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Analgesic and Anti-inflammatory Activity of Hydro-alcoholic Extract of *Juglans regia*

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Abstract
Analgesic and antipyretic effects of hydroalcholic extract of fruits of *Juglans regia* (Juglandaceae) were investigated at doses 150 mg/kg b.w. and 300mg/kg b.w. using acetic-acid induced writhing, hot- plate, tail-clip, formalin and yeast-induced pyrexia tests. Oral administration *Juglans regia* hydro-alcholic extract produced significant (P<0.0001) reduction in no. of writhes induced by acetic-acid. Moreover, in hot-plate test, *Juglans regia* hydro-alcholic extract significantly (P<0.0001) raised the pain threshold at different time of observation (0-60min) in comparison with control. In tail-clip test also the extract caused a significant (P<0.0001) inhibition of pain at both the doses used. There was a significant dose-dependent inhibition of both phases of the formalin induced pain response in mice. Tested on yeast-induced pyrexia in rats, *Juglans regia* hydro-alcholic extract significantly (P<0.0001) reversed hyperthermia at either dose. The results of pharmacological tests performed in the present study suggest that *Juglans regia* hydro-alcholic extract possesses potent analgesic and antipyretic effects.
Key words: *Juglans cinerea*, Analgesic activity, Antipyretic activity

Introduction

Pain is an unpleasant sensation no doubt, but on the whole it is usually beneficial to man (or animal). It is mainly a protective mechanism for the body, occurs whenever any tissues are being damaged, and it causes the individual to react to remove the pain stimulus[1]. Typically, it is a direct response to an untoward event associated with tissue damage, such as injury, inflammation or cancer, but severe pain can arise independently of any obvious predisposing cause (e.g. trigeminal neuralgia), or persistent long after the precipitating injury has healed (e.g. phantom limb pain). It can also occur as a consequence of brain or nerve injury (e.g. following a stroke or herpes infection). With many pathological conditions, tissue injury is the immediate cause of the pain, and this results in the local release of a variety of chemical agents, which are assumed to act on the nerve terminals, either activating them directly or enhancing their sensitivity to other forms of stimulation[2]. Pyrexia or fever is caused as a secondary impact of infection, malignancy or other diseased states[3]. It is the body's natural function to create an environment where infectious agents or damaged tissues cannot survive. Normally, the infected or

damaged tissue initiates the enhanced formation of proinflammatory mediators (cytokines, such as interleukin 1β, α, β, and TNF- α), which increase the synthesis of prostaglandin E2 (PgE2) near hypothalamic area and thereby trigger the hypothalamus to elevate the body temperature[4]. When body temperature becomes high, the temperature regulatory system, which is governed by a nervous feedback mechanism, dilates the blood vessels and increases sweating to reduce the temperature. When the body temperature becomes low, hypothalamus protects the internal temperature by vasoconstriction. High fever often increases faster disease progression by increasing tissue catabolism, dehydration, and existing complaints, as found in HIV[5]. Most of the antipyretic drugs inhibit COX-2 expression to reduce the elevated body temperature by inhibiting PGE2 biosynthesis[6].

Juglans regia has been documented growing in East and South Asia (China, Tibet, Nepal, Pakistan, and India), Central Asia (Kyrgyzstan, Uzbekistan, Tajikistan), Iran, the Balkans, the Southern Caspian region, the Caucasus, Azerbaijan, and Turkey. Juglans regia leaves have been used mostly in worldwide traditional medicines as antimicrobial, antihelmintic, astringent, keratolytic, antidiarrhoeal, hypoglycaemic, depurative, tonic, carminative, and for the treatment of sinusitis, cold and stomach ache. In Turkish folk medicine, fresh leaves are applied on the naked body or forehead to reduce fever or on swelled joint to alleviate the rheumatic pain. The kernel of Juglans regia has been used for the treatment of inflammatory bowel disease in Iranian traditional medicine. In Palestine, it is used for treatment of diabetes and asthma and to treat prostate and vascular disturbance.

