours post-injection falling to 340±18 bpm following administration of *B. vulgaris* and *P. kurroa* at 0.001 mg/kg each (Fig. 5).

Testing of the baroreflex in vehicle-injected animals during the transient ischemia protocol demonstrated a significant decrease in baroreflex sensitivity (BRS) within 5 minutes following MCA occlusion (Fig. 6). BRS fell from 0.59±0.05 bpm/mmHg prior to MCA occlusion to 0.34±0.06 bpm/mmHg following the onset of ischemia (Fig. 6). The attenuated BRS was maintained throughout the 30 minute period of ischemia and the entire 5.5 hours of reperfusion. Pretreating rats with *B. vulgaris* and *P. kurroa* (0.001 mg/kg each) as a combined intravenous injection 30 minutes prior to MCA occlusion did not affect BRS at any time point when compared to vehicle-injected controls.

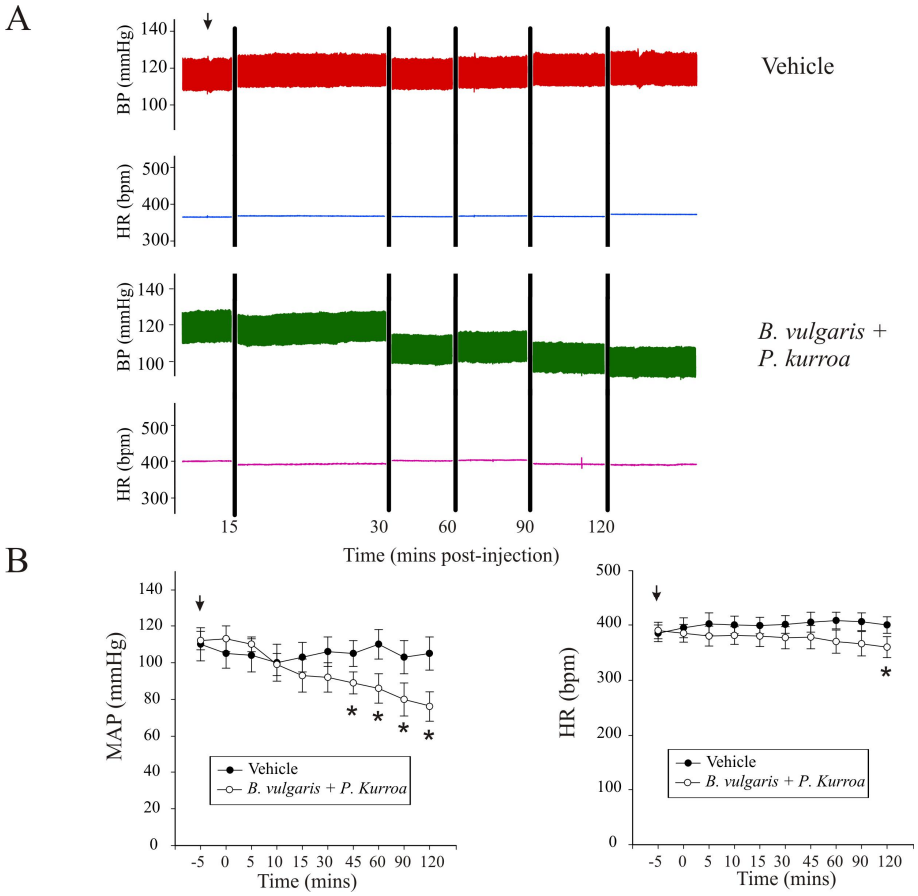

Fig. 5. Effect of *B. vulgaris* and *P. kurroa* co-administration on baseline mean arterial blood pressure and heart rate in anaesthetized rats

A) Raw data tracing representative of baseline MAP and HR following injection of either Vehicle (0.15% DMSO) or B. vulgaris with P. kurroa (0.001 mg/kg each). Downward arrow indicates time of drug injection (i.v.) with animals being observed for 120 minutes thereafter. B) Line graphs demonstrating the effect of either Vehicle or B. vulgaris + P. kurroa (0.001 mg/kg each) on MAP and HR. Downward arrow indicates time of drug injection (i.v.). Each data point represents the mean±S.E.M. and an asterisk (*) indicates a significant difference (p≤0.05) between Vehicle (0.15% DMSO) and drug-treated groups at that time point

