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ANTI-NOCICEPTIVE EFFECT OF GLYCYRRHIZA GLABRA ROOT EXTRACT ON CHRONIC CONSTRICTION INJURY OF SCIATIC NERVE INDUCED NEUROPATHIC PAIN AND SOME SELECTED INFLAMMATORY BIOMARKERS IN EXPERIMENTAL ANIMALS

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Running title: Glycyrrhiza glabra root extract elicits anti-nociceptive effect on rat induced neuropathic pain

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ABSTRACT:

Multiple causes of neuropathic pain have been identified and its incidence is likely to increase owing to the ageing global population. Glycyrrhiza glabra (licorice) is a medicinal plant known to be a highly efficacious medicinal herb with several pharmacological effects. Few researchers have demonstrated anti-nociceptive activity of licorice acute pain. The aim of this study was to investigate the anti-nociceptive effect of prepared aqueous extract of Glycyrrhiza glabra root administration on chronic constriction injury (CCI) of sciatic nerve induced neuropathic pain and some selected inflammatory biomarkers in adult male wistar rats. Seven groups of 5 rats per group were used. Groups 1 and 2 were controls. Administration started in groups 3, 4, and 5 three days after surgery and continued for 18 days. Group 3 received 10mg/kg of Imipramine. Groups 4 and 5 received 75mg/kg and 150mg/kg of licorice respectively. Groups 6 and 7 received 75mg/kg and 150mg/kg respectively for 10 days before surgery. Paw withdrawal thresholds were assessed using hot plate method on days 3, 7, 14, and 21. On day 21, plasma level of tumor necrotic factor (TNF-α) and C-reactive protein (CRP) were determined using appropriate ELISA kits. There was significant change in pain threshold in the extract treated ameliorative groups when compared with the control and the ameliorative reference drug. TNF-alpha and CRP concentrations were significantly reduced in groups 6 and 7, compared with groups 1, 2 and 3. In conclusion, anti-nociceptive activity of licorice and its effect on TNF-α, and CRP are dose dependent and administration before surgery was more effective.

Keywords: Glycyrrhiza glabra; Pain threshold; Tumor Necrotic Factor (TNF-α); C-reactive protein

INTRODUCTION:

Chronic pain is a debilitating condition that commonly impairs activities of daily living and health-related quality of life, and its prevalence is around 7–8% in the world population [1]. Neuropathic pain is also usually associated with increased drug prescriptions and visits to health care providers [2]. Some individuals experience distinct set of symptoms, such as, burning and electrical-like sensations, and pain resulting from non-painful stimulations (such as light touching); these symptoms usually persist and have a propensity to become chronic and less responsive to pain medications [3]. In spite of rigorous research over the last 30 years, the nature of neuropathic pain is still not clear [4]. These controversies include debate on the nature of neuropathic pain, whether such pain is peripheral or central in origin, and whether its etiology is inflammatory or non-inflammatory [5]. Increasing evidence has provided better understanding of the roles of both immune and pro-inflammatory mediators (e.g., the interleukins, TNF-α, complement components, ATP and the chemokines) in the mechanisms of both peripheral and central neuropathic pain [6]. Conversely, medicinal plants and the active principles sequestered from them are of vast importance to researchers in their fight against diseases [7]. Licorice obtained from the dry roots and rhizomes of licorice plant have been shown to be widely used in clinical prescriptions [7]. The pharmaceutical importance of licorice however lies in its capacity to yield a great variety of secondary substances. According to recent studies, the most important bioactive compounds in licorice are triterpenes, flavonoids and polysaccharides [8]. These compounds are reported to have biological activities such as: antitumor [9], anti-microbial [10], antiviral [11], anti-inflammatory [12], anti-diabetic [13], immunoregulatory [14], hepatoprotective [15], neuro-protective activities [16] and adrenal cortical hormone kind functions [17].

Previous study by Bhandage et al. [18] demonstrated effect of Glycyrrhiza glabra on acute pain using different models of pain assessment. From their results, the extract had anti-nociceptive activity via central and peripheral mechanisms. However, there are few studies that have investigated the use of this extract on chronic pain.

The aim of the present study was to further investigate the anti-nociceptive effect of the extract on chronic constriction injury of sciatic nerve model of neuropathic pain in rats and to assess some selected inflammatory biomarkers.