The plant is used as a topical remedy for dermal inflammation and excessive perspiration of the hands and feet. It is also a common home remedy for the treatment of chronic eczema and scrofula. The leaves of this plant are used topically to treat scalp itching and dandruff, sunburn and superficial burns as well as an adjunctive emollient in skin disorders. It also has high anti-atherogenic potential and a remarkable osteoblastic activity that adds to the beneficial effect of a walnut enriched diet on cardioprotection and bone loss. The bark, branches and exocarp of the immature green fruit of this medicinal plant have been used to treat gastric, liver and lung cancer a long time in China. It is used by traditional healer in northeastern region of Mexico to protect against liver damage. The bark is used as miswaks for teeth cleaning. In Nepal the bark paste is useful in arthritis, skin diseases, toothache, and hair growth. Seed coat is used for healing wounds. The shell of Juglans regia is used in Calabria folk medicine to heal malaria.[7]

Materials and Methods

Plant Material

The Fruit of *Juglans regia* were collected from local market of authenticated at Rajmata Vijayraje Scindia Krishi Vishwavidhalaya, College of Agriculture, Indore, India. The fruits were broken and inner soft material were then dried in shade at temperature between 21-30°C for 15 to 30 days, after which were chopped and ground. Finally extraction was carried out by the following procedure.

Preparation of the extract

The powdered crude drug (800g) was subjected for extraction process by maceration with 70%-30% hydro-alcoholic at room temperature for 7 days. The extract was filtered and concentrated to dryness at

room temperature to avoid the decomposition of natural metabolites. The yield was found to be approximately 5.18% w/w.

Experimental animals

Swiss Albino Mice (25-30g) and Wister Albino Rats (180-210g) of either sex were used in the study. The rats of either sex were isolated and housed in separate cages during the course of experimental period and kept them at room temperature (24± 2°C) with a 12 : 12 h light/dark cycle. The animals were fed with standard pellet diet and provided water *ad libitum.* All the procedures and protocols were reviewed and approved by the Institutional Animal Ethics Committee of MIPS, Indore

Acute toxicity studies

Swiss albino mice of either sex (18-22g weight) were used for acute oral toxicity study. The study was carried out as per the guidelines set by OECD and no adverse effects or mortality were detected in the mice up to 4g/kg, p.o., during the 24h observation period. Based on the results obtained from this study, the dose for anti-inflammatory activity was fixed to be 150mg/kg b.w. and 300mg/kg for dose dependent study.

Analgesic activity

The animals were divided into four groups (n=6). Group I served as Control, received the vehicle only (1% Carboxymethylcellulose, CMC, 10ml/kg p.o.). Group II served as Standard, received Indomethacin or Morphine (10mg/kg b.w.) or Paracetamol (200mg/kg b.w.) Group III and IV served as test, received hydroalcholic extract of *Juglans regia* at doses of 150mg/kg and 300mg/kg b.w. p.o. respectively.

Writhing test

The test was carried out according to Koster R et al[8]. Animals were administered orally with Indomethacin (10mg/kg b.w.) as the standard drug, hydroalcholic extract of *Juglans regia* at doses of 150mg/kg and 300mg/kg b.w and vehicle. Thirty minutes after treatment, the mice were given an intraperitoneal (i.p.) injection of 0.6% v/v acetic acid in a volume of 10ml/kg to induce the characteristic writhings. The no. of writhings occurring between 5 and 15 min. after acetic acid injection was recorded. The response of the extract treated animals was compared with that of control.

Hot-plate test

Mice were placed on an aluminium hot plate kept at 55±0.5ºC for a maximum time of 30s[9]. Reaction time was recorded when the animals licked their fore and hind paws and jumped; at before (0) and 15, 30, 45 and 60 min after i.p. administration of hydroalcholic extract of *Juglans regia* at doses of 150mg/kg and 300mg/kg b.w to different groups. Morphine (10mg/kg b.w.) as the standard drug.

