Nature Environment and Pollution Technology
An International Quarterly Scientific Journal

Vol. 9	No. 2	pp. 371-378	2010

Original Research Paper

Evaluation of Anti-nociceptive and Anti-inflammatory Effects of Hydroalcoholic Extract of Leaves of Oak, *Quercus brantii* by Formalin Test and Carraggeenan Model in Rat

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Nat. Env. Poll. Tech.
ISSN: 0972-6268
www.neptjournal.com

Key Words:
Quercus brantii
Antinociceptive effect
Anti-inflammatory effect
Rat

ABSTRACT

The aim of the present study was to evaluate the anti-nociceptive and the anti-inflammatory activity of hydroalcoholic extract of *Quercus brantii* leaves by formalin test and carrageenan model in rat. In this work, 80 male-Wistar rats weighting about 210 ± 20 g were divided into ten groups of eight. Five groups were used for formalin test, and five groups for carrageenan model. For evaluation of anti-nociceptive effects the formalin test was used. Anti-nociceptive effect was determined in two phases. The minutes 0-5 and 16-60 were considered as acute and chronic phases of pain in the formalin test, respectively. The animals of experimental groups 1, 2 and 3 were pre-treated with oral doses of the extract at 200, 400, 600 (mg/kg) doses, 60 min before administration of formalin. For anti-inflammatory effects, the carrageenan-induced hind paw oedema model in rats was used and the animals of experimental groups 1, 2 and 3 were pre-treated with oral doses of the extract at 200, 400, 600 (mg/kg), 30 min before administration of carrageenan. The control group without receiving any drug and the sham group receiving an equal volume of distilled water. The paw volume was measured in the mercury from 0 to 2 h and 30 min after carrageenan injection. Data were statistically analysed by ANOVA and *t*-tests ($p<0.05$). The results showed that the highest concentration of the extract 600 (mg/kg) decreased pain and inflammation in formalin test and carrageenan model in comparison with the control group ($P< 0.05$). The present study demonstrated that probably the anti-inflammatory and anti-nociceptive effects of oak leaf hydroalcoholic extract is related to available flavonoids and tannins presence in this extract.

INTRODUCTION

No steroidal anti-inflammatory drugs (NSAIDs) are among the most prescribed pharmacologic agents in medicine. The ability of these drugs to decrease inflammation is linked to their inhibitory effect on the synthesis of prostaglandins. This mechanism also results in toxicity that can cause gastrointestinal ulceration and bleeding, renal failure, and worsening of preexisting congestive heart failure (Berger 1994).

WHO estimated that 80% people of the world living in developing countries rely on medicinal plants for primary healthcare. The high cost of acquiring synthetic drugs, their inadequate supplies, the side effects associated with their uses, and the belief that plants hold cure to many disease conditions (including painful and inflammatory conditions) have led to a reawakening of interest in the utilization of plants and plant products in recent years (Magaji et al. 2008) .

Oak (*Quercus* sps.) belongs to the family Fabaceae. The large part of this country, Iran such as Zagros mountains is covered with Oak forests. The area in the Farse Province of Iran is characterized by the presence of *Quercus brantii* (Derakhshanfar et al. 2008). In traditional medicine, various parts of oak (fruit, peel, leaves and gall) were used to treat acne, ulcers, diarrhoea and respiratory diseases (Chiej 1984).

The results from the other studies by various workers showed that oak has antimicrobial and antibacterial activities (Sakar et al. 2005). Leaves of oak contain large quantities of phenolic compounds, particularly tannins and flavonoids. The young oak leaves are much richer in hydrolysable tannins (gallotannin and ellagitannin) and flavonoid glycosides than old leaves (Salminen et al. 2004). Quercetin is the major flavonoid which belongs to the class called flavonols. This is known to be highly active flavonoid (Lakhanpal & Rai 2007).

In the present work, anti-nociceptive and anti-inflammatory effects of hydroalcoholic extract of *Quercus brantii* leaves were evaluated by using formalin test and carrageenan model. Obtained results would be useful in clinical and treatment centres.

MATERIALS AND METHODS

In this work, 80 adult Wistar rats 210 ± 20 g were used. They were housed in standard polypropylene cages and kept under controlled room temperature 23 ± 2°C in a 12 h light-dark cycle (Magaji et al. 2008). The animals were fed on standard laboratory diet and water. Food and water were withdrawn during the experimental hours. The animals were divided into ten groups of eight (n = 8). Five groups each were used for formalin test and carrageenan test.

