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PROTECTIVE EFFECT OF AQUEOUS EXTRACT OF CALEA URTICIFOLIA IN A MOUSE MODEL OF DIABETIC NEUROPATHY

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Abstract: Diabetes mellitus is a disease that causes microvascular alterations and degeneration in sensory nerves; The symptoms result in the presence of pain and functional impairments regulated by the peripheral nervous system. These conditions are referred to as diabetic peripheral neuropathies. One medicinal plant for the prevention and treatment of diabetes-associated neuropathic changes is *Calea urticifolia (CuAqE)*, which is utilized by the Xi'uy ethnic group in México. In this study, we aimed to evaluate the therapeutic potential of CuAqE using a dose of 11 mg/kg in type-I diabetic neuropathy model induced by low doses of streptozotocin to 50mg/kg in C57BL/6 mice, characterized by fasting blood glucose >200 mg/dL on C57BL/6 mice. The objective of this study was to evaluation of CuAqE using non-stimulus evoked nociception methods, including the Mouse Grimace Scale and Body Condition Score as well as stimulus-evoked pain-like behaviors, specifically the mechanical paw withdrawal threshold. CuAqE administration resulted in a decrease in hyperglycemia levels; however, it failed to maintain physiological glucose levels and produced pain attenuation without affecting constipation behavior ($p<0.005$). In diabetic mice, thresholds for mechanical hypersensitivity were reduced, and CuAqE treatment did not show a significant change ($p<0.001$). These findings reveal protective action of CuAqE against pain hypersensitivity in mouse models of type I diabetes and provide insights for the development of novel approaches to manage diabetes using traditional medicine.
Keywords: Diabetic peripheral neuropathy, DM-1, aqueous extract

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by insulin resistance that induces hyperglycemia. Type 1 diabetes mellitus (DM-I), also known as insulin-dependent, accounts for approximately five percent of all diabetes cases worldwide. It is characterized by a loss of insulin production and impaired insulin signaling pathway. DM-I is primarily caused by the autoimmune destruction of pancreatic β cell responsible for insulin production, leading to insulin deficiency and elevated blood glucose levels throughout the body (King & Bowe, 2016).

Approximately 60% of all diabetic patients develop diabetic peripheral neuropathy (DPN), which is the most common microvascular complication. DPN is characterized by the progressive degeneration of peripheral nerves, starting from the distal areas and extending towards the proximal regions. This degeneration leads to sensory symptoms such as pain, weakness, and/or loss of sensation. The clinical manifestations, including numbness, tingling, pain, or weakness, vary depending on the specific nerve fibers affected. Initially, neuropathy affects the smaller nerve fibers, resulting in hyperalgesia and allodynia (increased sensitivity to painful or nonpainful stimuli, respectively). Dysfunction of the large myelinated nerve fibers is reflected in electrophysiological changes and alterations in the density of myelinated fibers in the sural nerve (O'Brien *et. al.* 2014).

Neuropathic pain is a pain persistent, severe, and debilitating form of chronic pain that arises from a lesion or disease affecting the somatosensory system. It can be triggered by physical trauma or diseases like diabetes mellitus, which cause damage to peripheral nerves, the spinal cord, and the brain, where somatosensory information processed (Adamson *et al.,* 2016; Jensen *et al.*, 2011). Clinical characteristics of neuropathic pain include pain in areas with partial or complete sensory loss, various types of evoked pain, specific descriptors like burning pain, increased pain following repetitive stimulation, and pain

that persists after stimulation ceases. However, two particularly distressing and prominent symptoms in neuropathic pain are allodynia and hyperalgesia (Jensen & Finnerup, 2014).