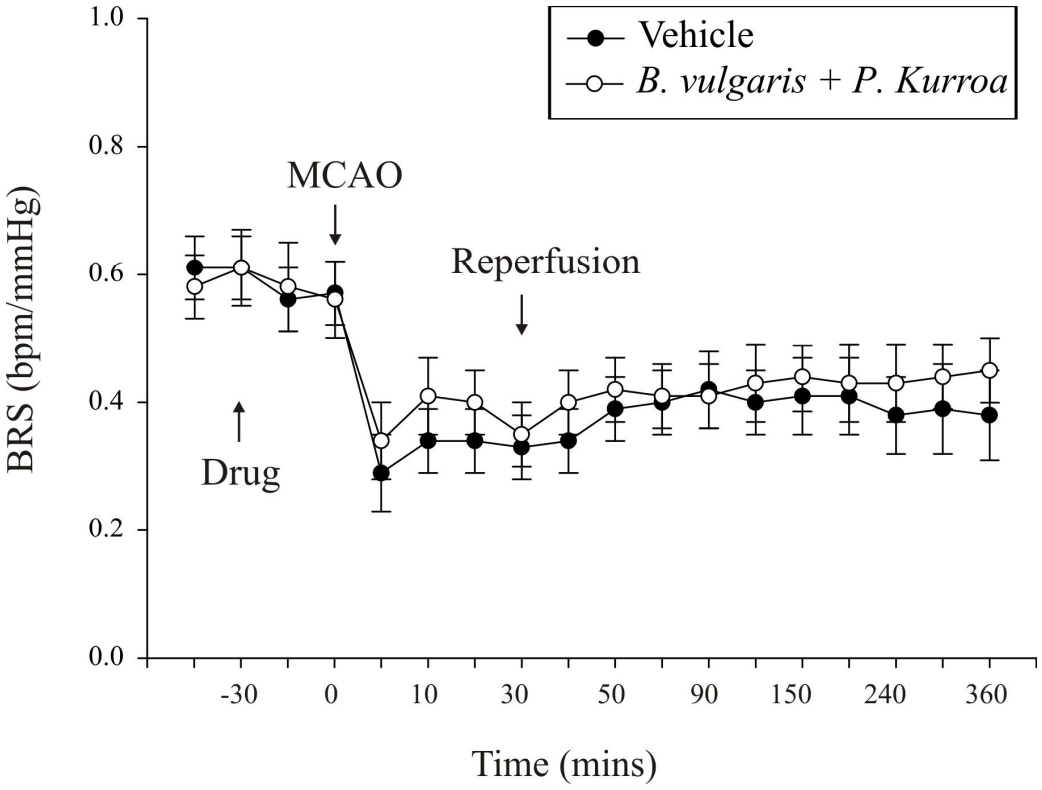

Fig. 6. Effect of *B. vulgaris* and *P. kurroa* co-administration (0.001mg/kg each) on the baroreceptor reflex

Bar graph illustrating the average change in baroreflex sensitivity (BRS) before and during ischemia-reperfusion (tMCAO). Upward arrow indicates time of drug injection (i.v.). Downward arrows indicate points at which ischemia (MCAO) and reperfusion commence. Each data point represents mean±S.E.M. No significant difference (p≤0.05) was measured between Vehicle (0.15% DMSO) and drug-treated groups (n=5/group)

4. DISCUSSION AND CONCLUSION

The re-establishment of blood flow to ischemic tissue paradoxically contributes to further cell death by a multifaceted process described as reperfusion injury. In patients treated for ischemic stroke, neurological deficits may present for up to 1 month following restoration of blood flow using routine thrombolytic therapy such as tissue plasminogen activator (t-PA) [2]. The mechanisms of cell death include such hallmark cellular events as cell swelling leading to necrosis, as well as activation of caspases leading to more controlled death by apoptosis [18]. At the molecular level, the increased presence of reactive oxygen and nitrogen species [19,20] contributes to lipid peroxidation, DNA damage, and depletion of energy molecules such as ATP [21]. Finding treatments which serve to mitigate the deleterious consequences arising from reperfusion is of paramount importance.

To this end, our results demonstrate that both *B. vulgaris* and *P. kurroa* were capable of producing neuroprotection in our rodent model of transient ischemia-reperfusion injury (tMCAO). Furthermore, combining these natural compounds at sub-threshold doses provided significant neuroprotection in addition to lengthening the therapeutic window in which drug treatment was capable of significantly attenuating cortical damage following tMCAO. There is accumulating evidence demonstrating the neuroprotective effects of *B. vulgaris* in animal models of ischemia-reperfusion. Using an intraluminal thread model of MCAO in mice, Zhou and colleagues [11] demonstrated that an intragastric injection of *B. vulgaris* (20 mg/kg) either 30 minutes prior to, or 24 hours following a 90 minutes period of MCAO, decreased infarct volume after 48 hours of reperfusion. In addition, these authors showed that *B. vulgaris* administration decreased cerebral ROS generation and inhibited MCAO-induced release

of cytochrome c and apoptosis inducing factor from the mitochondria. Using a model of global cerebral ischemia in mice by obstructing blood flow through the common carotid arteries, Hu and colleagues [9] demonstrated that intravenous injection of *B. vulgaris* contributed to enhanced Akt phosphorylation concomitant with attenuated caspase-3 activation. Taken together, these effects suggest an anti-apoptotic component to the neuroprotection afforded by *B. vulgaris* pre-treatment during the reperfusion period following transient ischemia. Furthermore, daily injection of *B. vulgaris* for 7 days prior to 20 minutes of occlusion of the common carotid arteries in mice protected hippocampal CA1 and CA2 regions from reperfusion–induced injury demonstrating the potential prophylactic application of *B. vulgaris* treatment in disease states having a significant oxidative component [8].