The Animals of Control and Experimental Groups in Formalin Test

Control group: 0.05 mL of a freshly prepared 2.5% solution of formalin was injected subcutaneously under the plantar surface of the right hind paw of these animals.

Sham group: These animals were received 1mL oral distilled water, 1 hour before formalin injection.

Experimental groups 1, 2 and 3: These animals were pre-treated with different doses of the extract 200, 400,600 (mg/kg) in groups 1, 2, 3 respectively, 60 min before administration of formalin.

On the day of testing, each rat was moved to the laboratory and placed in a plexi glass chamber (30 × 30 × 30 cm), 30 min before test performance to acclimate it to the experimental environment. One hour after administration of the extract or distilled water, 0.05 mL of a freshly prepared 2.5% solution of formalin was injected subcutaneously under the plantar surface of the right hind paw of rat, and immediately the rat was carried in to the test chamber. A mirror was positioned below the chamber at a 45° angle for unobstructed observation of the rat paws. Within each 15 second, pain behaviour response was recorded based on according to the method of Dubuisson & Dennis (1977). Pain score was measured during 60 minutes as 12 blocks 5 minutes. Average of pain score in each block was measured according to the formula as below (Dubuisson & Dennis 1977).

$$\text{Pain Score} = \frac{0T_0 + 1T_1 + 2T_2 + 3T_3}{300\ S}$$

T_0, T_1, T_2, T_3 are the number of 15 seconds that animal in a 5 minutes period shows numbers 0, 1, 2, 3 that was based on behaviour.

The formalin test has a distinctive biphasic nociceptive responses termed early and late phases. The first or acute phase begins immediately after formalin subcutaneously was injected into the animal hind paw. It continues for 5 to ten minutes. This phase is probably a direct result of stimulation of nociceptors in the paw. Stimulation of opioid receptors has also been suggested as a possible mechanism of action against neurogenic pain. This phase, therefore, reflects centrally mediated pain. The second or chronic phase occurs about 15 minutes after formalin injection. This phase is due to inflammation with the release of serotonin, histamine, bradykinin and prostaglandins and at least to some degree, the sensitization of central nociceptive neurons (Magaji et al. 2008, Dubuisson and Dennis 1977).

The Animals of Control and Experimental Groups in Carrageenan Model

Control group: 5 mL of a freshly prepared 1% solution of carrageenan was injected into the sub plantar region of these animals hind paw.

Sham group: These animals received 1mL oral distilled water, 30 min before carrageenan injection.

Experimental groups 1, 2 and 3: These animals were pretreated with different doses of the extract 200, 400, 600 (mg/kg) in groups 1, 2, 3 respectively 30 min before carrageenan injection.

Carrageenan-induced hind paw oedema is the standard experimental model for inflammation. Carrageenan is the phlogistic agent of choice for testing anti-inflammatory drugs as it is not known to be antigenic and is devoid of apparent systemic effect. Moreover, the model exhibits a high degree of reproducibility. The probable mechanism of action of carrageenan-induced inflammation is biphasic; the first phase is attributed to the release of histamine, serotonin and bradykinins in the first

Table 1: The anti-nociceptive activity of hydroalcoholic extract of oak leaves assessed using the formalin test.

Groups	Pain score of chronic phase (Mean ± SEM)	Pain score of acute phase (Mean ± SEM)
Control	4.19 ± 0.084	3.997 ± 0.047
Sham	4.41 ± 0.082	3.957 ± 0.037
Experimental Group 1200 mg/kg	3.899 ± 0.131	3.911 ±0.065
Experimental Group 2400 mg/kg	3.906 ± 0.081	3.883 ± 0.084
Experimental Group 3600 mg/kg	3.552 ± 0.136*	3.512 ± 0.092*

* $P < 0.05$

The average ± Standard Error of Mean (X ± SEM). The average amounts marked by * have a significant difference with the control group.

Table 2: The anti-inflammatory activity of oak leavf hydroalcoholic extract assessed using the carrageenan model.

Groups	Paw volume (cm^3) (Man ± SEM)
Control	3.952 ± 0.045
Sham	3.921 ± 0.042
Experimental Group 1200 mg/kg	3.843 ± 0.036
Experimental Group 2400 mg/kg	3.813 ± 0.034
Experimental Group 3600 mg/kg	3.124 ± 0.030*

* $P < 0.05$

The average ± Standard Error of Mean (X ± SEM). The average amounts marked by * have a significant difference with the control group.

