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Analgesic Effect of *Citrullus Colocynthis* (L.) Schrad. On Chronic Constriction Injury and Spared Nerve Injury Models of Neuropathic Pain, an In Vivo Study

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Abstract

Objective: *Citrullus colocynthis* (L.) Schrad. (Cucurbitaceae) has many therapeutic effects such as anti-diabetic, anti-inflammatory, and analgesic effects. In this study, the effectiveness of fruit pulp of this plant on peripheral neuropathy in two models of sciatic nerve pain.

Materials and Methods: The fruit of plant, once collected, dried, then powdered and extracted by ultrasonic using ethanol solvent and condensed and dried for further steps. Pharmacologically, the analgesic effect of mentioned extract was evaluated. The chronic constriction injury (CCI) was inducing by placing four loose ligatures around the sciatic nerve, proximal to its trifurcation and spared nerve injury (SNI), Cut off one branches of the three root of the sciatic nerve output and pull it forward. Then, in both cases CCI and SNI muscle and skin suture separately. Locomotors activity, paw withdrawal cold allodynia and paw withdrawal thermal latency (thermal hyperalgesia) of rats were measured on 3, 7,10 and 14 post operated day and calcium content and pathologic were measure in nerve tissue.

Results: A 14-day treatment with extract (1, 2, and 3mg/kg, i.p.) for different days starting from the 1st day after CCI operation significantly attenuated locomotors activity and paw withdrawal thermal latency. Ethanol extract also decreased lipid peroxidase and myeloperoxidase activity. However, administration of the extract for 14 day significantly attenuated chronic constriction injury-induced neuropathic pain and also decreased the oxidative stress and calcium level.

Conclusion: The results of this study suggest that *Citrullus colocynthis* extract could be useful in the treatment of different kinds of neuropathic pains and as an adjuvant to conventional medicines.

Keywords: *Citrullus colocynthis* • Analgesic • Neuropathy model • Allodynia • Hyperalgesia

Introduction

Pain is a protective mechanism for the body and often occurs when a tissue is damaged. Pain forces the person to react to stimuli and remove the pain. Pain is known as a research subject for many physicians and researchers in biological and medical sciences. From antiquity, medical plants in ancient civilizations have been applied in various forms of herbal products or total extracts to cure numerous ailments. In this regard, and with respect to the expansion of demands for herbal therapy, study and research in this area is very essential. An abundance of research on analgesic effect of extract is done in different countries [1].

Citrullus colocynthis (L.) Schrad. (C. colocynthis) is a worth cucurbit plant from the family Cucurbitaceae and is extensively distributed in the desert areas. C. colocynthis is a desert plant that grows throughout the year, usually in sand and tropical areas. This plant is native to central and southern regions of India, but grows as a non-native plant in Arabic countries, West Asia, tropical Africa and Mediterranean countries. In Iran, the plant is found in the south, central, and an eastern region grows [2]. It is also a well-known medicinal plant in traditional Persian medicine (TPM) and is used alone or in combination with other herbs for many medicinal purposes. The namesmentioned in traditional and folk Persian manuscripts are as kabast, Sharang (bitter thing), Khiar-e-Talkh (bitter cucumber),Kharbozetalkhak (bitter melon), Kharbozeroubah (foxmelon), Hanzal, HindavaneAboujahl (Aboujahl's melon), and

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Algham(bitter thing). Many of these names imply theextremely bitterness of this plant [4].

C. colocynthis Fruits are usually recognized for the wide range of medicinal uses as well as pharmaceutical and nutraceutical potential. The fruit is commonly called Colocynth/ Bitter Apple in English. This remedy is well-known for the treatment of diabetes, jaundice, inflammatory disorders, sciatic nerve pain and gout and asthma, traditionally. Recently, a number of studies have been conducted on the phytochemistry, toxicology, and pharmacology of *C. colocynthis*. Phytochemistry analysis of leaves, roots, flowers, fruits, has indicated that different classes of metabolites such as alkaloids, carbohydrates, tannins, flavonoids, terpenoids, gum, mucilage are present in fruits [2].

Acceptable dose of the fruit of *C. colocynthis* in Iran traditional medicine is from 6.0 to 75.1 gr/day. The LD50 in unripe fruits is reported as 7.553 mg/kg. According to the findings, immature seeds and fruits possess more analgesic activity in comparison to other parts of the plant [2].