METHODOLOGY:

Extract Preparation:

Licorice root powder was purchased from Amazon and was sold by Herbs and Crops Overseas, India with batch no: LRP-2017/02. Portion of the powder (50 g) was mixed with

100 ml of sterile distilled water in a flask with occasional shaking. The extract was then filtered through a muslin cloth for coarse residue and finally through Whatman No. 1 filters paper and kept in an airtight amber colored container [19].

Animals:

Thirty - five male 6 week-old Wistar rats bought at the Animal House of College of Medicine, Ekiti State University, (weight 200 ± 20 g) were used for the study. The rats were housed and maintained in standard conditions of light, feeding and temperature in the Animal House of College of Medicine, Ekiti State University. The study was conducted in accordance with the standards established by the Guide for the Care and Use of Laboratory Animals [20]. Rats had unrestricted access to standard rat chow and tap water. After one week of acclimatization, the animals were randomly assigned to one of the following experimental groups (n = 5 per group), ligated and were treated accordingly.

Group I: received distilled water (10ml/kg, orally) daily; designated as non - ligated vehicle-treated.

Group II: received distilled water (10ml/kg, orally) daily; designated as ligated vehicle-treated

Group III: received imipramine (40mg/kg, orally); designated as reference drug treated.

Group IV: received licorice extract (75mg/kg, orally); post-surgery; designated as low dose treated (LDL).

Group V: received licorice extract (150mg/kg, orally); post-surgery; designated as high dose treated (HDL).

Group VI: received low dose of licorice extract (75mg/kg, orally); pre surgery; designated low dose pre-treated (LDLp).

Group VII: received high dose of licorice extract (150mg/kg, orally); pre surgery; designated high dose pre-treated (HDLp).

Administrations of treatment began in group III, IV, and V three days after surgery and continued for 18 days. Group VI and VII received treatment for 10 days before surgery and treatment continued three days after surgery for another 18 days. All vehicle and licorice were administered parenterally. Blood plasma levels of tumor necrotic factor (TNF-α) and C-reactive protein (CRP) were also determined using an ELISA kit on day 21.

Nerve injury pain model:

Chronic constriction injury (CCI) of sciatic nerve was used to assess neuropathic pain according to the method described by Bennett and Xie's [21]. The rats were anesthetized using sodium pentobarbital via intraperitoneal (i.p.) administration. Neuropathic pain was thereafter induced by chronic constriction (CCI) of sciatic nerve using a chromic suture. The suture was tightly tied around the sciatic nerve located in

the right hand paw side making a diameter of approximately 0.33 to 0.50mm.

Hot plate latency test:

This procedure was carried out in accordance with the technique used by Eddy and Leimbach [22] as modified subsequently in another study by Gupta et al. [23]. Baseline values for hot plate latency test were obtained prior to surgery. Thermal hyperalgesia was assessed by placing animals on a hot plate (maintained at 55^0c) on the 3rd, 7th, 14th and 21st day after partial sciatic nerve ligation. The latency of first sign of jumping off or paw licking by the animals from the hot plate to avoid thermal pain was taken as an index of pain threshold. A cut off time of 30 secs was maintained. At no time was an animal allowed to stay on the hot plate for more than 30 secs to avoid tissue damage. The mean of the latencies of the animals on the hot plate was determined.

Determination of biochemical parameters:

At the end of the treatment period, the rats were anaesthetized using a mixture of 25% (w/v) urethane and 1% (w/v) alpha chloralose (5ml/kg; i.p., BDH chemicals Ltd., Poole, England). Blood samples were obtained from cannulated carotid artery into heparinized centrifuge tubes. Plasma was extracted by centrifugation at 3000 rpm for 15min. Plasma level of tumor necrotic factor (TNF-α) and C-reactive protein (CRP) were determined by using an Enzyme Immunoassay (EIA) kit from Randox laboratory Ltd. Co (Antrim, UK).

