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Evaluation of anti-inflammatory and analgesic activities of aqueous methanolic extract of *Ranunculus muricatus* in albino mice

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Abstract: *Ranunculus muricatus* (Ranunculaceae) is commonly used by inhabitants of Pakistan for the treatment of gout and rheumatism, both of which are inflammatory disorders. The present study attempts to evaluate the anti-inflammatory and analgesic activities of aqueous methanolic extract of *R. muricatus* in mice. The plant extract at doses of 50, 100 and 150 mg/kg was tested for anti-inflammatory activity against carrageenan and egg albumin induced paw edema in mice and analgesic activity was appraised against acetic acid induced writhing and formalin induced paw licking in mice models. The results designate that extract at the highest dose of 150 mg/kg significantly ($p<0.001$) and dose dependently inhibited carrageenan induced and egg albumin induced paw edema. Similarly, extract at the same dose of 150 mg/kg showed potent and dose dependent ($p<0.001$) suppression of formalin induced paw licking and abdominal constrictions / stretching of hind limbs induced by acetic acid. The anti-inflammatory and analgesic activity of plant extract was comparable to standard drug ibuprofen in all models. This study thus supports the use of *R. muricatus* in traditional medicine for conditions associated with inflammation and analgesia which might be attributed to its previously proven high alkaloid, flavonoids, phenol, tannins content and free radical scavenging activity.

Keywords: *Ranunculus muricatus,* anti-inflammatory, analgesic, ibuprofen.

INTRODUCTION

In the modern medical science, inflammation is regarded as a normal physiological protective response to noxious chemicals, microbes and tissue injury, more precisely considered as primary defense mechanism of body against these agents (Kumar *et al.,* 2004). It involves activation of various enzymes, discharge of mediator, ejection of fluid, cell migration, tissue breakdown and repair. However, inflammation would perform as an etiologic factor for numerous other chronic ailments, if it is not treated (Alamgeer *et al.,* 2015). At present, inflammatory disorders and associated pain are managed either by narcotics (opioids) or non-narcotics (salicylates and corticosteroids) (Gaddi *et al.,* 2004). Nonsteroidal anti-inflammatory drugs (NSAID's) are the most clinically imperative medicines used for treatment of inflammatory maladies such as arthritis, asthma and cardiovascular diseases, attributable to their efficiency for management of pain and inflammatory illnesses. Nevertheless, prolong treatment with NSAID's may instigate gastro-intestinal bleeding, ulcers and renal disorders because of their nonselective inhibition of COX-1 and COX-2 enzymes and are also quite expensive and not easily accessible to common populace (Kaushik *et al.,* 2012). Therefore, many researchers have thus focused on medicinal plants for finding natural anti-inflammatory and analgesic drugs, without those adverse effects and also they are cheaper i.e., within the reach of poor people.

Different communities of world have been making use of phytopharmaceuticals for centuries. This trend is well recognized in Pakistan by the name of Hikmat and around 600–1000 medicinal plants of country have been utilized for the control of several pathological conditions by above 40,000 registered and a huge number of unregistered Hakims/Tabib. These practices are without any scientific confirmation and hence, it is needed to explore the potency of traditionally used flora by scientific techniques (Saeed *et al*., 2011). Such studies can help to define their therapeutic worth.

Ranunculus muricatus, (RM) belonging to family Ranunculaceae, is a species of buttercup and known by the common name spiny fruit buttercup. It is native to Europe, but it also found in Pakistan, India, Iran and countries of West Asia, Western Australia and some states of America. In Pakistan, it is distributed in Swat, Hazara, Kashmir, Jhelum and Domel. Around 22 genera and 114 species including *R. muricatus* are existing in Pakistan. It is commonly used by local people for the treatment of gout and rheumatism, both of which are inflammatory disorders (Khan *et al.,* 2016; Yasari and Vahedi, 2011). The present study was undertaken to provide the scientific evidence for analgesic and anti-inflammatory activities of aqueous methanolic extract of *R. muricatus* using mice models.

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MATERIALS AND METHODS

Plant material

The whole plant was collected from marshy areas of Talash, district Dir (lower), Khyber Pakhtunkhwa (KPK) Pakistan. The plant was identified and authenticated by Dr. Ali Hazrat (Taxonomist), research officer Shaheed Benazir Bhutto University, Sheri glen Dir (Upper). A voucher specimen has been kept for future reference in the Herbarium of Faculty of Pharmacy, Department of Pharmacognosy, with the number of RM-1089.