Neuropathic pain from nerve under pressure or being cut off, is one of the common clinical features of pain which may be manifest as hyperalgesia and allodynia. The mechanism of neuropathic pain is not well known, extensive research is being done in this area to find various ways to relieve this pain [1,3].

There are many reports on the applications of *C. colocynthis* as an analgesic in traditional medicine. Accordingly, current study aimed to assess the analgesic effect of plant pulp extract on chronic and neuropathic pain in an animal model.

Materials and Methods

Animals

All experiments were performed on adult female Wistar rats, weighting from 200 to 250 g, randomly obtained from the animal house of Shiraz University of Medical Sciences, Iran. Animals were kept in a 12 h light–dark cycle environment. Tap water and standard food pellets were available ad libitum. In order to minimize circadian rhythm influence, all experiments were conducted between 08:00 a.m. to 1:00 p.m. All procedures were approved by Shiraz University of Medical Sciences and followed the internationally accepted principles for laboratory animal use and care.

Plant material and extraction

C. colocynthis fruit was collected in 2015 of Gachsaran county, Kohgiluyeh and Boyer-Ahmad Province in south of Iran (The mountain range of Dena, over 4000 meters high above the sea level) and authenticated by Sedigheh Khademian (botanist at Department of Traditional Pharmacy, School of Pharmacy, Shiraz of Medical Sciences, Shiraz, Iran).

The pulp was then separated, dried and powder. To produce ethanol extract of the plant, ethanol 80% was used. Using an ultrasonic device, the pulp was extracted for 30 minutes at 40°C. The extract was subsequently filtered, concentrated and dried using rotary evaporator and speed-vacuum devices, respectively.

Experimental Design

Animals were randomly divided 12 groups, where each group 6 rats as the following: Chronic Constriction Injury (CCI) models include: Sham group, positive control, group Gabapentin 100 mg/kg/ i.p.; groups received various doses of the extract 1, 2, 3 mg/kg/i.p.

Spared Nerve Injury (SNI) models include: Sham group, positive control, group Gabapentin 100 mg/kg/i.p.; groups received various doses of the extract 1, 2, 3 mg/kg/i.p.

In the sham group, after appearing the sciatic nerve, muscle, and skin the skin and muscle in the upper area of the thigh were cut up without manipulation by means of a 4.0 silk-sutured [4].

Based on weight ratio of 1 to 1, animals were anesthetized by ketamine and xylazine via intraperitoneal injection, before surgery. Then the behavioral study was conducted according to the protocol. In the CCI groups, the sciatic nerve of left foot animals were put under pressure and in the SNI groups, the sciatic nerve of left foot animals were put under Axotomy.

Experimental model of CCI

Bennett and Xie method was used to create the model of CCI [5]. Neuropathic pain was induced in rats by performing chronic constriction injury (CCI) model on the sciatic nerve in the left hind paw of animals. Intraperitoneally, ketamine and xylazine were used for anesthesia. After fixation of animal, the hair of thigh region on the left hind paw was shaved. Then, the left common sciatic nerve was exposed at the mid-thigh with the incision of skin and dissection of biceps femoris muscle. Until a slight twitching was observed in the expected hind limb, the common sciatic nerve (proximal to trifurcation) was loosely ligated by four ligature using 4.0 chrome sutures with 1 mm spacing. The muscle and skin were then closed separately with 4.0 silk sutures. Rats with deficiency in movement after operation were excluded from the test [6].

Experimental model of SNI

This is an animal model of neuropathic pain developed by Decosterd and Woolf. In this model, the rats were anesthetized and the skin of the lateral left thigh was incised. The cranial and caudal parts of the biceps femoris muscle were separated and held apart by a retractor to expose the sciatic nerve and terminal branches (the sural, common peroneal and tibial nerves). The tibial and common peroneal nerves were tightly ligated with 4.0 silk; 2 mm of the nerve distal to the ligation was removed. Any stretching or contact with the intact sural nerve was avoided. The muscle and skin were closed in two layers and the skin was sutured together with hidden stitches to avoid any opening of the wound by biting [7].

Behavioral testing (Locomotor activity)

Motor coordination test was performed to determine the side effects of tested medicines on the exploratory behavior of animals, 30 min after administration of agents, before above stated tests. The apparatus was a white-painted wooden square box (100 × 100 × 30); the area was divided into small equal (20 × 20 cm) squares separated with red lines. Rats were placed on the central square and as soon as animal entered a square with four legs, the number of

squares that animal crossed was recorded during a 5 min session. At the end of each test, the whole area was cleaned. All of the tests were performed one day prior to surgery as referred to day 0 and 3, 7, 10 and 14 days thereafter, 30 min after injection of agents [5].

