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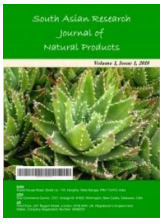

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Modulation of Inflammatory and Pain Pathways in Trigeminal Ganglion Neurons and Glial Cells by Plant Extracts

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Authors' contributions

This work was carried out in collaboration among all authors. Author YF wrote the original draft of the manuscript and was responsible for generation of the cell culture results. Author HS was responsible for the isolations and polyphenolic assays and also helped to write those sections of the manuscript. Authors LC and SW were responsible for establishing the cultures, contributed to the experimental design, data analysis, and editing of the manuscript. Author PLD was involved in all aspects of the study design, analysis and preparation of final manuscript.

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ABSTRACT

Aims: To evaluate the modulatory effect of plant extracts on the expression of mitogen-activated protein kinase phosphatase-1 (MKP-1) and the protein kinases P-ERK and P-p38 in primary cultures of trigeminal ganglion from Sprague Dawley rats.
Study Design: *In vitro* analysis of methanol extracts on proteins implicated in inflammation and pain signaling.
Place and Duration of Study: Center for Biomedical and Life Sciences, Missouri State University, between September 2017 and December 2018.
Methodology: Methanol extracts were prepared from *L. barbarum, S. canadensis, R. copallinum*, and *V. missurica*, while an aqueous extract was prepared from *V. californica*. Primary cultures of

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rat trigeminal ganglion cells were utilized to investigate cellular changes mediated by the extracts on basal levels of MKP-1 and sorbitol-stimulated expression of the signaling proteins P-ERK and P-p38 using immunocytochemistry and fluorescent microscopy. Toxicity of each extract was evaluated by trypan blue exclusion and polyphenol levels were determined using the Folin-Ciocalteu reagent and reported as gallic acid equivalents.

Results: Each extract tested caused a significant increase (P<.05) in basal levels of MKP-1 in trigeminal neurons and satellite glial cells when compared to untreated cells. Sorbitol treatment of cultured cells stimulated expression of the inflammatory signaling proteins P-ERK and P-p38 in neurons and glial cells. This stimulatory effect on ERK and p38 was significantly inhibited (P<.05) to near basal levels in both neurons and glia by overnight incubation with the extracts. None of the extracts caused cell toxicity and all extracts were determined to contain polyphenols, with *V. californica* and *V. missurica* exhibiting the highest levels.

Conclusions: The findings of this study provide evidence of a cellular mechanism by which plant extracts modulate trigeminal ganglion neurons and glial cells to inhibit inflammatory and pain signaling, and thus may be beneficial in managing orofacial pain conditions such as migraine and temporomandibular disorder.

Keywords: Trigeminal; phosphatase; kinase; inflammation; neuron; glia.

1. INTRODUCTION

Chronic pain associated with prevalent, disabling inflammatory diseases involving the head and face including migraine and temporomandibular disorder (TMD) is mediated by the trigeminal or fifth cranial nerve [1-4]. The trigeminal nerve provides the pathway for the transmission of painful stimuli from the peripheral tissues to the ganglion and central nervous system [5]. In response to tissue injury, inflammatory molecules are released from the terminals of trigeminal fibers to promote an inflammatory response and initiate pain signaling [6]. Activation of the trigeminal nerve also causes release of inflammatory molecules in the ganglion that increases signaling between the neurons and satellite glial cells to promote an inflammatory loop that lowers the activation threshold of neurons resulting in peripheral sensitization [7-9].

The development of peripheral sensitization, which is implicated in the underlying pathology and enhanced pain state associated with migraine and TMD, involves increased expression of the mitogen-activated protein (MAP) kinases [10,11]. This family of intracellular signaling proteins functions to promote an inflammatory response by stimulating the synthesis and release of pro-inflammatory molecules from trigeminal neurons and satellite glial cells in response to chemical, mechanical, and thermal stimuli as well as osmotic stress. The members include the extracellular regulated protein kinase (ERK), c-Jun nuclear protein kinase (JNK), and p38. The activity of the MAP kinases to promote inflammation and pain signaling is regulated by the MAP kinase phosphatases, which are a family of proteins known to protect against uncontrolled inflammation that could lead to tissue damage and chronic pain [12,13].

In a previous study from our laboratory, daily inclusion of a grape seed extract from *V. californica* as a dietary supplement was shown to increase basal levels of MKP-1 and repress stimulated expression of active p38 in trigeminal neurons and satellite glial cells in Sprague Dawley rats [14]. *V. californica* is used as a natural antioxidant [15] and is enriched in bioactive phenolic compounds that decrease inflammatory signaling [16]. Many health benefits are attributed to *V. californica* including its ability to improve liver function, protect against development of cardiovascular disease and cancer, and neuroprotection [17,18].

In addition, other traditional plants have been reported to possess diverse medicinal properties. For example, *L. barbarum* is the most popular variety of goji berry or wolfberry, is the legal resource recorded in the Chinese Pharmacopoeia, and is listed as one of the geographical indication products in China. *L. barbarum* possesses numerous properties that contribute to human health including antioxidant potential, regulation of immune cell function, inhibition of tumor growth, and protection of the liver and vision [19, 20]. Similarly, many health benefits are credited to consumption of elderberries (*Sambucus nigra, subsp. canadensis* L.) such as protection against viral infections, reduced risk of cardiovascular disease, neurological diseases, and cancer [21].

Rhus (winged sumac) has a long history as a folk medicine with many health benefits ascribed to its consumption including its ability to function to protect the nervous, digestive, immune, and cardiovascular systems [22-24]. Iron weed (*Veronica*) has been used as a medicinal plant to protect against diabetes, parasites, and malaria, and more recently to reduce fever, headache pain, inflammatory skin diseases, and joint pain [25-29]. Taken together, the reported health benefits of each of these plants is likely due to their potent anti-inflammatory and anti-oxidant activities mediated by secondary plant products. However, there is less direct evidence for the ability of these plants to regulate cellular pathways involved in pain signaling.