Haffner's tail clip method

A metal artery clip was applied to the root of the mouse's tail to induce pain[10].A sensitivity test was carried out and animals that did not attempt to dislodge the clip within 10s were discarded. The responsive mice were allotted to groups of six animals each. The tail clip was applied 60min after oral administration of extract (150mg/kg b.w and 300mg/kg b.w.), morphine (10 mg/kg b.w.). Whereas vehicle treated group served as control.

Formalin test

The method used was similar to that described previously[11]. 20µl of 1% formalin was injected subcutaneously into the right hind paw of mice. The time (in seconds) spent in licking and biting responses of the injected paw was taken as an indicator of pain response. Responses were measured for 5min after formalin injection (first phase) and 15–30min after formalin injection (second phase). Extract (150mg/kg b.w. and 300mg/kg b.w.) and Indomethacin (10 mg/kg b.w.) were administered 60min, before formalin injection. Control animals received the vehicle.

Antipyretic activity

Antipyretic activity was measured by slightly modifying the method described by Adams et al[12]. Rats were fasted overnight with water *ad libitum* before the experiments. Pyrexia was induced by subcutaneously injecting 20% w/v brewer's yeast suspension (10ml/kg) into the animal's dorsum region. 17h after the injection, the rectal temperature of each rat was measured using a digital thermometer.Only rats that showed an increase in temperature of at least 0.7 ℃ were used for experiments. *Juglans regia* at doses of 150mg/kg and 300mg/kg b.w, aspirin, (200mg/kg b.w.) or vehicle were administered orally and the temperature was measured at 1, 2, 3, 4, and 5h after treatment.

Statistical analysis

Results are expressed as Mean ± S.E.M. The difference between experimental groups was compared by One-way Analysis of Variance (ANOVA) followed by Dunnett's test. The results were considered statistically significant when $P<0.0001$.

Results

The effects of extract on acetic-acid induced writhes in mice have been shown in Table 1. *Juglans regia* at either dose (150mg/kg b.w and 300mg/kg b.w.) produced a significant ($P<0.0001$) decrease in no. of writhes in comparison with the control group. Indomethacin (10mg/kg b.w.) also showed significant ($P<0.0001$) decrease in no. writhes

Hot-plate test was also assayed to characterize the analgesic activity of the *Juglans regia*. The results presented in Table 2 show that the oral administration of the *Juglans regia* at doses 150mg/kg b.w and 300mg/kg b.w. significantly ($P<0.0001$) raised the pain threshold at different time of observation (0- 60min) in comparison with control. Morphine (10mg/kg b.w.), used as standard drug, also produced a significant analgesic effect during all the observation times when compared with control values ($P<0.0001$).

The effect of *Juglans regia* on tail clip test is shown as in Tables 3. The extract caused a significant ($P<0.0001$) inhibition of pain at both the doses used (150mg/kg b.w and 300mg/kg b.w.), Morphine (10mg/kg b.w.), a standard drug, was highly effective ($P<0.0001$).

There was a significant dose-dependent inhibition of both phases of the formalin induced pain response in mice (Table 4), with a more potent effect on the second than the first phase. Morphine (10mg/kg b.w.) also inhibited both phases of the pain significantly ($P<0.0001$) when compared to control group.

Tested on yeast-induced pyrexia in rats, *Juglans regia* significantly reversed hyperthermia at either dose (150mg/kg b.w and 300 mg/kg b.w.). Time of peak effect obtained were 1 to 3h after oral administration. The standard drug, Paracetamol (200mg/kg b.w.) also suppressed hyperthermia induced by yeast significantly ($P<0.0001$) during all the observation times when compared with control values (Table 5).

Table 1 : Effect of hydroalcholic extract of fruits of *Juglans regia* on acetic-acid induced writhes in mice

Treatment	Dose (mg/kg)	No. of writhes (Mean ± S.E.M)	Inhibition %
Control		47.67±2.69	
Standard	10	12.67±1.11^{c}	73.42
Hydroalcholic extract of fruits of *Juglans regia*	150	30.67±2.84^{c}	35.66
Hydroalcholic extract of fruits of *Juglans regia*	300	19.33±1.56^{c}	59.45

Each value is the Mean ± S.E.M. for 6 rats, $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.0001$ compared with control, Data were analyzed by using One-way ANOVA followed by Dunnett's test, Standard: Indomethacin (10mg/kg b.w.)