Behavioral testing (Assessment of cold allodynia)

The examination of cold allodynia was performed about 15 min after termination of mechanical allodynia test for the animals that acclimatized to Plexiglas chambers as described by Choi et al. method. We applied acetone drop with a syringe to the plantar surface of the operated hind paw of animal via the mesh floor without allowing access of syringe to the skin of hind paw. This procedure was repeated five times with an interval of 3 min. Any reaction including licking, shacking or rubbing was considered as positive response.

The reaction to acetone test was calculated on the basis of frequency of withdrawals.

Frequency of withdrawals=(Number of positive responses)/ (Number of stimulation) × 100 (9827)

Assessment of thermal hyperalgesia

In the thermal hyperalgesia test, the animal was placed in a water bath at 42°C. The length of time that it takes an animal to bring the feet out of the water and the action recorded for healthy feet and foot surgery, per animal were repeated five times at intervals of 5 min. Paw withdrawal latency calculation method is as follows:

Paw withdrawal latency=Average withdraw foot surgery-Average withdraw healthy feet

Biochemical parameters

By cervical dislocation, all groups of animals were sacrificed after the 14th day, and immediately, the sciatic nerve was isolated. The excised sciatic nerve was stored in PBS buffer (pH 7.4, 37°C) and was subjected to total calcium content assessment. Also samples kept in formalin 10% for histopathology tests.

The total calcium levels were estimated in the sciatic nerve as described earlier (Severinghaus and Ferrebee 1950 and Muthuraman et al. 2008 (18, 19). Briefly, homogenate sciatic nerve was mixed with 1 ml of trichloroacetic acid (4%) in ice cold and then centrifuged at 2000 g, for 10 min. The clear supernatant was used to estimate the total calcium ion by atomic emission spectroscopy at 556 nm.

Histopathology tests

For tissue histo-pathological evaluation, samples of sciatic nerve were fixed in buffered formalin solution (0.4% sodium phosphate monobasic, NaH2PO4, 0.64% sodium phosphate dibasic, Na2HPO4, and 10% formaldehyde in distilled water). Paraffin embedded sections of liver were prepared and stained with haematoxylin-eosin (H&E) prior light microscope viewing.

Statistical analysis

Outcomes are presented as Mean ± SEM in each group (six animals in each). Comparisons between multiple groups were made by a one way analysis of variance (ANOVA). Differences were considered significant when $P<0.05$.

Results

Results of behavioral tests

Doses of 1, 2, 3 mg / kg from extract on 3, 7, 10 and 14 days after surgery-CCI model was injected. Subsequently, 30 min after injection locomotor activity tests were performed on different days. According to, sham operated group's respond on all days were better than that of other groups. Also, Gabapentin possessed significant difference as compared to CCI group. Results of CCI group show that group operated sham and gabapentin exhibited more activity than other groups while the extract (3 mg/kg) was at the next grade. Extracts (1, 2 mg/kg) responses were approximately equal to each other, and less than other groups (No significant differences).

Doses of 1, 2, 3 mg/kg extract on 3, 7, 10 and 14 days after surgery-CCI model was injected. Then, 30 min after injection locomotor activity tests were performed on different days, sham operated group on 3, 7 and 14 days exerted less pain compared to SNI group. Only Gabapentin-receiving group on days 10 and 14 could have significant analgesic effect. All extract receiving groups exhibited significant effect in this model.

Doses of 1, 2, 3 mg/kg extract on 3, 7, 10 and 14 days after surgery-CCI model was injected. Approximately, 60 min after injection, Acetone test were performed on different days, sham operated group on days 3, 7 and 10 had less pain compared to the CCI group Also, Gabapentin or extract-receiving groups showed significant effect in CCI model.

Doses 1, 2, 3 mg/kg of extract on 3, 7, 10 and 14 days after surgery-CCI model was injected, then 60 min after injection, Acetone test were performed on different days, sham operated group on days 3, 7 and 10 had less pain compared to the SNI group. On the third day after administration, Sham group had significant difference with other groups, on the seventh day and the fourteenth day, in group received 1 mg/kg extract, pain decreased significantly prior CCI group.