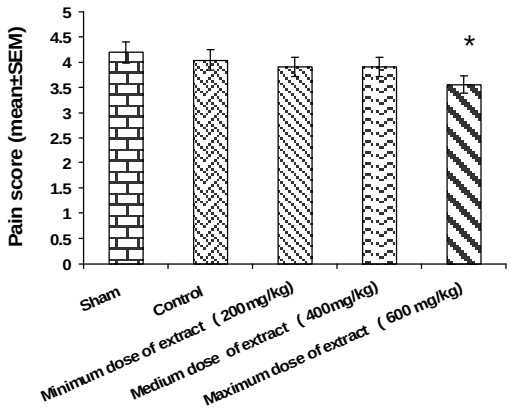

Fig 1: The comparison of pain score (Mean ± SEM) of formalin test acute phase (0-5 min) in pre-treated groups with various doses of oak leaf hydroalcoholic extract with the control group.

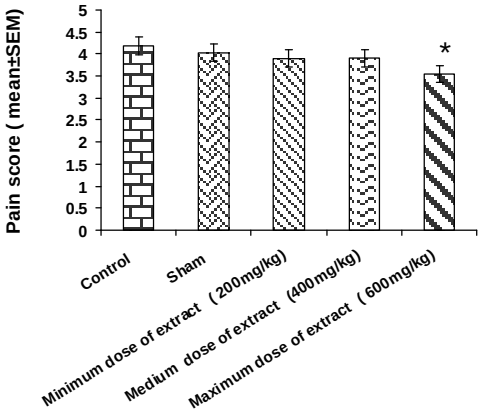

Fig 2: The comparison of pain score (Mean ± SEM) of formalin test chronic phase (16-60 min) in pre-treated groups with various doses of oak leaf hydroalcoholic extract with the control group .

hour; while the second phase is attributed to the release of prostaglandins and lysosome enzymes in the second to third hour (Magaji et al. 2008).

In carrageenan test, 5 µL of a freshly prepared 1% solution of carrageenan was injected into the subplantar region of animal hind paw. The paw volume was measured in the mercury from 0 to 2 h and 30 min after carrageenan injection. In order to measure paw volume, 300 g mercury should be split in a beaker and should be put on a digital scale so the scale became zero. The rat was held in hand and by right hand the rat paw was entered in mercury dish. In order to be correct and low error in this test, the rat paw was sunk in to mercury to malleolus and the number related to exerted force was registered in a table. This number showed paw weight according to gram. To measure paw volume in mercury, paw weight in mercury was divided into mercury density. The mercury density had been considered 13.6 g/cm^3 (Fereidoni et al. 2000). All the results were expressed as mean ± SEM. Data were analysed using ANOVA and *t*-tests. P values < 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

The mean of pain scores of chronic phase (16-60 min) and acute phase (0-5 min) of formalin test in sham, control and pre-treated groups with various doses of oak leaf hydroalcoholic extract are shown in Table 1**.** As also observed in Figs. 1 and 2, the results obtained indicate that oak leaf extract with highest dose (600 mg/kg) decreased pain significantly, in both the formalin test phases in comparison to control group (P< 0.05).

The carrageen induced hind paw oedema with various doses of oak leaf hydroalcoholic extract in pre-treated groups, sham and control group is given in Table 2. As also observed in Fig. 3, the results obtained indicate that oak leaf hydroalcoholic extract with highest dose (600 mg/kg) decreased carrageen induced paw oedema in comparison to control group (P< 0.05).

The obtained results showed that the highest concentration of oak leaf hydroalcoholic extract of 600 mg/kg in comparison to the lower doses of the extract at 200 mg/kg and 400mg/kg decreases

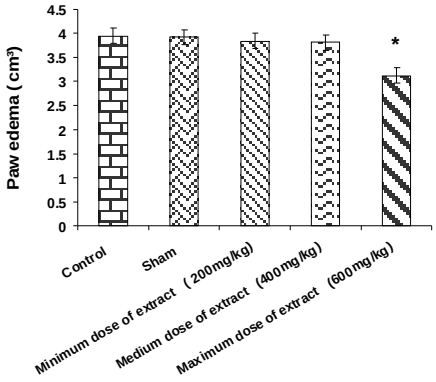

Fig 3: The comparison of carrageenan-induced paw edema (mean ± SEM) in pre-treated groups with various doses of oak leaf hydroalcoholic extract with the control group.

pain and inflammation in formalin test and carrageenan model significantly relative to control group. The main compounds of oak leaves are tannins and flavonoids. Tannins and flavonoids have anti-nociceptive and anti-inflammatory effects (Mule et al. 2008).