Given the prevalence, significant morbidity, and major social and economic ramifications of chronic orofacial pain conditions [30-32], there is a critical need for improved therapeutic and preventative measures. Hence, identification of novel alternatives to pharmaceutical drugs to modulate inflammation and pain is warranted. The use of natural products found in plants provides the potential for managing inflammatory responses and pain signaling. In this study, the hypothesis that extracts from several traditional medicinal plants contain active biological molecules to increase expression of MKP-1 and subsequently repress induction of P-ERK and P-p38 in cultured trigeminal ganglion cells was investigated. Our findings provide evidence that each of the plants tested may be beneficial in the treatment of orofacial diseases by inhibiting MAP kinase signaling, a key pathway involved in the initiation and maintenance of inflammation and pain [33].

2. MATERIALS AND METHODS

2.1 Plant Materials

Grape seed extract made from California grapes (*Vitis californica* Benth) was purchased from Healthy Origins (MegaNatural-BP Grape Seed Extract 300 mg, Pittsburgh, PA, USA). Dried goji berries (*Lycium barbarum* L.) were provided as a gift from Ningxia University School of Agriculture (Zhongning County, Ningxia Province, P.R.China). Elderberry (*Sambucus canadensis* L.), winged sumac (*Rhus copallinum* L.), and ironweed (*Veronica missurica* Raf.) were collected from a farm in Christian County (Southwest Missouri, USA). All plant names were checked using the website http://www.theplantlist.org.

2.2 Animals and Reagents

Adult female Sprague Dawley rats (Charles River Laboratories Inc.; Wilmington, MA) were housed in clean plastic cages on a 12-hour light/dark cycle with unrestricted access to food and water. All animal protocols and procedures were approved by the Institutional Animal Care and Use Committee at Missouri State University. MKP-1 rabbit antibody (Catalog: ab61201; Lot # GR140703-4) was purchased from Abcam (Cambridge, MA). P-p38 rabbit antibody (Catalog: AF869; Lot # DJA 1115011) was purchased from R&D Systems (Minneapolis, MN). P-ERK rabbit antibody (Catalog: BS5016; Lot # CN893300) was purchased from Bioworld Technology, Inc. (St. Louis Park, MN). Alexa Fluor® 488-conjugated AffiniPure Donkey anti-rabbit IgG was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Methanol, ethanol, gallic acid, Folin-Ciocalteu reagent, sorbitol, ascorbic acid, glutathione, glutamine, glucose, poly-D-lysine, penicillin, streptomycin, and trypan blue were all purchased from Sigma Aldrich (St. Louis, MO). Fetal bovine serum was obtained from Atlanta Biologicals, Inc., Flower Branch, GA), 2.5 S nerve growth factor from Alomone Laboratories (Jerusalem, Israel), and glass coverslips from BD Biosciences (Bedford, MA).

2.3 Preparation of Plant Extracts

Dried goji berries (*L. barbarum*), elderberries (*S. canadensis*), winged sumac (*R. copallinum*) berries, and ironweed (*V. missurica*) flower petals were collected and stored at 4°C prior to preparation of extracts. The raw material was frozen in liquid nitrogen and then ground to a fine powder using a cooled mortar and pestle. Four grams of pulverized sample was suspended in 30 mL of 99.9% methanol and mixed for 5 minutes with a PRO250 homogenizer (Oxford, CT). Homogenized samples were then vortexed for 1 minute before being spun for 5 minutes at ~20°C at 3000 rpm in an Eppendorf 5810 R centrifuge. The supernatant was removed in 10 mL aliquots and placed into individual preweighed borosilicate tubes and dried using a GeneVac EZ-2 personal solvent evaporator.

2.4 Primary Culture of Trigeminal Ganglia

Primary cultures of trigeminal ganglia were established based on our previously published protocols [34- 36]. Briefly, trigeminal ganglia were obtained from 3- or 4-day old rat pups and placed in 15 ml polypropylene tubes containing

10 ml of ice-cold L-15 plating medium (Leibovitz; Sigma; St. Louis). Following centrifugation and removal of the supernatant, ganglia were suspended in L-15 medium containing 10 mg/ml Dispase II (Invitrogen Corp., Carlsbad, CA) and 1 unit/µl RQ1 DNase (Promega; Madison, WI). The tubes were rotated at 37°C at 15 rpm for 30 minutes to facilitate digestion of the tissues, and then centrifuged for 3 minutes at 150 × *g* to pellet the tissues. The supernatant was discarded, and the tissue pellets were combined and resuspended in L-15 medium. The tissues were dissociated into a single-cell suspension by vigorous pipetting. The cells were pelleted by centrifugation at 300 × *g* for 3 minutes. The resulting cell pellets were resuspended in L-15 complete medium (50 mM glucose, 250 µM ascorbic acid, 8 µM glutathione, 2 mM glutamine) supplemented with 10% fetal bovine serum, 10 ng/ml mouse 2.5 S nerve growth factor, 100 units/ml penicillin, and 100 µg/ml streptomycin and plated at a density of 0.33 ganglia per poly-D-lysine coated glass coverslip, which corresponds to ~10,000 cells per coverslip. The coverslips were placed in 24 well tissue culture plates and incubated at 37°C.