Doses of 1, 2, 3 mg/kg extract on 3, 7, 10 and 14 days after surgery-CCI model was injected, then 90 min after injection Paw withdrawal latency test were performed on different days. Based on, sham operated group possessed less pain compared to the CCI group. Sham operated groups on day 3 and 7 show the maximum time difference.

Doses 1, 2, 3 mg/kg of extract on 3, 7, 10 and 14 days after surgery-SNI model was injected, then 90 min after injection Paw withdrawal latency test were performed on different days. According to the SNI group felt more pain than the control group and with gabapentin and 2 and 3 mg / kg extract groups significant difference is observed.

Calcium test results

Results showed increased levels of calcium in CCI group and Sham operated (Table 1).

Granulation tissue	Edema	Inflammation chronic	Vacuolization nerve	Groups
++	++	++	++	CCI
++	++	++	++	CCI
++	++	++	++	CCI
+	+	+	+	Sham
+	+	++	+	Sham
++	+	++	+	Sham
+++	+++	+++	+++	Ga 100mg/kg
+++	+++	+++	+++	Ga 100mg/kg
++	+++	+++	+++	Ga 100mg/kg
+++	++	+++	++	Extract 1mg/kg
+++	++	+++	+	Extract 1mg/kg
++	+	++	+	Extract 1mg/kg
++	+	++	+	Extract 2mg/kg
++	+	++	+	Extract2mg/kg
++	+	++	+	Extract2mg/kg
+++	++	+++	++	Extract3mg/kg
++	++	++	++	Extract3mg/kg
++	++	++	++	Extract3mg/kg

Table 1. Increased levels of calcium in CCI group and Sham operated.

There is significant difference between CCI group and Gabapentin group. The differences between 1 and 2 mg/kg extract and CCI group indicate that in CCI group and Sham operated, more degenerated nerve is under pressure. This degeneration was in lowest level in groups of different extracts and gabapentin.

Pathology test results

Pathology results showed that the 1 and 2 mg kg of extract vacuolization and edema less than the control group and there was a significant difference compared to those of the CCI group. However, no significant difference was seen between other groups.

Discussion and Conclusion

Suffering from pain for long times may cause adverse mental effects, so researches are always in investigation for a solution to reduce or eliminate the pain [2].

There are two stages of acute and chronic pain; acute pain is related to non-inflammatory and neurogenic pain through nerve pathways. At this stage, a specific nerve pathway through the pain message is transmitted. The chronic stage, on the contrary, is related to inflammatory pain. Chronic inflammatory pain is related to the stage pain and at this stage, the pain message transfers due to inflammatory reactions. With clear adverse and harmful effects of chemical drugs, considering the use of herbal and natural medicines may lead researchers to new approaches [1]. In other word, one of the possible choices to achieve new and effective analgesic drugs with fewer side effects is to seek and evaluate medicinal herbs or natural compounds.

Today, the study of plant species with traditionally used as an analgesic, a rewarding research strategy on the way to the preparation of new analgesic drugs can be considered [3]. Due to the active ingredients in herbal medicines and association with other materials, there is a biological balance state that prevents accumulation in the body and exerting unwanted effects. These parameters cause considerable superiority of herbal preparations in analgesic effects in comparison with chemical drugs [2].

Various active ingredients in the plant have shown analgesic effects. These compounds including terpenoids, alkaloids, flavonoids, tannins, and essential oil may exhibit analgesic affects via inhibiting prostaglandin synthesis and in central nervous system or in the body. Flavonoids can possess analgesic and anti-inflammatory activities by different mechanisms such as effecting on GABA-A, Opioids, α-adrenergic receptor, and inhibition of nitric oxide synthesis (NOS) enzymes as well as inhibition of inflammatory-involved enzymes in the brain including inhibition of cyclooxygenase and tumor necrosis factor (TNF) production. Other studies have shown that flavonoids, via inhibition of N-methyl-D-aspartate (NMDA) receptors, reduce intracellular calcium, nitric oxide synthesis (NOS) enzyme activity and calcium-dependent phospholipase A2, possess the analgesic effect [4].

Among the essential oils,the composition of alpha-eudesmolcauses inhibition of the P/Q-type calcium channel sensitive to Omega-toxin and subsequently, neurotransmitter releases of the end of pain fibers in the dorsal horn of the spinal cord fibers [3].

The pulp of *C. colocynthis* contains colocynthin, a bitter compound, resins such as colocynthein and colocynthetin, pectin, and gum. Phenolic compounds isolated from *C. colocynthis* have shown hypoglycemic, antioxidant and anti-carcinogenic activities [5].