Preparation of extract

The dried and coarsely powdered plant (1kg) was extracted by cold maceration to prepare aqueous methanolic extract using methanol and distilled water (70:30). For extraction, the powder was soaked in solvent (70:30 v/v methanol and distilled water) for 72 hours with occasional shaking. Afterwards, it was passed through muslin cloth and filtered through the Whatman filter paper I. The extract was concentrated with the help of rotary evaporator and dried by lyophilizer and a yield of 8% of extract was obtained (Alamgeer *et al*., 2015).

Experimental animal

Albino mice of either sex weighing 22-25g were used in all experiments. Mice were housed in polypropylene cages at animal house of Faculty of Pharmacy, University of Sargodha. The animals were kept in controlled ventilated conditions (25°C and light/dark cycles i.e. 12/12h) and were fed with standard food and water ad libitum. All the animals were treated according to the guidelines of National Institute of Health (NRC, 1996).

Chemicals and drugs

Carrageenan (Sigma, USA), fresh egg-albumin, formalin (Merck, Germany), acetic acid (Sigma, USA) and ibuprofen

Anti-inflammatory activities

Carrageenan induced paw edema in mice

Anti-inflammatory activity of aqueous methanolic extract of *R. muricatus* (AMERM) against carrageenan induced paw edema in mice was evaluated according to the method described by Nawafor and Okwuasaba (2003). Animals were divided into 5 groups of 5 mice each. Group I served as control group and received normal saline, 2ml/kg p.o. Group II, III and IV were given AMERM at doses of 50 mg/kg, 100 mg/kg and 150 mg/kg p.o. respectively and Group V was treated with standard drug, ibuprofen at a dose of 40 mg/kg p.o. One hour after the treatment, edema was induced by injecting carrageenan (0.1 ml, 1%, w/v in saline) into the sub-planar surface of right hind paw. The linear circumference of injected paw was measured at 0, 1, 2 and 3 hours of administration of phlogistic agent, with the help of Vernier caliper. The percent inhibition of edema was calculated by formula:

$$\text{Percent inhibition} = 100\,\frac{(\text{Vc} - \text{Vt})}{\text{Vc}}$$

Where, Vc = mean paw edema of control group and Vt = mean paw edema of drug treated group.

Egg albumin-induced paw edema in mice

Anti-inflammatory activity of AMRM against carrageenan induced paw edema in mice was appraised following the method described by Alamgeer *et al* (2015). Briefly, adult albino mice of either sex were divided into 5 groups of 5 mice each. Group I served as control group and received normal saline, 2ml/kg p.o. Group II, III and IV were given AMERM at 50mg/kg, 100mg/kg and 150 mg/kg p.o. respectively. Group V was treated with ibuprofen, standard drug at dose of 40mg /kg p.o. After 1 hr. of treatment, 0.1 ml of fresh egg albumin was injected into the paw as phlogistic agent. The linear circumference of the injected paw was measured at 0, 1, 2 and 3 hours of administration of phlogistic agent, with the help of Vernier calipers. The ability of extract to subdue paw inflammation was stated as percent inhibition of paw edema and was calculated according to the following equation:

Percentage of inhibition (%) = 100 x (1 - x/y)

Where, x = mean increase in paw thickness of treated mice; y = mean increase in paw thickness of control mice.

Analgesic activities

Acetic acid induced writhing in mice

Analgesic activity of AMRM against acetic acid induced writhing was conducted according to method of Koster *et al.* (1959), with some modifications. Concisely; after an overnight fasting, mice were divided into 5 groups of 5 mice each. Group I served as control and treated with 2 ml/kg of distilled water p.o. Group 2, 3 and 4 were given AMERM at doses of 50, 100 and 150mg/kg p.o. respectively. Group 5 was treated with standard drug, ibuprofen (40 mg/kg p.o.). After 30 minutes of treatment, acetic acid solution (0.6% in normal saline, at the dose of 10ml/kg body weight) was administered i.p. to every mouse in order to induce writhing. The number of abdominal constrictions (writhing) and stretching with a jerk of hind limb were counted between 5 and 15 minutes, after injection of acetic acid. The response of animals in extract and ibuprofen treated groups were compared with control group animals.