Table 2 : Effect of hydroalcholic extract of fruits of *Juglans regia* on mice subjected to the hot-plate test

Treatment	Dose (mg/kg)	Reaction time in seconds at time (minutes)				
		0	15	30	45	60
Control		7.71±0.33	7.75±0.34	8.07±0.13	9.03±0.18	8.62±0.21
Standard	10	7.77±0.37	11.15±0.37^{c}	13.67±0.44^{c}	18.21±0.60^{c}	20.90±0.40^{c}
Hydroalcholic extract of fruits of *Juglans regia*	150	7.73±0.28	9.28±0.40^{a}	11.26±0.26^{c}	12.95±0.34^{c}	14.88±0.32^{c}
Hydroalcholic extract of fruits of *Juglans regia*	300	7.78±0.39	10.10±0.37^{c}	12.10±0.16^{c}	15.19±0.24^{c}	17.58±0.29^{c}

Each value is the Mean ± S.E.M. for 6 rats, $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.0001$ compared with control Data were analyzed by using One-way ANOVA followed by Dunnett's test, Standard: Morphine (10mg/kg b.w.),

Table 3 : Effect of hydroalcholic extract of fruits of *Juglans regia* **on tail-clip test in mice**

Treatment	**Dose (mg/kg)**	**Reaction time (in Sec)**	**Inhibition %**
Control		1.17±0.12	
Standard	10	7.65±0.27^{c}	84.70
Hydroalcholic extract of fruits of *Juglans regia*	150	2.74±0.24^{c}	57.27
Hydroalcholic extract of fruits of *Juglans regia*	300	3.75±0.24^{c}	68.80

Each value is the Mean ± S.E.M. for 6 rats, $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.0001$ compared with control Data were analyzed by using One-way ANOVA followed by Dunnett's test

Table 4 : Effects of the hydroalcholic extract of fruits of *Juglans regia* on formalin- induced pain in mice

Treatment	**Dose (mg/kg)**	**0–5 min**	**% Inhibition**	**15–30 min**	**% Inhibition**
Control		**1.17±0.12**		**94.33±1.764**	
Standard	**10**	**7.65±0.27^{c}**	**84.70**	**27.50±2.045^{c}**	**70.84**
Hydroalcholic extract of fruits of *Juglans regia*	**150**	**2.74±0.24^{c}**	**57.27**	**55.67±1.585^{c}**	**40.98**
Hydroalcholic extract of fruits of *Juglans regia*	**300**	**3.75±0.24^{c}**	**68.80**	**43.17±1.167^{c}**	**54.23**

Each value is the Mean ± S.E.M. for 6 rats, $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.0001$ compared with control

Data were analyzed by using One-way ANOVA followed by Dunnett's test, Standard: Morphine (10mg/kg b.w.)

Table 5 : Effect of hydroalcholic extract of fruits of *Juglans regia* on brewer's yeast induced pyrexia in rats

Treatment	Dose (mg/kg)	Mean ± S.E.M. Rectal temperature (ºC)					
		0h	1h	2h	3h	4h	5h
Control		37.02±0.11	37.10±0.10	37.07±0.08	37.05±0.11	37.07±0.16	37.07±0.21
Standard	10	37.10±0.10	35.82±0.08 c	35.72±0.13 c	35.80±0.09 c	35.75±0.15 c	35.92±0.06 c
Hydroalcholic extract of fruits of *Juglans regia*	150	37.08±0.12	36.72±0.10 a	36.57±0.10 b	36.68±0.11 a	36.72±0.13	36.70±0.18
Hydroalcholic extract of fruits of *Juglans regia*	300	37.13±0.12	36.37±0.10 c	36.15±0.06 c	36.45±0.09 b	36.43±0.12 a	36.68±0.07