Investigation of antinociceptive activity of oak leaf hydoalcoholic extract: The hydroalcoholic extract of oak leaves of 600 mg/kg inhibited both the phases of formalin test (acute and chronic phases), similarly, so the hydroalcoholic extract of oak leaves has central anti-nociceptive function. The hydroalcoholic extract of oak leaves has also shown anti-inflammatory activity and inflammation is a peripheral process in formalin-induced pain, therefore, it can be concluded that the hydroalcoholic extract of oak leaves also has peripheral anti-nociceptive function. Tannins dose-dependently inhibited both the phases of the formalin test (Souza et al. 2007, Viana et al. 1998) and central anti-nociceptive function.

The antinociceptive effect of tannins is reverted by naloxone (opioid receptor antagonist) (Viana et al. 1998). So part of tannins antinociceptive effects and the hydroalcoholic extract of oak leaves are related to activate opioid system. Opioid receptors are present in many regions of the nervous system that are involved in pain transmission and control, including primary afferent neurons, spinal cord, midbrain and thalamus (Chahl 1996). Opioid receptors are apparently coupled via G-proteins directly to K^+ channels and voltage-sensitive Ca^{2+} channels. Uploads also interact with other intracellular effector mechanisms, the most important being the Aden late cycles system. Opioids have actions at two sites, the presynaptic nerve terminal and the postsynaptic neuron. Opioids inhibit N-type Ca^{2+} channels in presynaptic site, and thus, decrease neurotransmitter release (substance P) from C fibres. Opioids open voltage-sensitive K^+ channels in postsynaptic site, and thus, increase outward movement of K^+ from neurons. Increased outward movement of K^+ is the most likely mechanism for the postsynaptic hyperpolarisation and inhibition of neurons induced by opioids throughout the nervous system (Chahl 1996). These two mechanisms together inhibit transmission of pain impulse to brain ascending pathway. The other compound of oak leaves is flavonoids; quercetin is one of the most active flavonoids. Quercetin appears to act as calmodulin antagonist. Through this mechanism, quercetin functions at cell membrane level with a membrane stabilizing action. Quercetin inhibits calmodulin dependent enzyme present at cell membrane such as ATPases and phospholipases thereby influencing membrane permeability. The enzyme inhibitory action of quercetin extends to

phospholipases which catalyses the release of arachidonic acid from phospholipids stored in cell membranes. Arachidonic acid serves as a key substrate for substances such as thromboxane, inflammatory prostaglandins and leukotrienes. Quercetin also inhibits the enzymes cyclooxygenase and lipooxygenase, which catalyse the conversion of arachidonic acid to its metabolites. So quercetin inhibits pain mediators such as prostaglandins. Flavonoids also decrease intracellular calcium by inhibiting NMDA receptors and following that NOS enzyme activity and phospholipase A_2 decrease (Sucher 2006).

Anti-nociceptive function mechanism of flavonoids can be explained that N-methyl-D-aspartate (NMDA) receptors are stimulated due to primary afferent neurons of pain stimulation and release substance P and glutamate as pain pathway neurotransmitters in synapse between primary neurons and secondary neurons of spinothalamic pathway (Petrenko et al. 2003). Activation of the NMDA-receptors increases intracellular calcium. On the other hand it has given rise to activate the enzymes that their activities are dependent on calcium. Among these enzymes are NOS and phospholipase A2. Flavonoids inhibit these enzymes by inhibiting NMDA receptors.