Formalin induced paw licking in mice

The method defined by Nwafor and Okwuasaba (2003) was used to assess the analgesic activity of AMRM against formalin induced paw licking. The albino mice of either sex were divided into 5 group of 5 mice each. The mice were kept fasted for 24 hours prior to experiment with free access to water. Group I, which served as control was treated with normal saline, 2 mg/kg p.o. Group II, III and IV were orally given AMERM in doses

of 50,100 and 150 mg/kg respectively. The group V was treated with ibuprofen in dose of 40 mg/kg p.o. After 1 hour of treatment, 20 ul of 2.5% formalin solution was injected subcutaneously under the surface of left hind paw of each mouse and responses were observed for 30 minutes immediately after injection of formalin. The time spent by mice to lick the injected paw was noted which indicates pain (Nwafor and Okwuasaba, 2003).

STATISTICAL ANALYSIS

The values were expressed as mean ± SEM and data was analyzed by one-way ANOVA and two-way ANOVA followed by Bonferroni post-test using Graph Pad Prism 5. A value of $p<0.05$ was considered as statistically significant.

RESULTS

Effect of aqueous methanolic extract of R. muricatus on carrageenan induced paw edema in mice

Mice administered with AMERM at 50 mg/kg, 100mg/kg and 150 mg/kg doses recorded a constant reduction in paw swelling i.e., 4.00 mm ± 0.25, 3.97 mm ±0.157 and 3.84mm ±0.14 respectively, compared to those that served as the control (4.51±0.09 mm) at 3rd hr. of treatment. However, extract at 150 mg/kg produced maximum effect ($p<0.001$) against paw edema induced by carrageenan. Changes in the paw sizes of mice treated with standard drug, ibuprofen at 40mg/kg (4.32 mm ±0.14) were also significantly ($p<0.001$) lower when compared to the control mice but reduction in edema formation observed for AMERM groups was found to be greater than that obtained for ibuprofen. These result depicted in table 1 direct a dose-dependent effect of AMERM.

Effect of aqueous methanolic extract of R. muricatus on egg albumin-induced paw edema in mice

In egg albumin-induced edema test, the changes in paw diameter by AMERM and standard drug, ibuprofen are shown in table 2. The plant extract at all the doses showed a substantial reduction of paw edema ($p<0.001$), especially at 150 mg/ kg and it also produced protective effect in a dose-dependent manner. Nonetheless, paw sizes of ibuprofen treated mice were significantly ($p< 0.001$) smaller than extract treated mice at 3rd hour of treatment. The paw circumference of mice at 50, 100 and 150 mg/kg of AMERM and 40mg/kg of ibuprofen were found to be 3.88 mm ±0.12, 3.80 mm ±0.06, 3.66 mm ± 0.31 and 3.29 mm ±0.31 respectively as compared to the control (3.92 ± 0.14).

Effect of aqueous methanolic extract of R. muricatus on acetic acid induced writhing in mice

In acetic acid induced writhing model for evaluation of analgesic activity, plant extract at the doses of 50, 100 and 150 mg/kg significantly ($p<0.001$) and dose-dependently decreased the number of abdominal constriction induced in mice from 19.4±0.927 (50.51%) to 18.2±0.88 (53.57%) to 13.4±0.509 (65.81%) respectively, akin to ibuprofen, 17.2±0.80 (56.12%). Though, 150mg/kg of extract produced greater analgesic effect than ibuprofen. The summary of results is shown in table 3.

Effect of aqueous methanolic extract of R. muricatus on formalin induced paw licking in mice

The mice administered with AMERM at 50, 100 and 150 mg/kg demonstrated significantly ($p< 0.001$) shorter mean itching period i.e., 58.8±2.33 sec (81.12%), 54.2±2.95 sec (82.60%) and 12.8±1.57 sec (95.89%) respectively, compared to the control group mice (311.5±10.1 seconds). Besides, mice administered with ibuprofen exhibited longer mean itching period of 105.6±2.84 sec (66.09%), compared to the crude extract treated mice. The results represented in table 4 reveal that AMERM at all the doses displayed comparatively significant and a greater analgesic activity to ibuprofen, with 150 mg/kg showing strongest effect.

DISCUSSION

Findings from this study presented that aqueous methanolic extract of *R. muricatus* has strong anti-inflammatory and analgesic properties. This was confirmed by observations from all the models of inflammation and analgesia used in this investigation.