In addition to being a proven antioxidant, *B. vulgaris* has also been shown to have anti-inflammatory properties. Using a 5 minute occlusion of the common carotid artery in gerbils, Yoo and colleagues [10,22] demonstrated that the prior oral administration of *B. vulgaris* decreased hippocampal cell death. The authors reported increased COX-2-like immunoreactivity as well as an attenuation of the ischemia-induced elevation in prostaglandin E2 levels following *B. vulgaris* pretreatment. In a rat model of permanent occlusion induced by inserting a nylon monofilament to the base of the middle cerebral artery, *B. vulgaris* was shown to decrease infarct volume [12], an effect related to both increased activation of the Akt/GSK (glycogen synthase kinase) apoptotic signaling pathway together with decreased expression of the inflammatory mediator NF-KB (nuclear factor kappa B). Lastly, it is tempting to speculate that the neuroprotective potential of *B. vulgaris* may arise in part from direct effects on neuronal excitability. Bath application of *B. vulgaris* was demonstrated to block outward voltage-dependent potassium currents in isolated hippocampal CA1 pyramidal neurons [23], and in parabrachial and cerebellar cells in rat brain slices *in vitro* [24]. *Berberis vulgaris* has also been demonstrated to inhibit the release of glutamate from cerebral cortex nerve terminals [25]. At the electrophysiological level, these effects likely contribute to an overall dampening of neuronal excitability thereby limiting the extent of ischemia related spreading depression in the ischemic cortex.

Picrorhiza kurroa is comprised of several constituent compounds, the major ones being apocynin, picrosides 1, 2 and 3 and the kutkin group of kutkoside and iridoid glucoside [26]. Our laboratory, among others, has demonstrated the neuroprotective effect of apocynin [27,28]. The proposed mechanism by which apocynin confers neuroprotection in animal models of ischemia includes inhibition of NADPH-oxidase, a key mediator of oxidative stress-induced neuronal damage [28]. Picroside-2 has been shown to reduce infarct volume in rat models of both global and focal cerebral ischemia [29,30]. Specifically, picroside-2 treatment decreased the number of apoptotic cells and the expression of caspase-3 and PARP, 24 hours following 2 hours of MCA occlusion [30]. Guo and colleagues [29] using a similar paradigm of 2 hours ischemia followed by 24 hours reperfusion, demonstrated decreased expression of several inflammatory mediators including NFKB, TNFα and TLR4. In the present study, animals were pretreated with a whole leaf extract of *P. kurroa*, thereby employing the greatest potential for neuroprotective effects owing to all the individual components of the plant being present as is the case currently with *P. kurroa* use in human naturopathic medicine.

Measurement of baroreflex sensitivity is a strong prognostic tool providing valuable information regarding hemodynamic stability under a given set of circumstances [31]. Clinically, impaired BRS has been observed in patients presenting with acute ischemic stroke [32]. Additionally, impaired BRS following stroke is associated with poor prognosis [33]. Among the factors influencing BRS, stroke volume and insular cortex involvement were identified as being of particular importance [32]. In our rat model of transient ischemia-reperfusion, neuronal damage is localized to the insular region of the cortex in a consistent and reproducible manner and associated with a depressed BRS [17]. While infarct volume to this area following tMCAO was measurably reduced in rats pre-treated with a combination of *B. vulgaris* and *P. kurroa*, there was no significant effect on BRS compared to vehicle-injected controls. Under non-ischemic conditions, we measured a significant decrease in mean arterial pressure and heart rate following co-administration of *B. vulgaris* and *P. kurroa*. *Berberis vulgaris* is used primarily as an anti-hypertensive therapy and similar cardiovascular effects have been reported elsewhere [34]. The decrease in both MAP and HR induced by these compounds in non-ischemic animals suggests a

centrally-mediated effect, possibly via inhibition of sympathetic outflow from medullary nuclei or the intermediolateral cell column (IML) of the spinal cord. However, similar measurements made following co-administration of *B. vulgaris* with *P. kurroa* in our model of tMCAO had no effect on MAP or HR suggesting that at these low doses, *B. vulgaris* and *P. kurroa* do not significantly attenuate sympatho-excitation produced by ischemia-reperfusion injury.

In the pursuit of adjuvant therapies in the treatment of ischemic stroke, natural products have garnered a great deal of attention. Thrombolytic therapy using t-PA remains the treatment of choice for patients presenting with symptoms of acute ischemia in spite of its limited application potential and narrow therapeutic window [6,35]. Recognizing that the reperfusion period following re-establishment of blood flow is critically involved in both the propagation of tissue damage as well as the healing and recovery of tissue function, strategies to navigate the complex interplay of these factors are essential.

In conclusion, natural products such as *B. vulgaris* and *P. kurroa* show great promise as neuroprotective agents capable of attenuating post ischemic damage at several levels possibly at doses significantly lower than currently available options.

ACKNOWLEDGEMENT

The authors wish to thank the funding support of the Atlantic Canada Opportunity Agency's Atlantic Innovation Fund (ACOA-AIF) project #199294.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the University of Prince Edward Island's Animal Care Committee and were in compliance with the requirements of the Canadian Council on Animal Care (protocol #14-041) and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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