In this study, the analgesic effect of hydroalcoholic extract of *C. colocynthis* pulp was evaluated in animal models of neuropathic pain. Extract in doses of 1 and 2 and 3 mg was compared to a standard medication (Gabapentin 100 mg) in chronic constriction injury (CCI) and spared nerve injury (SNI) - female rat model of neuropathic pain.

In this study, the results of behavioral tests showed that both Gabapentin and hydroalcoholic extract (dose of 3 mg/kg) increase locomotor activity, as compared to control group in different CCI test groups. However, the extract represented no significant differences in other doses, as compared to the control group. For SNI group, the results of behavioral tests revealed a significant difference between control group and gabapentin-receiving group. However, this difference was not significant in groups receiving the extract.

The results of acetone test in CCI group significantly were difference between control and the sham group. Whereas it was not significant in other groups. The results of the acetone test in SNI

Group showed significant differences between control, gabapentin-receiving group, and 1, 3 mg extract groups.

The results of thermal hyperalgesia in CCI group showed significant difference between control and other groups, while there was no difference between gabapentin-receiving and extract groups. In addition, results of thermal hyperalgesia in SNI group showed significant difference between gabapentin-receiving group and positive control groups, as well as between doses of 2 and 3 mg of extract with positive control group.

Findings of this study are in accordance with results from other researchers. Studies have shown an important feature of SNI model, extreme sensitivity to non-painful mechanical stimulation. This fact indicates a reduction in threshold level of sural nerve receptors in hind leg. Some studies believe that Aβ fibrils are main causes of allodynia in neuropathic pain, as it was revealed that by blocking these fibers, allodynia eliminates [6]. Following this action through communication between neurons in the dorsal horn of the spinal ganglia and the neuroma, activity exacerbated C fibers, which leads to understanding a non-painful stimulus as painful stimuli [7].

Based on the presented reports, surgery, alone can be effective and can increase sensitivity to sensory messages. In addition, changes in the dorsal horn of the spinal cord are a main cause of hyperalgesia and allodynia in recurrent and spontaneous pain which is a symptoms of neuropathic pain [1].

Data obtained from histopathological evaluation shows that, despite evidences based on which, in gabapentin-receiving and extract groups in comparison with CCI group, the inflammation reduced, however, this reduction was not significant. The results of these data need to be investigated further and perhaps be assumed that major analgesic effect of the extract and gabapentin might undergo via non-inflammatory mechanism.

The difference in dose, time and route of administration as well as the duration of action for the effect of extract are the majority of variables which are to evaluate more comprehensively. In this current study, the duration of the test was 14 days, which is less than the duration used in other neuropathic studies. It seems that this period of time was not sufficient for extract and standard drug torepair the nerve tissue. Also, measuring the muscle activity associated with myeloperoxidase enzyme around the nerve and histopathological examination can be helpful to arrive at a desirable outcome.

Results of calcium test show that 1 and 2 mg kg of extract have decreased calcium level, compared to that of the control group. Moreover, decrease by these doses was significantly different in comparison with CCI group. Calcium is a key regulator of cell for many important processes. It has been proven that calcium concentration increases in neuropathic pain and damaged tissues. Furthermore, calcium ion concentration is a secondary messenger which has an important role in the homeostasis of the nervous system [3].

Several studies have shown that calcium and free radicals are responsible for oxidative stress and inflammation. These conditions have an important role in the pathogenesis and neurodegeneration which may lead to CNS diseases such as neuropathic pain, Alzheimer, and Parkinson's diseases. An increase in the concentration of intracellular calcium is a main cause for various neuronal functions such as membrane excitability, release of neurotransmitters, synaptic plasticity, gene expression, and excitotoxicity. Parts of the analgesic effect of gabapentin is related to calcium channel antagonistic activity, by which the level of calcium decreases in the place of pain and subsequently, reduces the sense of pain.

In our study, a significant reduction in calcium ion was observed in Gabapentin-receiving group compared to the extract-receiving. Obviously, the analgesic properties of extract are shown that this property is less significant than the analgesic properties of Gabapentin (200 mg/kg). On the other side, the analgesic property of the extract at 2 mg/kg was significantly higher than 1 mg/kg.

Taken as a whole, the results of this study suggest that *Citrullus colocynthis* extract could be useful for the management of different kinds of neuropathic pains, mainly as an adjuvant to conventional medicines.

Conflicts of interest

Authors of this manuscript have no conflicts of interest.

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