Carrageenan and egg albumin-induced paw edema are the pertinent animal models to evaluate acute inflammation (Mossai *et al.,* 1995; Sawadogo *et al*., 2006).The inflammation induced by carrageenan and egg albumin is biphasic in nature, in which a wide array of mediators are discharged that induce pain and inflammation, ultimately resulting in edema formation. In the first phase of inflammation i.e., 1 hr after the administration of irritant, serotonin and histamine are liberated whereas, in the second phase (over 1 hr.) bradykinin, prostaglandins and cyclooxygenase products are discharged (Silva *et al*., 2008; Wallace, 2002). Edema is believed to result from augmented tissue water, plasma protein and neutrophil ejection and combined response of inflammatory intermediaries at the site of inflammation thus, leading to increased vascular permeability and blood flow (Yankanchi and Koli, 2010; Kang *et al.,* 2008). In the current investigation, plant extract exhibited a dose-dependent anti-inflammatory effect, with the highest concentration of 150 mg/kg body weight being most effective. AMERM significantly suppressed paw edema induced by carrageenan and egg albumin, both in early and late phase but more pronounced effect during late phase and effect was comparable to ibuprofen. This proposes that extract might exhibited anti-inflammatory action by possibly deterring the synthesis and release of inflammatory intermediaries (histamine, serotonin, prostaglandins, cyclooxygenase) and this effect is akin to

Table 1: Effect of *R. muricatus* on carrageenan induced edema in mice

Treatment	Paw circumference in mm at indicated time			
	0 hr.	1 hr.	2 hr.	3 hr.
Control (2ml/kg)	3.6 ± 0.1	4.09 ± 0.06	4.30 ± 0.1	4.51 ± 0.09
AMERM (50mg/kg)	4.47 ± 0.158	4.64 ± 0.53 ^{ns}	4.00 ± 0.172 ^{ns}	4.00 ± 0.25 ^{ns}
AMERM (100 mg/kg)	4.59 ± 0.099	4.41±0.148**	4.22±0.175**	3.97± 0.157**
AMERM (150 mg/kg)	4.72 ± 0.129	4.28 ± 0.165***	4.17±0.158 ***	3.84± 0.14***
Ibuprofen (40 mg/kg)	4.9±0.025	4.69±0.13***	4.54±0.22***	4.32±0.14***

Values are expressed as mean ± SEM. $p < 0.05$ was considered as significant. *p < 0.05, **P<0.01, ***P<0.001 and ns = non-significant compared to control

Table 2: Effect of *R. muricatus* against egg albumin induced edema in mice

Treatment	Paw circumference in mm at indicated time			
	0 hr.	1 hr.	2 hr.	3 hr.
Control (2ml/kg)	3.56 ± 0.14	3.57 ± 0.15	3.71 ± 0.15	3.92 ± 0.14
AMERM (50mg/kg)	4.82 ± 0.13	4.55± 0.1^{ns}	4.1 ± 0.06 ^{ns}	3.88 ± 0.12**
AMERM (100mg/kg)	4.66± 0.15	4.36 ± 0.13**	4.09 ± 0.14**	3.80 ± 0.06^{ns}
AMERM (150mg/kg)	4.52 ± 0.13	4.29 ± 0.22**	4.02 ± 0.34***	3.66 ± 0.31***
Ibuprofen (40mg/kg)	4.00±0.12	3.70±0.34**	3.50±0.11***	3.29±0.31***

Values are expressed as mean ± SEM. $p < 0.05$ was considered as significant. *p < 0.05, **P<0.01, ***P<0.001 and ns = non-significant compared to control

Table 3: Effect of *R. muricatus* on acetic acid induced writhing in mice.

Treatment	No. of Writhing	Inhibition (%)
	Mean ± SEM	
Control (2ml/kg)	39.2 ± 1.35	
AMERM (50mg/kg)	19.4 ± 0.92***	50.51
AMERM (100mg/kg)	18.2 ± 0.88***	53.57
AMERM (150mg/kg)	13.4 ± 0.50***	65.81
Ibuprofen (40mg/kg)	17.2 ± 0.80***	56.12

Values are expressed as mean ± SEM. $p < 0.05$ was considered as significant. ***P<0.001 compared to control.

Table 4: Effect of *R. muricatus* on formalin induced paw licking in mice.