2.5 Determining Effect of Plant Extracts on Primary Trigeminal Ganglion Cultures

Following overnight incubation of the cells in L15 complete medium, cells were left untreated (control) or incubated with extracts prepared as a 0.05% (w/w) solution with sterile phosphate buffered saline (PBS) from *V. californica*, *L. barbarum, S. canadensis R. copallinum*, or *V. missurica*. The extracts were added to the cells at a final dilution of 1:100 in L-15 complete medium and the plates placed in the 37°C incubator for 24 hours. Following the overnight incubation, some cells were incubated for 30 minutes at 37^{o}C in HEPES-buffered saline (HBS; 22.5 mM HEPES, 135 mM NaCl, 3.5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 3.3 mM glucose, and 0.1% BSA, pH 7.4) containing sorbitol at a final concentration of 0.6 M to stimulate MAP kinase expression [37]. To prepare cells for immunostaining, untreated naïve control cells and treated cells were rinsed with PBS and incubated in 4% paraformaldehyde for 15 minutes at room temperature, rinsed with PBS, and stored at 4°C. To test for possible cytotoxic effects of the various treatments, neuronal and glial cell viability was assessed in trigeminal cultures 24 hours after incubation with extracts using the trypan blue exclusion method (Fisher Scientific, Waltham, MA, 15250061) and visualized under a Motic AE31 microscope equipped with a Leica EC3 camera using LAS EZ software (Leica, Wetzlar, Germany). Each experimental condition was performed in duplicate on the plate and repeated in at least three independent experiments.

2.6 Immunocytochemistry

After fixation in paraformaldehyde, cells were incubated for 20 minutes in a PBS solution containing 0.1% triton and 5% donkey serum (Jackson ImmunoResearch Laboratories) to permeabilize cell membranes and block non-specific antibody binding. The primary antibodies MKP-1 (1:500), P-p38 (1:100), or P-ERK (1:40) were diluted in PBS containing 5% donkey serum and added to cells for overnight incubation at 4°C. Cells were washed in 0.1% Tween in PBS and rinsed in PBS prior to incubation for 1 hour at room temperate with Alexa Fluor® 488-conjugated AffiniPure Donkey anti-rabbit IgG secondary antibodies (1:200). Cells were again rinsed and then mounted using Vectashield media containing 4', 6-diamidino-2- phenylindole (DAPI, Vector Laboratories; Burlingame, CA) as a nuclear marker. Multiple image alignment of 200X magnifications were obtained using a ZEISS AxioCam MRm camera mounted on a ZEISS Axio Imager Z2 fluorescent microscope (Carl Zeiss MicroImaging GmbH, Gottingen, Germany). Image acquisition was performed using ZEISS ZEN PRO 2012 processing software. The fluorescence intensity of immunostained cells was determined using Image J Image Analysis Software. The relative fluorescence intensity was reported as the intensity of the fluorescence signal divided by DAPI stained nuclei fluorescence intensity.

2.7 Total Phenolic Content Assay

The total phenolic content of plant extracts was determined using the Folin-Ciocalteu method [38]. Briefly, the dried extracts were reconstituted in distilled water heated to 55°C to achieve a 10% solution (w/w). The 10% extract solutions were further diluted to 1:1000, 1:2000, and 1:4000 in 40% ethanol and added in triplicate to a 96-well plate. Folin-Ciocalteu reagent (0.25N) was added to each well and allowed to incubate for 2 minutes at room temperature before the addition of 1M sodium carbonate and further incubation with mixing on an orbital shaker for 2 hours at room temperature. The absorbance at

760 nm was determined using a Spectra Max® Plus 384 Microplate Reader. Polyphenolic levels are reported as mg/g gallic acid equivalents (GAE) for each extract.

2.8 Statistical Analysis

Data are presented as the mean±standard error of the mean (SEM). Each experimental condition was repeated in at least three independent experiments. Analysis was performed using a Mann-Whitney U test (SPSS Statistics Data Editor Version 24). *P*<.05 was considered to represent a statistically significant difference.

3. RESULTS

3.1 Plant Extracts Increased Basal MKP-1 Expression

As seen in Fig. 1, overnight incubation with extracts from *V. californica* or *L. barbarum* correlated with a significant increase (*P*<.05) in the basal level of expression of MKP-1 in primary trigeminal ganglion cultures when compared to the average level in untreated cells (1.00±0.005). The stimulatory effects mediated by *V. californica* (1.31±0.064) and *L. barbarum* (1.22±0.043) on MKP-1 levels were observed in the cytosol and nuclei of Aδ and C-fiber neurons as well as in the satellite glial cells when compared to naïve cells (Fig. 2). Similar stimulatory effects on the relative basal staining intensity of MKP-1 were observed in all three cell types following incubation with extracts from *S. canadensis*, *R. copallinum*, and *V. missurica* (Table 1).

Table 1. The effect of plant extracts on the relative intensity of MKP-1 in trigeminal ganglion cell cultures (N = 4)