Currently recommended pharmacological treatments for neuropathic pain have limited efficacy and often results in a range of side effects that can make them intolerable for patients (Dworkin *et al.*, 2010). Therefore, there is a need to identify new therapeutic targets that offer improved effectiveness and a better balance between pain relief and side effects. Complementary and alternative medicines show great promise for the prevention and treatment of diabetes-related neuropathic changes. One such example is *Calea urticifolia*, a medicinal plant used by the Xi'uy ethnic group in San Luis Potosí, Mexico. Traditional healers in this community employ it as an herbal remedy for the management of diabetes mellitus within their rudimentary health system (Torres *et al.,* 2016). The objective of this study was to evaluated the impact of a lyophilized aqueous extract of *Calea urticifolia* (CuAqE) on behavioral tests assessing peripheral nerve function in a Low-Dose Streptozotocin (STZ) model of type 1 diabetes mellitus (DM-1) to induce neuropathic pain.

MATERIALS AND METHODS

HARVEST OF HERBAL MATERIAL

The fresh leaves of *Calea urticifolia* were harvested in the Xi'uy ancient native community of Potrero del Carnero (21°52'27.6" N y 099°27'00.6", to 901 msnm, San Luis Potosí, Mexico) in July 2021. The plant material was authenticated by an herbalist from Isidro Palacios Herbarium (IIZD-UASLP, San Luis Potosí, México). After separation, the herbal material was placed on absorbent paper for drying and stored at room temperature until ready for use.

PREPARATION OF THE AQUEOUS EXTRACT

The dry leaves of *Calea urticifolia* were crushed using an electric mill prior to extraction. A mixture of 100g of crushed leaves and 1L of distilled water was boiled for 5 min. After the aqueous extract was cooled down, it was filtered and subjected to freeze-drying using a Freeze-dryer TFD5505. The yield of extract was 21.80% of the residue which was stored at 4˚C until it was ready for use.

ANIMAL PROCUREMENT AND HOUSING

Male C57BL/6 mice aged four to six weeks were purchased from CINVESTAV-IPN (Mexico City, Mexico). Upon arrival, the mice were housed in a controlled environment with a temperature of 21±2°C, humidity of 50±10%, and a 12-hour light/dark cycle (7:00 am to 7:00 pm). They were provided with standard rodent food (Formulab Chow 5001) and water ad libitum. The mice were allowed to acclimate for 5 days before the start of the experiment and were randomly assigned to treatment groups. Each group consisted of thirty animals, and all animals were individually housed in acrylic cages. The animal study protocol was reviewed and approved by the local Animal Ethical Review Committee (FCQ-UASLP, San Luis Potosí, Mexico), with registration code CEID2018015R2, and the handling of mice was carried out in accordance with animal ethics guidelines, following the Mexican Norm for Animal Care and Handling (NOM-062-ZOO-1999).

INDUCTION OF THE TYPE-I DIABETIC NEUROPATHY MODEL

Four groups were intraperitoneally injected (IP) with low doses for four or five consecutive days of STZ (50mg/kg, Sigma Aldrich, St. Louis, MO), while the control mice were given vehicle citrate buffer (pH 4.4-

4.5) at a dose volume of 0.25 mL/kg IP. The body weight of the mice was recorded weekly. After the STZ injection, all mice underwent a 12-hours fasting period, and fasting blood glucose (FBG) analysis was conducted. The success rate of the diabetic model (DSR) was calculated as a percentage of mice with FBG >200 mg/dL after one week of STZ injection.

ANIMAL TREATMENT

After six months of measuring blood glucose levels, the mice were randomly assigned to six groups (n=5/group) and given unrestricted access to water, food, and six different treatments. Two groups received distilled water and served as controls (CTL-DM and CTL-NOM), while the other two groups were administered doses of 11 mg/kg of CuAqE (CTL-EA and DM-EA) corresponding to the traditional oral doses. CuAqE was orally administered daily using an intra-gastric probe (0.1 mL/10 g body weight) to each mouse at the same timing and order for 60 days. CuAqE was dissolved in distilled water to prepare stocks and diluted as needed to achieve the desired final concentrations. All solutions were prepared fresh.