Statistical analysis:

All data are expressed as means ± standard error of the mean (SEM) for 5 rats per group. Statistical group analysis was performed with graph pad (Prism 7) statistical software. Test of variance was done using ANOVA, followed by Tukey's multiple comparisons test and Bonferroni's multiple comparisons test. Statistically significant differences were accepted at $p < 0.05$. Ethical Approval Protocol number: EKSU/A67/2018/02/009

RESULTS:

Hot plate latency Test:

Thermal threshold of ipsilateral hind paw of animals across the groups were shown in table 1. Group VI and VII demonstrated significant increase in pain threshold when compared with group I in which other groups maintained close range values at the baseline, after which the surgeries were performed on groups II through to group VII. On day 3 post-surgery, the animals in group I demonstrated a significant pain threshold difference when compared with all other groups. Conversely, groups V, VI and VII demonstrated significant difference in pain threshold when compared with group II. On day 7, group VII demonstrated a slight significant difference in pain threshold when compared with group I, likewise, groups III, V, and VII also demonstrated significant difference in pain

threshold when compared with group II. Furthermore, on day 14, groups V, VI and VII also demonstrated increased significant difference in pain threshold compared with group II. Finally, on day 21, groups VI and VII demonstrated increased significant difference when compared with groups I and II, similarly, group VII demonstrated increased significant difference in pain threshold when compared with groups III.

Tumor Necrotic Factor (TNF-α)

Changes in serum concentration level of TNF-α among the groups was shown in table 2. There was significant increase in change in concentration in groups II and group III when compared with group I, while group VI and group VII showed a significant decrease in change in concentration when compared with group I, II and III. Conversely, group V showed significant decrease in concentration when compared to group II and III.

C - reactive protein (CRP):

Changes in serum CRP concentration across the groups are shown in table 3. There was a significant increase in change in CRP concentration in groups III and IV when compared with group I. The results showed significant decrease in change in CRP concentration in groups VI and VII when compared with groups I, II, III, IV, and V. Similarly, group V showed a significant decrease in change in CRP concentration when compared with groups III and IV.

Table 1: Effect of licorice extract on pain threshold during CCI induced neuropathy in male Wistar rats.

	Pain threshold (Seconds)				
Rat groups	**Base line**	**Day 3**	**Day 7**	**Day 14**	**Day 21**
Control	7.3 ± 0.4	6.5± 0.35	7 ± 0.6	4.6 ± 0.5	3.3 ± 0.2
Control ligated	8.4 ± 0.3	10.9 ± 0.8a	3.8 ± 0.53	4 ± 0.2	2.5 ± 0.2
Imip treated	7 ± 0.7	13.1 ±0.5a	6.7 ± 0.5b	5.1 ± 0.7	3.8 ± 0.2
LDL treated	7.9 ± 0.78	10.9 ± 0.8a	4.5 ±0.45	4.6 ± 0.7	3.6 ± 0.3
HDL treated	8 ± 0.63	13.3 ± 1.7a,b	6.9 ± 0.6b,d	5.1 ± 0.4b	4.4 ± 0.3
LDLp treated	9.6 ± 0.2a,b	14.8 ± 0.6a,b	5.7 ± 0.3	5.7 ± 0.2 b	5.7 ± 0.3a,b
HDLp treated	1.7 ± 0.7a,b,c,d,e	16.3 ± 0.6a,b,c	8.5 ± 0.7a,b,c	6.8 ± 0.3 b	7 ± 0.1a,b,c

Data expressed are means ± SEM, n = 5.

Data were analysed by two-way ANOVA followed by Turkey's multiple post hoc test.

a,b,c,d,e, $p < 0.05$ vs Control, Control ligated, Imipramine treated, LDL treated and HDL treated respectively.

Key: Imipramine (Imip); Low dose licorice (LDL); High dose Licorice (HDL); Low does Licorice pre-treated (LDLp); High dose Licorice pre-treated (HDLp)

Table 2: Effect of licorice extract on serum TNF-α during CCI induced neuropathy in male Wistar rats

Rat groups	Serum TNF-α (ng/ml)
	Mean ± SEM
Control	443.3 ± 14.2
Control Ligated	557.7 ± 7.3a
Imipramine Treated	555.3 ± 18.4a
LDL treated	518.2 ± 16.6
HDL treated	401.3 ± 18.6b,c
LDLp treated	329.2 ± 9.6a,b,c
HDLp treated	285.1 ± 11.2a,b.c

Data expressed are means ± SEM, n = 5
Data were analysed by one-way ANOVA followed by Turkey's multiple post hoc test.
a,b,c, p<0.05 vs Control, Control ligated, Imipramine treated.
Key: Imipramine (Imip); Low dose licorice (LDL); High dose Licorice (HDL); Low does Licorice pre-treated (LDLp); High dose Licorice pre-treated (HDLp).