Investigation of anti-inflammatory activity of oak leaves hydoalcoholic extract: Peripheral inflammation involves an increase in cyclooxygenase-2 (COX-2)-mediated prostaglandin (PG) synthesis in the central nervous system (CNS), which contributes to allodynia and hyperalgesia (Guay et al. 2004). Several lines of evidence indicate that the COX-2-mediated increase in prostaglandin (PG) E2 production in the central nervous system (CNS) contributes to severity of the inflammatory and pain responses in carrageenan model. COX-2 is rapidly induced in spinal cord and other regions of the CNS following carrageenan injection in the paw. The administration of selective COX-2 inhibitors, but not COX-1 inhibitors, reduces levels of PGE2 in cerebrospinal fluid (CSF) and hyperalgesia. The central effects of PGE2 appear to be mediated via EP3 receptor based on observations that the microinjection of an agonist of the EP3 receptor in brain directly causes hyperalgesia, and inflammatory responses are strongly reduced in the mice deficient in EP3 receptor (Guay et al. 2004). Nitric oxide (NO) also plays an important role in carrageenan-induced paw oedema (Salvemini et al. 1996). The principal therapeutic effects of NSAIDs derive from their ability to inhibit prostaglandin G/H synthase (cyclooxygenase or COX) which convert arachidonic acid to the unstable intermediates PGG2 and PGH2 and leads to the production of thromboxane A2 and a variety of prostaglandins. Based on the obtained results in this study and other workers, it is suggested that anti-inflammatory activity of oak leaves hydroalcoholic extract is resulted from inhibiting release of PGE2 and NO as inflammatory mediators.

The previously studies showed that tannins inhibit carrageenan-induced paw oedema (Souza et al. 2007). Tannins could affect the inflammatory response via their radical scavenging activities. There is ample evidence supporting the connection between inflammation and free radical reactions. Production of super oxide, hydroxyl radical, and other oxidizing species initiates a feedback cycle, with the radical product of inflammatory reactions serving as inducing agents for further inflammation. For example, nitric oxide ($NO^{\cdot}$), a radical produced by nitric oxide synthase, acts as a second messenger during the process of inflammation (Jeffers 2006).

ROS (reactive oxygen species), which can directly damage protein, lipids and nucleic acids, are biologically produced either as a by-product of normal mitochondrial metabolism, or as components of the inflammatory pathway. The mechanism by which tannins inhibit inflammatory markers may be through oxidation of the tannin and reduction of ROS including free radicals (Jeffers 2006).

Mast cells are a principal source of IL-6, IL-1β, and TNF-α and histamine in inflammatory

reaction (Kim et al. 2006). cAMP and intracellular calcium pathways are critical to the degranulation of mast cells (Kim et al. 2006). Tannins inhibit histamine release from mast cells. Tannins act as cell membrane stabilizers as well as radical scavengers (Kanoh et al . 2000). The mode of action of gallic acid (structural unit of hydrolysable tannins) for histamine inhibition is likely related to the prevention of calcium release from the calcium store of mast cells due to elevation of the intracellular cAMP level by inhibition of cAMP phosphodiesterase (Kim et al. 2006).

It is reported that quercetin is an effective inhibitor of phospholipase A2 which catalyses the hydrolysis of phospholipids to release arachidonic acid as precursor of inflammatory response (Toker et al. 2004), therefore, quercetin inhibits PGE2 synthesis.

Tumour necrosis factor alpha (TNF-α) is one of the major proinflammatory cytokines involved in the pathogenesis of chronic inflammatory diseases and is modulated by oxidative stress. TNF-α also triggers cellular release of other cytokines, chemokines, or inflammatory mediators. Quercetin significantly inhibited TNF-α production and gene expression (Lakhanpal et al. 2007), and it could also suppress NO and inducible nitric oxide synthase (iNOS) gene (Toker et al. 2004). The action mechanism of quercetin for NO and TNF-α inhibition can be explained that it suppress lipopolysaccharide (LPS)-induced macrophages, LPS-induced TNF-α, NO production and inducible nitric oxide synthase (iNOS) gene. Inhibition of iNOS gene expression by quercetin is also mediated by inhibition of nuclear factor-kappa B (NF-κβ) and depends on heme oxygenase-1 induction (Chen et al. 2005). It could be concluded that available compounds in oak leaf extract inhibit calcium dependent enzymes as phospholipase A2 and iNOS via intracellular calcium reduction.

CONCLUSION

The present study demonstrated that the anti-inflammatory and anti-nociceptive effects of oak leaves hydroalcoholic extract are related to available flavonoids and tannins present in the extract. However, further studies are required in order to recognise effective compounds of oak leaf hydroalcoholic extract.

ACKNOWLEDGEMENT

The authors wish to thank the Research Secretary of Islamic Azad University of Kazeroun who made this study possible.

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