EVALUATION PAIN BEHAVIORS IN RODENTS NON-STIMULUS EVOKED NOCICEPTION

The body condition of mice was evaluated by palpating the sacroiliac bones (spine and hip bones) and assigning a score from one to five. A score of one indicates extreme thinness, while a score of five indicated obesity. Mice with an optimal body condition, scored a three, the bones are palpable but not prominent. Figure 1 depicts the visual and tactile characteristics observed during the palpation for the various levels of body condition. Body condition serves as a more sensitive indicator of welfare in mice compared to body weight (BW), as certain health conditions like DM can lead to weight gain simultaneously breaking down body fat and muscle. The body condition score (BCS) was conducted following the established procedures outlined by Burkholder *et. al.* 2012. Scores ranged from 1 to 5, with additional increments denoted by plus or minus signs (e.g., 2+). Each score was assigned a corresponding numerical value (e.g., BCS of 2+ =2.66 or BCS of 3– = 2.66). BCS assessments were performed by an experienced individual specialized in evaluating mouse body condition.

A study-specific pain scale was employed as an effective tool for assessing pain and distress in mice, providing a convenient means to monitor the deteriorating condition or clinical endpoint of DM. We adhered to a general clinical pain scoring system as reported by Burkholder *et. al.* 2012. To measure weight, mice were gently removed from their cages and placed in a small plastic dish on a scale, with their body weight recorded to the nearest 0.1 g.

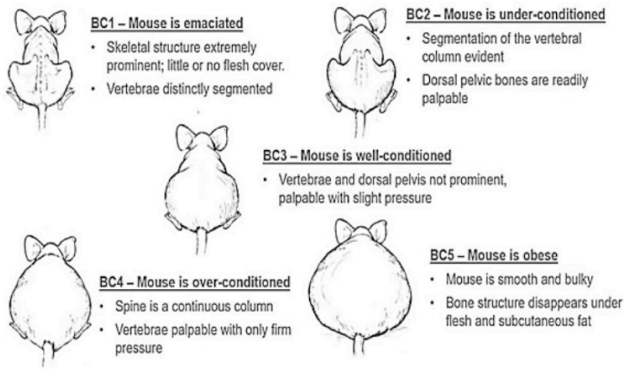

Figure 1. Body Condition Scoring (BCS) method for assessing mouse health. It utilizes a scoring system of 1 to 5 with 3 being the optimal condition, 1 being emaciated and 5 being obese. Information according to Burkholder *et. al.* 2012.

EVALUATION PAIN BEHAVIORS IN RODENTS' STIMULUS-EVOKED PAIN-LIKE BEHAVIORS

In this study, the manual Von Frey test was used to evaluated the effect of CuAqE in the type-1 diabetic neuropathic pain model. Mechanical allodynia, which is a characteristic of neuropathic pain, was measured under

low level white light conditions following the method described by Anderson *et al.* in 2014. Testing was conducted during the daytime (06:00-18:00 h) to align with the circadian cycle. Mice were placed in individual plexiglass boxes on stainless steel wire mesh floors and allowed to acclimatize for 10–30 min before testing, as described by Adamson *et al.* in 2016. Mechanical allodynia was assessed by measuring the mechanical paw withdrawal threshold (PWT) in response to stimulation with flexible Von Frey filaments. The filaments were applied perpendicularly to the plantar surface of hind paw with sufficient force to bend them, as illustrated in Figure 2.

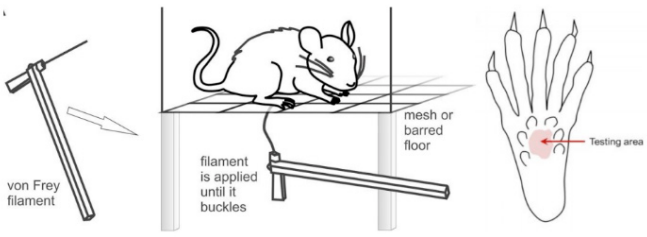

Figure 2. Method Von Frey filament used to assess mechanically evoked pain like behaviors in rodents. The illustration shows the proper use of the Von Frey filament on the plantar surface of a mouse's foot. Information according to Deuis *et. al.* 2017.