Groups	MKP-1
Naïve	1.00±0.005
SCE	1.20±0.037*
VME	1.21±0.062*
RCE	1.16±0.017*

**P < 0.05 when compared to naïve*

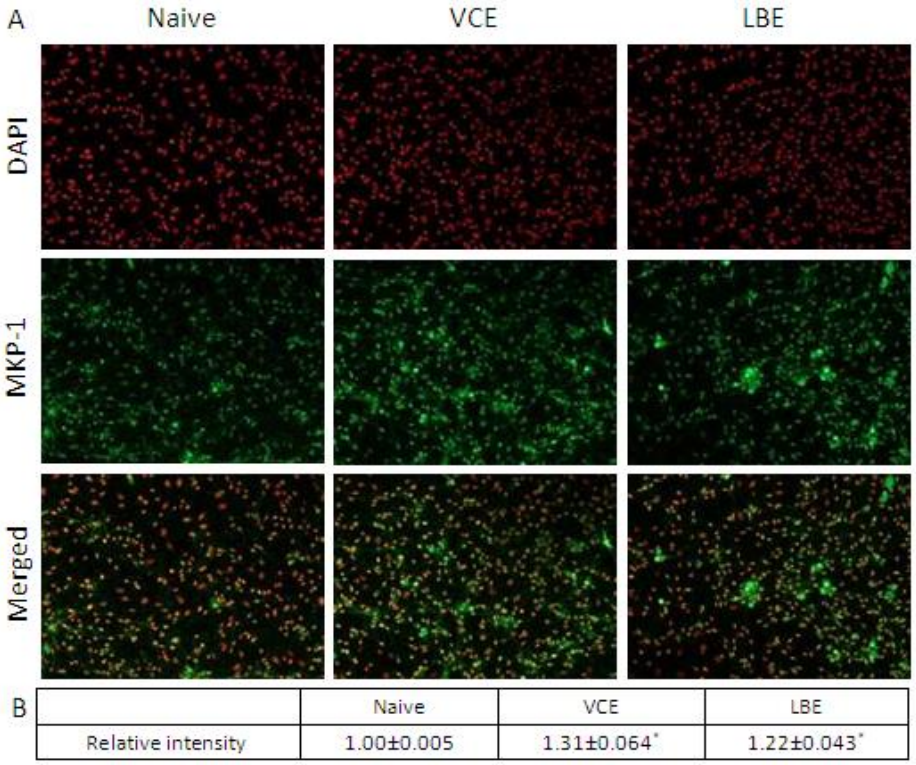


Fig. 1. Extracts from *V. californica* (VCE) or *L. barbarum* (LBE) increased basal MKP-1 expression in primary trigeminal ganglion cultures

*(A) Fluorescent images (200×) were obtained from naïve cells, VCE treated cells, and LBE treated cells. (B) The average relative intensity ± SEM of MKP-1 staining is reported. (N = 4 independent experiments performed in duplicate). *P<.05 when compared to normalized naïve levels.*

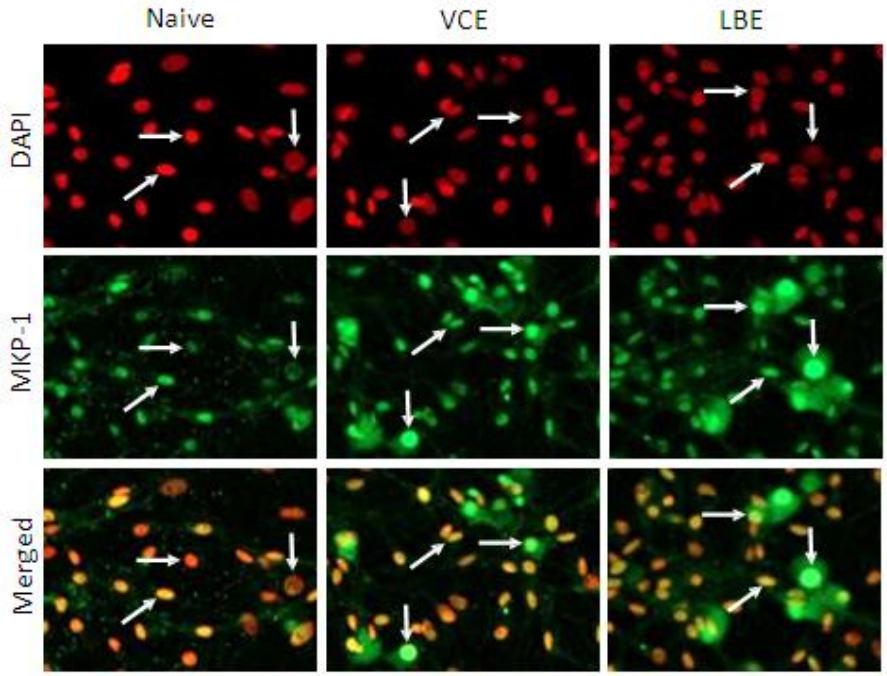


Fig. 2. Increased basal MKP-1 expression in Aδ and C fiber neurons and satellite glial cells in response to overnight incubation with extracts from *V. californica* (VCE) or *L. barbarum* (LBE)

Enlarged images from boxed regions shown in Fig. 1 that demonstrate immunolocalization of MKP-1 in neuronal cell bodies and satellite glial cells (N = 4). Vertical arrows indicate Aδ neurons. Horizontal arrows indicate C fiber neurons. Diagonal arrows indicate satellite glial cells

3.2 Plant Extracts Repressed Sorbitol-stimulated P-p38 Expression

In response to sorbitol, the level of expression as measured by a change in the relative staining intensity of the active, phosphorylated form of p38 (1.42±0.098) in trigeminal ganglion cultures was markedly increased (*P*<.01) compared to levels in naïve cells (1.00±0.018) (Fig. 3). However, the expression of P-p38 in sorbitol-stimulated cells was repressed in response to pretreatment with extracts from *V. californica* (1.12±0.042) or *L. barbarum* (1.14±0.033). As shown in Fig. 4, the P-p38 immunostaining intensity was greatly increased in the cytosol and nuclei of Aδ and C fiber neurons and satellite glial cells when compared to relative levels in naïve cultures. The inhibitory effects of *V. californica* and *L. barbarum* involved repression of sorbitol-stimulated P-p38 expression in both types of neurons and satellite glial cells. In agreement with the MKP-1 results, incubation with *S. canadensis, R. copallinum, and V. missurica* was sufficient to inhibit the stimulatory effects of sorbitol on P-p38 levels in neuronal and glial cells (Table 2).