Treatment	Licking time(s)	Inhibition (%)
	Mean ± SEM	
Control (2ml/kg)	311.5 ± 10.1	
AMERM (50mg/kg)	58.8 ± 2.33 ***	81.12
AMERM (100mg/kg)	54.2 ± 2.95 ***	82.60
AMERM (150mg/kg)	12.8 ± 1.57 ***	95.89
Ibuprofen (40mg/kg)	105.6 ± 2.84 ***	66.09

Values are expressed as mean ± SEM. $p < 0.05$ was considered as significant. ***P<0.001 compared to control.

that produced by NSAID's, whose mechanism of action is inhibition of prostaglandins and cyclooxygenase enzyme (Gupta *et al.,* 2006).

Pain is allied with the pathophysiology of several clinical disorders like arthritis, muscular pain, cancer and vascular diseases**.** Acetic acid induced writhing and formalin induced paw licking are appropriate methods for assessing analgesic activity. Acetic acid induced abdominal writhing response is used to evaluate peripheral analgesics and formalin induced paw licking is used to appraise whether analgesic acts peripherally/ centrally/both (Lingaraju *et al.,* 2014).

Acetic acid prompts inflammatory pain by persuading capillary permeability however, formalin exhibits

neurogenic and inflammatory pain (Vaz *et al.,* 1996). Injection of acetic acid elicits localized inflammatory reaction, which culminates in the liberation of free arachidonic acid from tissue phospholipids (Kaushik *et al.,* 2012), endogenous mediators i.e., bradykinin, serotonin, histamine, substance P, prostaglandins and some cytokines like TNF-α, IL-1β and IL-8 (Richard *et al.,* 2011). Furthermore, the two phase response in formalin test is generally caused by direct provocation of nociceptors in the paw, which ends in centrally mediated pain with the discharge of substance P in early phase and release of serotonin, bradykinin, histamine and prostaglandins in late phase. Earlier it has been stated that centrally acting drugs (opioid analgesics) inhibit both phases equally while, peripherally acting drugs (steroidal and non-steroidal anti-inflammatory drugs) inhibit only late phase (Zeashana *et al*., 2009). Drugs that prevent first phase of formalin test have the potential to relieve neurogenic pain whereas, those that avert second phase have the capability to subdue inflammatory pain (Tjølsen *et al.,* 1992). The extract at all the doses (50, 100, 150 mg/kg) significantly ($p<0.001$) and dose-dependently repressed licking time in both phases of formalin test as well, the specific writhing detected subsequent to i.p. injection of acetic acid but 150 mg/kg produced more pronounced analgesic effect which was analogous to ibuprofen. These experiments of analgesia further institute the plausible mechanism of anti-inflammatory and analgesic action of aqueous methanolic extract of *R. muricatus* as both centrally and peripherally mediated.

The results of present study have shown that crude extract of investigated plant exhibited preeminent anti-inflammatory and analgesic activities. These activities may be linked with the presence of various phytoconstituents, as have been earlier reported in *R. muricatus* i.e., alkaloids (root 0.967%, stem 0.801% and leaves 0.456%), flavonoids (root 0.403%, stem 0.699%, and leaves 0.313%), tannins (root 0.013%, stem 0.015% and leaves 0.009%), saponins (root 4.730%, stem 7.110% and leaves 7.350%) and phenols (root 0.0038%, stem 0.0044% and leaves 0.0031) (Khan *et al.,* 2016). In addition, free radical scavenging ability of *R. muricatus* has previously been measured by using DPPH method (Khan *et al.,* 2016). Phytochemicals like flavonoids and saponins are well known for reducing pain sensitivity owing to their free radical scavenging properties (Kaushik *et al.,* 2012), together with exert anti-inflammatory properties via inhibition of enzymes involved in the production of inflammatory mediators and arachidonic acid metabolism (Sawadogo *et al.,* 2006). Flavonoids also suppress prostaglandin synthesis (end products of cyclooxygenase and lipoxygenase pathways), which are involved in several immunological reactions (Jothimanivannan *et al.,* 2010). Likewise, phenols, tannins and alkaloids have also been reported to possess anti-inflammatory activity (Khan *et al.,* 2016).

CONCLUSION

The presence of alkaloids, flavonoids, phenols, tannins and the anti-oxidant effect of flavonoids might be responsible for anti-inflammatory and analgesic activity of *R. muricatus* in albino mice. However, advance studies are required to rationalize the traditional use and further activity-oriented fractionation of this extract to isolate the active constituents from plant and to elucidate their mechanism of action.

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