Table 3: Effect of licorice extract on serum CRP during CCI induced neuropathy in male Wistar rats

Rat groups	Serum CRP (ng/ml)
	Mean±SEM
Control	158.7±6.02
Control Ligated	206.6±7.29
Imipramine Treated	254.6±6.94a
LDL treated	255.8±5.96a
HDL treated	182.2±6.85c,d
LDLp treated	122.4±6.57a,b,c,d,e
HDLp treated	107.6±4.86a,b,c,d,e

Data expressed are means ± SEM, n = 5
Data were analysed by one-way ANOVA followed by Turkey's multiple post hoc test.
a,b,c,d,e, p<0.05 vs Control, Control ligated, Imipramine treated, LDL treated and HDL treated.
Key: Imipramine (Imip); Low dose licorice (LDL); High dose Licorice (HDL); Low does Licorice pre-treated (LDLp); High dose Licorice pre-treated (HDLp)

DISCUSSION:

This study investigates the anti-nociceptive effect of aqueous roots of Glycyrrhiza glabra in CCI induced neuropathic pain in male Wistar rats. Our study demonstrated that the aqueous extract increased pain threshold significantly in the licorice pre-treated ameliorative groups with most effect on group pre-treated with high dose of licorice as shown in table 1. At baseline, there was great significant increase in response

in both pre-treated licorice groups compared with all others across the group before the surgery demonstrating the analgesic role of licorice in these groups. Thermal painful stimuli are well known to be selective to centrally but not peripherally acting analgesic drugs [18]. Furthermore, on day 3 after the chronic constriction injury (CCI) of the sciatic nerve, the result of this study demonstrated a generalized increased in pain threshold across the groups except the group that was not subjected to surgery. The higher magnitude of pain threshold noticed in high dose licorice pre-treated group is suggestive of a cumulative effect of sensation lost in the limb and the anti-nociceptive effect of licorice. On day 7, all the animals across every group except group I (non-ligated vehicle) demonstrated the presence of neuropathic pain resulting from CCI procedure performed on them, as their pain thresholds were all reduced compared with that of day 3.

The result of this study also showed that the high dose licorice pre-treated (group VII) demonstrated best pain control over all the days when compared with low dose licorice pre-treated (group VI) and reference drug group (group III). However, ligated vehicle treated demonstrated worst response to pain stimuli. Furthermore, there was downward trend in the pain sensitivity (threshold) across all groups, suggesting the effect of receptor sensitization to pain stimuli over the days.

TNF-α plays a role in the peripheral mediation of neuropathic pain [24]; study by Tonini et al [25] reported that neuropathic pain is associated with massive release of TNF-α in serum, this is evident in this study where the ligated vehicle (group II) had the highest serum concentration of TNF-α as seen in table 2. This study also shows that, both vehicle treated (groups I and II), reference drug treated (group III) and the group that received low dose extract after surgery (group IV) had a significant increased change in TNF-α concentration in the serum compared with the high dose treated group after surgery (group V) and both pre-surgery licorice treated groups (groups VI and VII). The result obtained in this study is similar to the trend of result obtained by Yang et al. [7] that reported anti-inflammatory property of this extract in different models of inflammation as well as its ability to reduce the level of pro-inflammatory cytokines. However, in our current study the findings show that the effect of licorice was dose and duration dependent.

Previous study has shown a correlation between TNF-α production and the concentration of CRP [26]. TNF-α induces a dose-dependent secretion of CRP in hepatocytes [27]. Conversely, elevated CRP levels in atheroma also leads to the induction of TNF-α production by macrophages [28].

CRP is mainly classed as an acute marker of inflammation which exists in two different

isoforms and the levels are known to increase dramatically in response to injury, infection, and inflammation [29]. Our present study demonstrated that the pre-treated licorice groups (group VI and VII) had significantly reduced change in CRP concentration in the serum compared with all other groups. This may suggest that the long duration use of licorice not only plays an anti-inflammatory role but also cause better injury healing of the nerve process as noticed in this study. However, further studies are needed to assess the wound and nerve healing potential of licorice.

In conclusion, anti-nociceptive activity of Glycyrrhiza glabra (licorice) and its effect on TNF-α, and C-reactive protein (CRP) during CCI induced neuropathic pain is dependent on dose and duration.

Conflict of Interest:

The authors declare no financial or other conflicts of interests in the design and interpretation of study results.

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