A positive response was recorded if brisk withdrawal or paw flinching occurred. Flinching immediately upon removal of the filament was also considered a positive response. Pain-like behaviors such as flinching, licking, shaking, biting, jumping, stretching or squashing the abdomen, guarding of the hind paw, and changes in posture were assessed for their presence or absence.

To determine mechanical sensitivity using manual Von Frey test were employed 50% paw withdrawal threshold (PWT) testing and Up-down method was utilized to determine the mechanical force required to elicit a paw withdrawal response in 50% of animals. This method relies on a statistical formula commonly used to determine LD50s.

To calculate the PWT, the method of Dixon was used (Adamson et al., 2016) with the formula: PWT=log X+ (k) [δ], where X = represents the value (in log units) of the final Von Frey filament used; k = is a tabular value, and δ is the mean difference (in log units) between stimuli. In addition, the Simplified Up-Down method (SUDO) according to Bonin *et al.* (2014) was employed.

The ascending stimulus method was applied by gradually increasing the force on the mice´s paws until a withdrawal was elicited. The force of the Von Frey filament that triggered this positive response was designated as the mechanical withdrawal threshold. It was observed that using a cut-off force filament with 100% rate as the upper limit for testing was preferable, as stiffer filaments tended to raise the entire limb rather than causing buckling, thereby significantly altering the nature of the stimulus. For each mouse, ascending stimuli were tested until either until the maximum stimulus was reached or a filament strength was achieved that resulted in a 100% response. For example, if the buckling weight of the last filament, which produced six positive responses, was 1.5 g, and previous filaments elicited two positive responses to the 1.2 g filament and one positive response to the 1.0 g filament, then the tactile threshold would be as $[(1.5 \times 6) + (1.2 \times 2) + 1.0]/9 = 1.38$ g (Watcho *et al.*, 2010).

STATISTICAL ANALYSIS

The behavioral data were presented as means±SEM and analyzed using a significance criterion of $p< 0.05$. Data was assessed for differences among groups and across time using one- or two-way analysis of variance (ANOVA) for treatment and time, respectively. Post-test were conducted where appropriate. Significance for all data analysis was set at $p<0.05$. Statistical analysis was

performed using GraphPad Prism Version 6.0 (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS AND DISCUSSION

TYPE-I DIABETIC NEUROPATHY MODEL

Endpoints play a crucial role in monitoring the health status of animals during the development as such DM-1 and its complications, particularly DPN. In C57BL/6 mice with induced DM-I and DPN through STZ (50mg/kg), blood glucose levels significantly increased ($p < 0.001$). This clinical condition arises as a result of pancreatic deterioration and subsequent low insulin levels, which are key metabolic manifestations. While CuAqE demonstrated the ability to decreased hyperglycemia, it was unable to maintain physiological levels throughout the entire duration of the experiment. Furthermore, both BW and BCS were significantly ($p < 0.001$) in diabetic mice, whereas they remained unchanged in the other groups of mice. The reductions in BW and BCS are closely associated with pancreatic damage. Despite the hypoglycemic and neuroprotective effects of CuAqE, it did not significantly effect maintenance of BW and BCS in this mouse model. See table 1.

Treatment group	fasting blood glucose (mg/dL)	Body weight (g)	Body condition score (BCS)
CTL-NOM	106.3±8.73[c]	33.36±2.21[b]	3.06±0.26[a]
CTL-DM	395.6±28.5	28.35±1.13	2.26±0.25
DM-EA	267.9±78.8[c]	32.12±2.46[b]	3.02±0.29[a]
CTL-EA	116.7±6.34	32.12±2.46[b]	3.19±0.35[a]

Data shown are means ± SEM. [abc]Values differ significantly ($p < 0.001$) vs. CTL-DM

Table 1. Glycemia, BW and BCS on C57BL/6 mice with DM-I and DPN induced with STZ (50mg/kg) and later treated with CuAqE.

BCS is a valuable method for assessing he health status of mice in studies where wasting and mortability are potential endpoints due to the severe pancreatic damage caused by STZ in diabetic mice. In the evaluation period, C57BL/6 diabetic mice receiving CuAqE remained stable in terms of BW and BCS compared to severely ill mice with DM-1 The changes in BW and BCS were significantly correlated with loss of muscle mass and visceral fat. BCS offers advantages over BW, including its rapidity (30 to 60 seconds) and the absence of equipment or reference values requirement (Cowley *et. al.* 2019).