Each value is the Mean ± S.E.M. for 6 rats, $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.0001$ compared with control Data were analyzed by using One-way ANOVA followed by Dunnett's test

Standard: Paracetamol (200mg/kg b.w.),

Discussion

The data presented here suggests that the *Juglans regia* possesses anti-nociceptive and antipyretic activities. The extract at the doses tested was shown to possess anti-nociceptive activity evident in all the nociceptive models, signifying it possesses both central and peripherally mediated activities. The abdominal constriction response induced by acetic acid is a sensitive procedure to evaluate peripherally acting analgesics[13]. In general, acetic acid causes pain by liberating endogenous substances such as serotonin, histamine, prostaglandins (PGs), bradykinins and substance P, which stimulate nerve endings. Local peritoneal receptors are postulated to be involved in the abdominal constrictions response[14]. The method has also been associated with prostaglandins in general, that is, increased levels of PGE2 and PGF2α in peritoneal fluids[15], as well as lipoxygenase products[16]. The significant reduction in acetic acid-induced writhes by *Juglans regia* suggests that the analgesic effect may be peripherally mediated via the inhibition of synthesis and release of PGs and other endogenous substances. The hot-plate and tail-clip tests are useful in elucidating centrally mediated antinociceptive

responses, which focuses mainly on changes above the spinal cord level[17]. The significant increase in pain threshold produced by *Juglans regia* in these models suggests involvement of central pain pathways. Pain is centrally modulated via a number of complex processes including opiate, dopaminergic, descending noradrenergic and serotonergic systems[18-21]. The analgesic effect produced by the extract may be via central mechanisms involving these receptor systems or via peripheral mechanisms involved in the inhibition of prostaglandins, leucotrienes, and other endogenous substances that are key players in inflammation and pain.

The extract gave a similar effect on the formalin test inhibiting both the first and the second phase. Formalin test is biphasic, and measures pain of both neurogenic (first phase) and of inflammatory origin (second phase). The first phase (0 – 5min) being a result of direct stimulation of nociceptors measures centrally mediated effects and is insensitive to anti-inflammatory agents while the second phase (15 – 30 min) which is qualitatively different from the first phase is dependent on peripheral inflammation and changes in central procession due to chemical mediators release from damaged cells that stimulate nociception and thus induced pain [22]. In general, the test measures the response to a long lasting nociceptive stimulus similar to clinical pain[23] and is recommended as a tool in basic pain research for studying the mechanisms of analgesic agents because of its connection to tissue injury. Agents that act primarily on the CNS inhibit both phases equally while peripherally acting drugs inhibit the late phase. The ability of *Juglans regia* to inhibit both phases of the formalin test indicates its involvement in both central and peripherally mediated activity, probably by prostaglandin synthesis inhibition, as well as central inhibition mechanism.

Fever may be due to infection or one of the sequele of tissue damage, inflammation, graft rejection, or other disease states. Antipyretic are the agents, which reduce the elevated body temperature. Regulation of body temperature requires a delicate balance between production and loss of heat, and the hypothalamus regulates the set point at which body temperature is maintained. In fever this set point elevates and a drug like paracetamol does not influence body temperature when it is elevated by the factors such as exercise or increase in ambient temperature. Yeast- induced fever is called pathogenic fever. Its etiology includes production of prostaglandins, which set the thermoregulatory center at a lower temperature[24]. The present results show that *Juglans regia* possesses a significant antipyretic effect in yeast-provoked elevation of body temperature in rats, and its effect is comparable to that of paracetamol (standard drug). So inhibition of prostaglandin synthesis could be the possible mechanism of antipyretic action as that of paracetamol[25]. Also, there are several mediators or multi- processes underlining the pathogenesis of fever. Inhibition of any of these mediators may bring about antipyresis[26].

Conclusion

The results obtained in this study indicate that *Juglans regia* possesses potent analgesic and antipyretic properties, which are mediated via peripheral and central inhibitory mechanisms. This could provide a rationale for the use of this plant in fever, pain and inflammatory disorders in folk medicine.

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