Table 2. The effect of plant extracts on the relative intensity of P-p38 and P-ERK in trigeminal ganglion cell cultures (N = 4)

Groups	P-p38	P-ERK
Naïve	1.00±0.015	1.00±0.028
Sorbitol	1.42±0.098**	1.35±0.039*
Sorbitol+SCE	1.07±0.019$^{\#}$	1.19±0.027$^{\#}$
Sorbitol+VME	1.04±0.013$^{\#}$	1.17±0.031$^{\#}$
Sorbitol+RCE	1.11±0.019$^{\#}$	1.13±0.018$^{\#}$

** P<.05 when compared to naïve. ** P<.01 when compared to naïve. $^{\#}$P < 0.05 when compared to sorbitol*

3.3 Plant Extracts Repressed Sorbitol-stimulated P-ERK Expression

As seen in Fig. 5, sorbitol treatment caused a significant increase in the expression of the active, phosphorylated form of ERK (1.35±0.039) in trigeminal ganglion cultures was markedly increased (*P*<.01) compared to levels in naïve cells (1.00±0.028). In contrast, the intensity of P-ERK immunostaining in sorbitol-stimulated cells was greatly repressed in response to pretreatment with extracts from *V. californica* (1.05±0.036) or *L. barbarum* (1.07±0.011). As

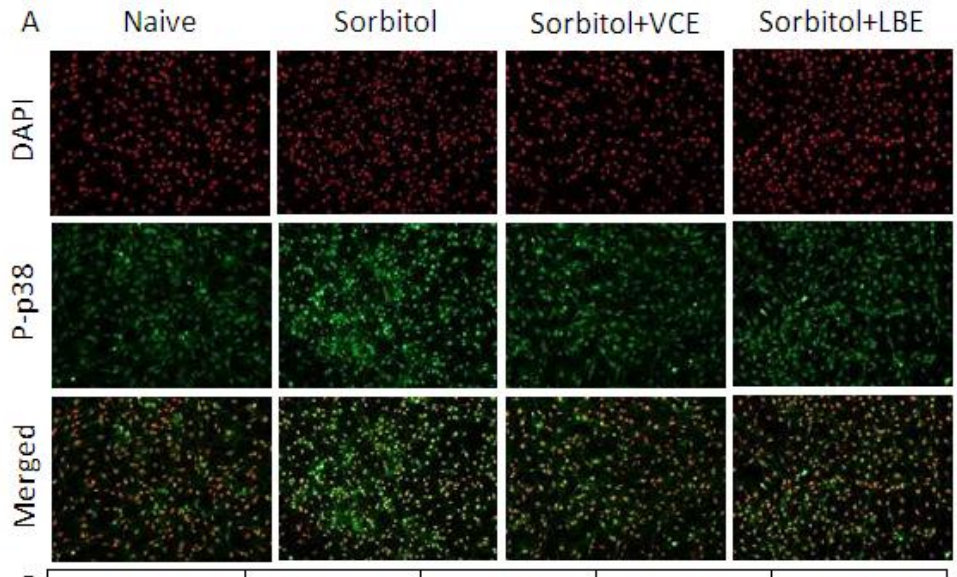
can be seen in Fig. 6, P-ERK expression was markedly enhanced in the cytosol and nuclei of Aδ and C fiber neurons and satellite glial cells when compared to relative levels in naïve cultures. The observed inhibitory effects of *V. californica* and *L. barbarum* involved suppression of sorbitol-stimulated P-ERK expression in both types of neurons and satellite glial cells. This inhibitory effect on P-ERK levels was also observed in sorbitol-treated cultures incubated overnight with extracts prepared from *S. canadensis, R. copallinum, and V. missurica* (Table 2).

3.4 Plant Extracts Are Not Toxic to Trigeminal Ganglion Neurons or Glia

Cell viability in response to overnight incubation with each of the plant extracts was determined using the trypan blue exclusion assay and phase microscopy. As shown in Fig. 7, the majority of cells are colorless and transparent, which is indicative of viable cells with intact cellular membranes. No significant differences were observed in the number of dead cells stained blue when comparing levels in naïve cultures to those treated with extracts. Overall, cell viability was ~98% based on cell counts. Thus, these results provide evidence that extracts from *V. californica, L. barbarum, S. canadensis, R. copallinum, and V. missurica* are not inducing cell toxicity at the concentrations shown to increase basal MKP-1 levels and suppress sorbitol-stimulated expression of P-p38 and P-ERK.

3.5 The Yield and Total Phenolic Content of Plant Extracts

The polyphenol levels in the extracts was determined with Folin-Ciocalteu reagent and reported as gallic acid equivalents. The yield and total polyphenolic content of the plant extracts is shown in Table 3. While *L. barbarum* had the highest yield (210.4 mg/g), the extract exhibited the lowest total phenolic content (72.5 GAE/g). In contrast, the yield of *S. canadensis* was the lowest (29.5 mg/g), but the total phenolic content was relatively high (256.8 GAE/g). The total phenolic content of *V. missurica* is particularly high (1037.7 GAE/g), which is close to that of commercial *V. californica* (1781.0 GAE/g).



B

	Naive	Sorbitol	Sorbitol+VCE	Sorbitol+LBE
Relative intensity	1.00±0.018	1.42±0.085**	1.12±0.042#	1.14±0.033#

Fig. 3. VCE and LBE repressed sorbitol-stimulated P-p38 expression in primary trigeminal ganglion cultures

*(A) Fluorescent images (200×) of naïve cells, cultures treated for 30 minutes with 0.6 M sorbitol, or sorbitol treated cells incubated overnight in the presence of extracts from V. californica (VCE) or L. barbarum (LBE). (B) The average staining intensity ± SEM for the active, phosphorylated form of p38 (P- p38) is reported. (N = 5 independent experiments performed in duplicate). * * P<.01 when compared to naïve levels. # P<.05 when compared to sorbitol-treated levels*