PAIN BEHAVIORS IN DPN RODENTS NON-STIMULUS EVOKED NOCICEPTION

Chemical lesioning by STZ in diabetic mice triggered changes in MGS scores compared to the group of apparently healthy mice ($p<0.001$), as show in Figure 3. However, CuAqE attenuated pain without affecting constipation behavior ($p<0.005$), indicating a reduction in emotional responses to pain. However, the pain thresholds did not reach normal levels, likely due to the damage caused by insulin deficiency and consequently neuropathic damage. In preclinical pain assays, noxious stimuli applied to deep tissues (joints and viscera) resulted in higher MGS difference scores superficially applied stimuli. This is due to the inability of sick mice to suppress painful facial expressions for stimuli of longer-duration stimuli, marking them more vulnerable to potential predators or aggressive conspecifics. In mice whit experimental nerve injury, neuropathic pain is of long duration and not associated with a pain face as observed in our study where the application of Von Frey filaments did not induced a pain face. These differences may reflect variations in the strength of the affective component. The MGS, a standardized behavioral coding system with high accuracy and reliability, allows the

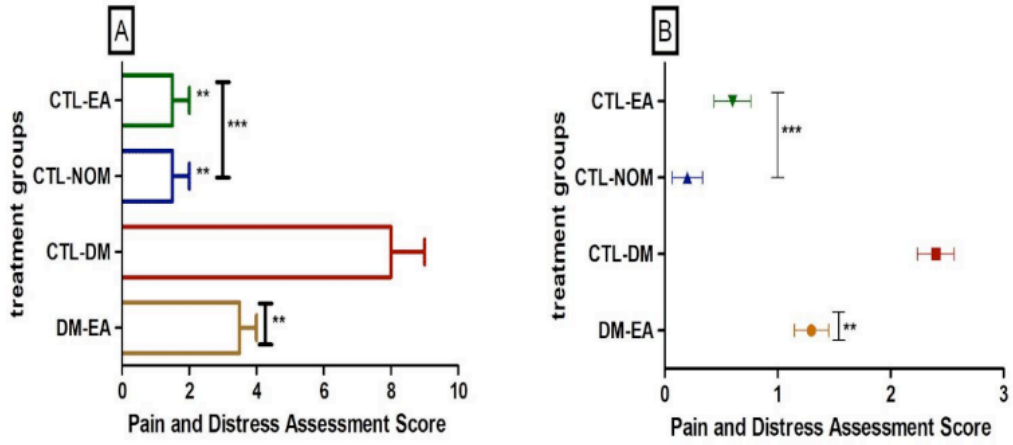

Figure 3. Score during the evaluation of pain and distress according to A) Langford, *et. al.* 2010 and B) Burkholder *et al.,* 2012; in C57BL/6 mice with DM-I and diabetic peripheral neuropathy induced with STZ (50mg/kg) and later treated with lyophilized aqueous extract of *Calea urticifolia* (CuAqE). Treatment groups are denoted as: normal control-vehicle (CTL-NOM), disease-vehicle control (CTL-DM), disease-CuAqE (DM-EA) group, and normal control-CuAqE (CTL-EA). Values represent mean ± SD (n = 5/group). ***p<0.001 vs CTL-DM, **p<0.005 vs CTL-NOM. Kruskal-Wallis test, Dunn's Test.

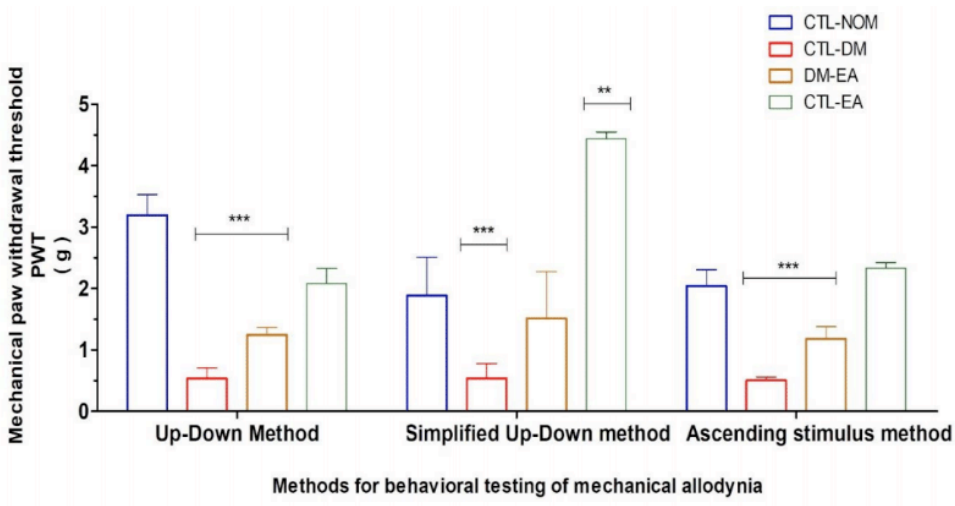

Figure 4. Paw withdrawal threshold during the evaluation of mechanical allodynia with Von Frey filaments in C57BL/6 mice with DM-I and diabetic peripheral neuropathy induced with STZ (50mg/kg) and subsequently treated with lyophilized aqueous extract of *Calea urticifolia* (CuAqE). Treatment groups are denoted as: normal control-vehicle (CTL-NOM), disease-vehicle control (CTL-DM), disease-CuAqE (DM-EA) group, and normal control-CuAqE (CTL-EA). Values represent mean ± SD (n = 5/group for each time point). ***p<0.001 vs CTL-NOM in each method. ANOVA, Bonferroni test.

assessment of facial expressions associated with pain during assays involving moderate-duration noxious stimuli. This measure of spontaneously of moderate duration are accompanied by facial expressions of pain. This measure of spontaneously emitted pain may provide insight into the subjective pain experience of mice.

PAIN BEHAVIORS IN DPN RODENTS' STIMULUS-EVOKED PAIN-LIKE BEHAVIORS

DPN in diabetic mice leads to significant mechanical hypersensitivity ($p < 0.001$). Figure 4 illustrates the comparison of thresholds computed using different methods in mice groups of this experimental protocol. It presents data from each group, including withdrawal thresholds from mechanical stimuli. For diabetic mice, thresholds were reduced by up to 70% across all methods. However, mice treated with CuAqE did not show a significant change in their thresholds. These findings confirm despite that CuAqE results in significant mechanical hypersensitivity attenuation; it is unable to modify this hypersensitivity in the DM-1 mouse model.

Despite advances in understanding the mechanisms of DPN in diabetic mice and significant attempts to develop therapies for it, there has been limited progress in exploring the effects of reported medicinal plants with antioxidant and neuroprotective properties, partly due to the difficulty in reliably assessing pain in animals. One of the major challenges is the reliable quantification of ongoing pain, which is a key feature in mice with DPN (Tappe & Kuner, 2014). In a similar manner to CuAqE, different organic extracts of *Salvia divinorum* were tested in a model of neuropathic pain by measuring the paw withdrawal threshold (PWT) as an indicator of changes in mechanical nociceptive response (Simón et. al. 2017). Additionally, the organic/aqueous extract of *Lawsonia inermis* demonstrated similar effects to CuAqE in mechanical allodynia-like behavior (Rakhshandeh et. al. 2021).

CONCLUSION

CuAqE administration regulated glycemia, BW, BCS, GMS and PWT, suggesting its potential neuroprotective effects in a murine model of DPN/DM-1. These effects can be attributed to its anti-oxidant, and anti-inflammatory properties. Therefore, further evaluation of CuAqE in other chronic pain models would contribute to a better understanding of its mechanism of action in diabetic neuropathy, potentially leading to its therapeutic use in traditional Mexican medicine.

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