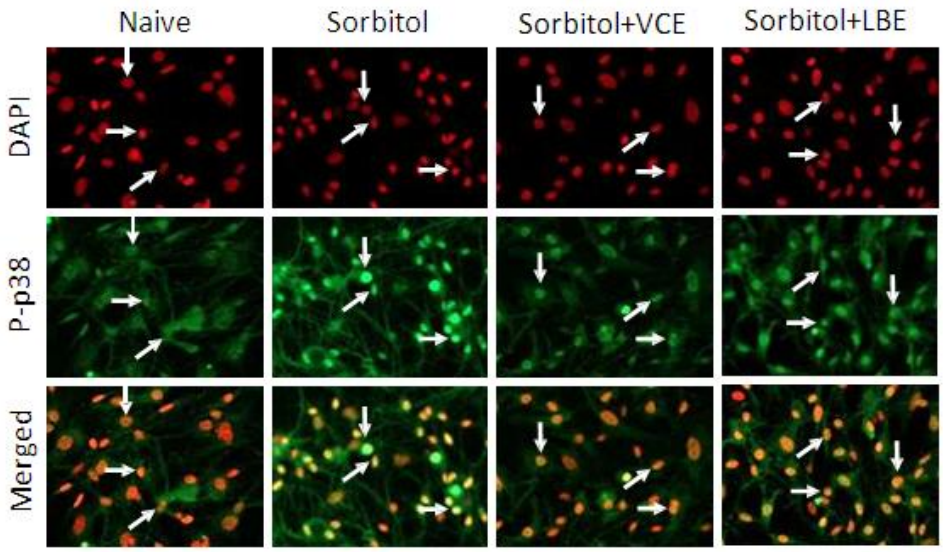
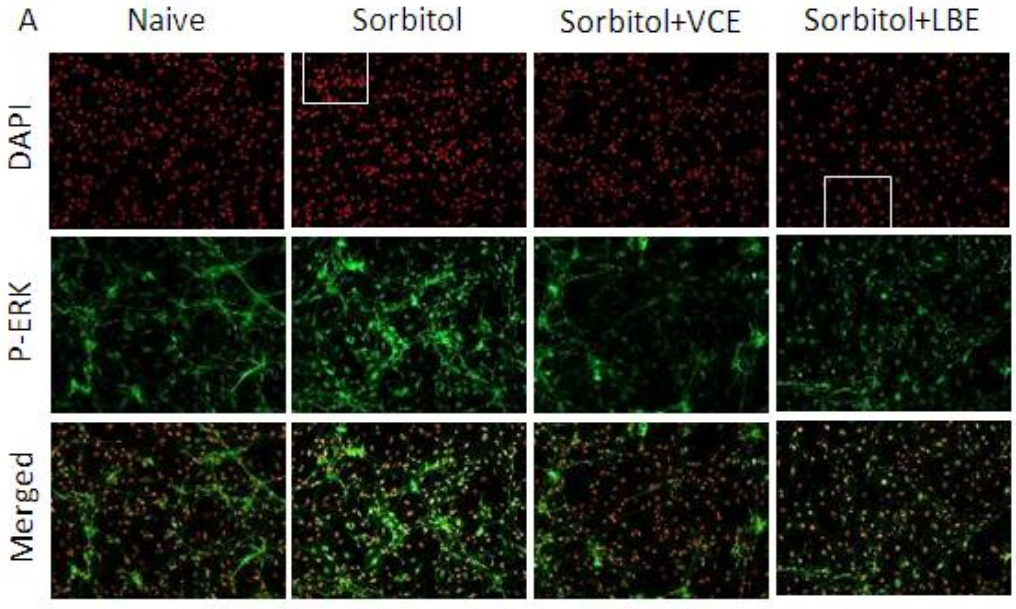


Fig. 4. Repression of sorbitol-stimulated P-p38 expression in Aδ and C fiber neurons and satellite glial cells by overnight incubation with extracts from *V. californica* (VCE) or *L. barbarum* (LBE)

Enlarged images from boxed regions shown in Fig. 3 illustrate localization of P-p38 staining in neuronal cell bodies and satellite glial cells (N = 5). Vertical arrows indicate Aδ neurons. Horizontal arrows indicate C fiber neurons. Diagonal arrows indicate satellite glia



B

	Naive	Sorbitol	Sorbitol+VCE	Sorbitol+LBE
Relative intensity	1.00±0.025	1.35±0.034*	1.05±0.036#	1.07±0.011#

Fig. 5. VCE and LBE repressed sorbitol-stimulated P-ERK expression in primary trigeminal ganglion cultures

*(A) Fluorescent images (200×) of naïve cells, cultures treated for 30 minutes with 0.6 M sorbitol, or sorbitol treated cells incubated overnight in the presence of extracts from V. californica (VCE) or L. barbarum (LBE). (B) The average staining intensity ± SEM for the active, phosphorylated form of ERK (P-ERK) is reported. (N = 5 independent experiments performed in duplicate). * * P<.01 when compared to naïve levels. # P<.05 when compared to sorbitol-treated levels*

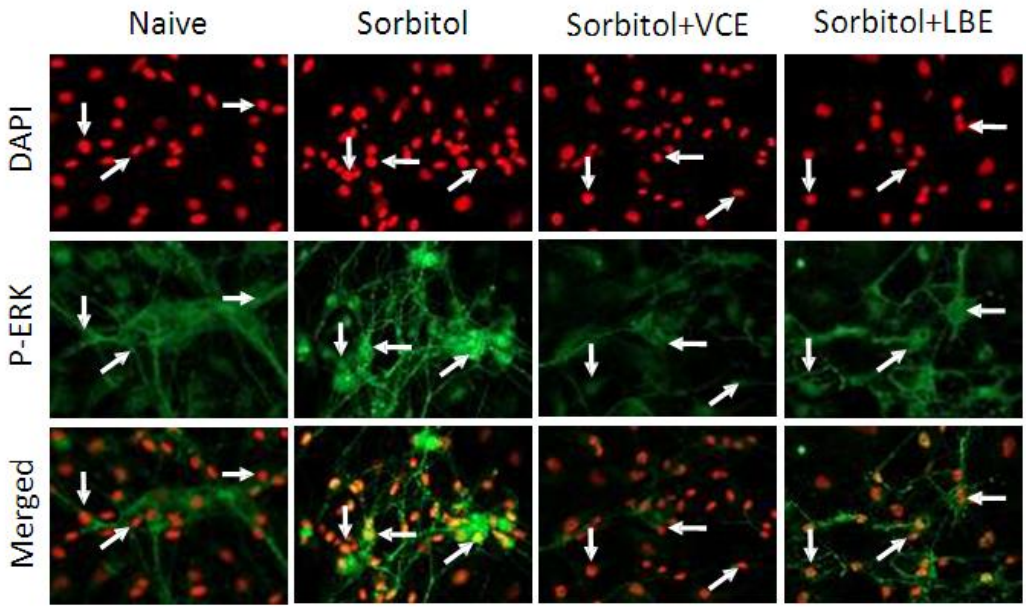


Fig. 6. Repression of sorbitol-stimulated P-ERK expression in Aδ and C fiber neurons and satellite glial cells by overnight incubation with extracts from *V. californica* (VCE) or *L. barbarum* (LBE)

Enlarged images from boxed regions shown in Fig. 5 demonstrate localization of P-ERK staining in neuronal cell bodies and satellite glial cells (N = 5). Vertical arrows indicate Aδ neurons. Horizontal arrows indicate C fiber neurons. Diagonal arrows indicate satellite glia

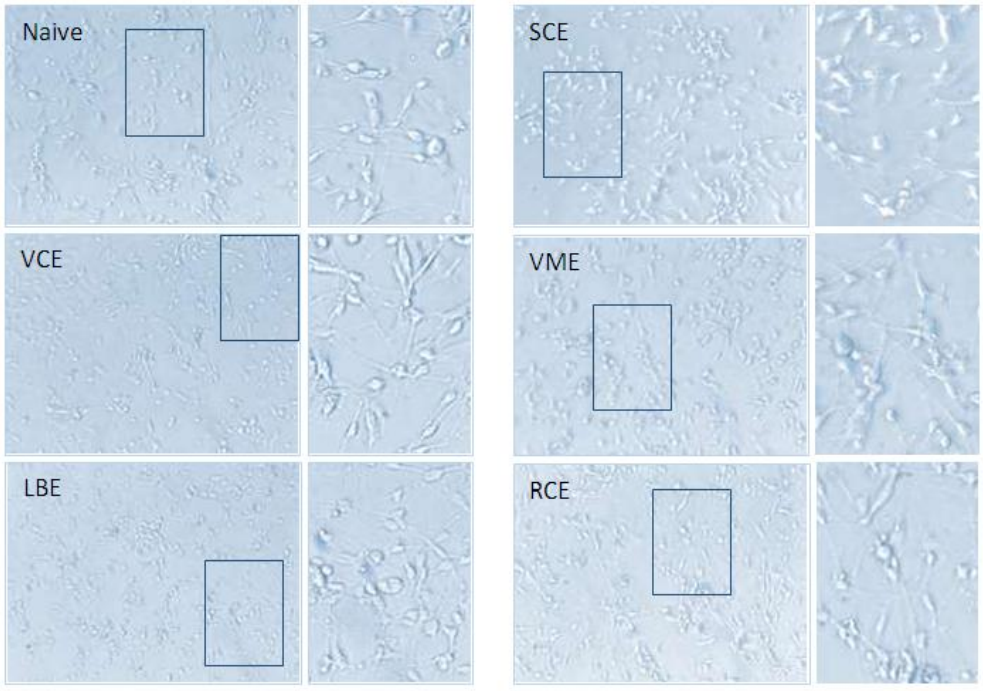


Fig. 7. Overnight incubation with extracts did not affect cell viability

Untreated cultures or cultures incubated overnight with extracts from V. californica (VCE), L. barbarum (LBE), S. canadensis (SCE), R. copallinum (RCE), and V. missurica (VME) were analyzed using phase microscopy (100X) to determine the presence of trypan blue as a marker of dead or dying cells. Cell viability was > 95% for each of the extracts when compared to naïve levels

Table 3. The yield and total phenolic content of plant extracts

Plant Extracts	Yield (mg/g raw material) (N = 2)	Total phenolic content (mg GAE/g) (N = 3)
VCE	--	1781.0 ± 66.51
LBE	210.4 ± 1.31	72.5 ± 1.05
SCE	29.5 ± 0.14	256.8 ± 1.6
VME	117.7 ± 1.06	1037.7 ± 53.05
RCE	100.6 ± 4.49	382.5 ± 11.6

4. DISCUSSION

Results from our *in vitro* study provide evidence that extracts from plants that have been used traditionally or as folk medicine to treat diverse human diseases can inhibit pathways in the trigeminal ganglion known to be involved in pain signaling and promoting inflammation. Sensitization and activation of trigeminal neurons is implicated in the underlying pathology of orofacial pain conditions including migraine [39], TMD [40], and tooth and gum inflammation and pain [6]. One of the extracts of interest in our study was the water-based grape seed extract prepared from *V. californica* that we had shown previously to increase MKP-1 expression and inhibit stimulated activation of P-p38 and P-ERK in the trigeminal ganglion of male Sprague Dawley rats [14]. In agreement with results from the *in vivo* study, overnight incubation with a water extract of *V. californica* resulted in a significant increase in the basal level of MKP-1 in cultured trigeminal neuronal and satellite glial cells. The stimulatory effects of *V. californica* on MKP-1 were observed in myelinated, fast conducting Aδ cell bodies as well as slower conducting C fiber neurons, and satellite glial cells, which function to modulate the basal excitability state of trigeminal neurons and their response to inflammatory stimuli [8,9,41]. MKP-1 is a member of the proteins that regulate the inflammatory response of MAP kinases to restore and maintain homeostasis in many diverse cell types including neurons and glial cells [13,42]. Similar to the effect of *V. californica*, overnight incubation with the extracts from *L. barbarum, S. canadensis R. copallinum*, and *V. missurica* stimulated increased expression of MKP-1 in neuronal cell bodies of Aδ and C fiber neurons, and satellite glial cells. While it is known that these extracts exhibit anti-inflammatory properties, to our knowledge, this is the first evidence that extracts from these plants promote upregulation of the basal levels of MKP-1 in trigeminal ganglion cells implicated in pain signaling during migraine, TMD, and other orofacial pain conditions. Interestingly, the anti-inflammatory effect of the steroid drug dexamethasone, which is used in the treatment of a multitude of inflammatory diseases, is reported to involve the upregulation of MKP-1 and suppression of MAP kinase inflammatory signaling [43-46]. Thus, our results provide evidence that extracts from *V. californica*, *L. barbarum, S. canadensis R. copallinum*, and *V. missurica* contain biological molecules that function in a similar manner to a clinically beneficial medication.

Having demonstrated that these extracts could increase neuronal and glial expression of MKP-1, a protein reported to dephosphorylate and hence inactivate the MAP kinases ERK and p38, their effect on stimulated trigeminal neurons and satellite glial cells was investigated. Initially, sorbitol was used to stimulate expression of the active, phosphorylated forms of the MAP kinases ERK and p38 [47, 48]. Upon activation, P-ERK and P-p38 translocate into the nucleus and phosphorylate specific transcription factors that bind to promoters of genes known to promote and sustain an inflammatory response and facilitate pain signaling [11]. As expected, treatment of cultured trigeminal ganglion cells with sorbitol resulted in a significant increase in the levels of P-ERK and P-p38 in Aδ and C fiber neurons and the satellite glial cells. While Aδ neurons are responsible for protecting tissues from harmful stimuli by mediating a rapid withdrawal reflex, C fiber neurons are responsible for a more sustained inflammatory and painful response to noxious thermal, mechanical, or chemical stimuli. Elevated levels of P-ERK and P-p38 in satellite glial cells are reported to increase the synthesis and release of inflammatory cytokines [49] and nitric oxide [50, 51] than can promote establishment of an inflammatory loop within the ganglion to mediate peripheral sensitization, a state characterized by a lowering of the activation threshold of Aδ and C fiber neurons. Peripheral sensitization of trigeminal neurons is implicated in the underlying pathology of migraine and TMD [3,40] and in support of this notion, drugs that target this hyperexcitable state are effective in inhibiting pain levels [52-54]. Importantly, in this study

overnight incubation with each of the five plant extracts was sufficient to significantly repress the sorbitol-stimulated expression of P-ERK and P-p38 and reduce the active levels observed in the nucleus of Aδ and C fiber neurons and satellite glial cells. Based on results from the trypan blue exclusion assay, the inhibitory effects of the extracts were not caused by cytotoxicity, but rather were likely mediated at least in part by the increased expression of MKP-1. Our findings are in agreement with results from other studies that extracts from these plants exhibit anti-inflammatory and anti-nociceptive responses. For example, our results are in agreement with findings that leaf extracts of *V. amygdalina,* which is used as a medicinal plant in certain regions of the world, possess anti-nociceptive properties as well as mediating anti-inflammatory effects by reducing migration of leukocytes and lipid peroxidation [27]. Taken together, there is a growing body of knowledge to support the use of these traditional plants in the management of human diseases by repressing inflammatory signaling in diverse cell types including immune, hepatic, cardiovascular, and intestinal cells. Based on our results, each of the plant extracts contain biologically active molecules that can inhibit phosphorylation, and hence activation of the MAP kinases ERK and p38 that are known to be involved in the initiation and maintenance of peripheral sensitization, inflammation, and pain signaling.

Many of the reported health benefits of plants are due to their secondary plant products, which mediate anti-inflammatory and anti-nociceptive responses in different cell types. Not surprisingly, all plant extracts evaluated in this study had detectable levels of polyphenolic compounds, reported as GAE, with *V. californica* exhibiting the highest level and *L. barbarum* the lowest level. Although not directly investigated in this study, the inhibitory effects of the extracts on MAP kinase signaling are likely to involve polyphenolic compounds. We can only speculate that other non-water-soluble compounds may also contribute to MKP-1 upregulation and suppression of P-p38 and P-ERK expression. Interestingly, *L. barbarum* has been reported to produce the phytosterol compound β-sitosterol, which is a potent anti- inflammatory compound and one of the main active compounds in *Aloe vera* and *Theobroma cacao*.

5. CONCLUSIONS

In summary, our findings provide evidence that extracts from 5 different plants including *V. californica*, *L. barbarum, S. canadensis R. copallinum*, and *V. missurica*, contain biologically active compounds that increase the basal level of MKP-1 in trigeminal ganglion neurons and glial cells and inhibit the stimulated upregulation of P-ERK and P-p38. Thus, these extracts can modulate the activity of key proteins to suppress trigeminal pain signaling pathways implicated in the underlying pathology of migraine and TMD. It will be of interest in future studies to incorporate these extracts into the diet either dissolved in their water or contained in the food pellets to determine if they can inhibit inflammation and pain in orofacial pain models. This approach was utilized in the characterization of a methanol extract from *Theobroma cacao* initially shown to inhibit trigeminal neurons activation in primary cultures [34] and then later incorporated as a nutritional supplement and found to suppress inflammation and pain in a model of TMD pathology [55,56]. Taken together, results from this study demonstrate that bioactive compounds are present in the extracts from plants used in traditional medicine to treat inflammatory and painful conditions involving the head and face including migraine, TMD, and toothache. Given that similar pathways are activated in the dorsal root ganglion to mediate inflammation and pain in other peripheral areas of the body [57,58], our findings may also help to explain why these extracts are reported to be beneficial in treating other human diseases.

ETHICAL APPROVAL

As per international standard or university standard written ethical permission has been collected and preserved by the author(s).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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