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HPLC analysis and pharmacological study of *Quercus dilatata* Lindle. ex Royle aqueous methanolic extract in Sprague Dawley rats

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Abstract: *Quercus dilatata* (QD) is traditionally used to treat inflammation and pain. In the present study, aqueous methanolic extract of QD was evaluated for anti-inflammatory and anti-arthritic and anti-nociceptive potential by using *in-vitro* (protein denaturation and Human red blood cell membrane stabilization) and *in-vivo* (carrageenan induced inflammation and formaldehyde induced arthritic) assays. More so, analgesic potential was determined by hot plate and acetic acid induced writhing methods. High performance liquid chromatography (HPLC) analysis was performed for quantitative estimation of phenol and flavonoids in the QD extract. HPLC analysis of QD extract indicated the presence of quercetin, sinapic acid, and gallic acid. The extract (250 and 500 mg/Kg) exhibited significant ($p<0.001$) *in-vitro* and *in-vivo* anti-arthritic, anti-inflammatory and analgesic potential as compared to standard (diclofenac sodium) group mainly at higher (500 mg/Kg) dosage level. The QD extract exhibited good potential for treating inflammation, pain and arthritis.

Keywords: *Quercus dilatata,* anti-inflammatory, anti-arthritic, analgesic, high performance liquid chromatography.

INTRODUCTION

Inflammation is a normal physiological, reversible, protective response of body against harmful stimuli i.e. physical agents, chemicals, microbial attack, immune reactions etc. (Ismail *et al*., 2017). If inflammation persists for long time or get uncontrolled, it may cause serious ailments, hypersensitive reactions, organ damage and life threatening event. The inflammation of joints in chronic cases leads to arthritis. Rheumatoid arthritis (RA) is most common type of arthritis affecting about 1% of world's population (Saleem *et al*. , 2020a). The women are more susceptible than men (Yin *et al*., 2019). The risk factors of RA are, age, gender, genetics, smoking, use of oral contraceptives, hormone replacement therapy. RA is also responsible for co-morbidities i.e. cardiovascular diseases, infections, malignancies, mental health conditions as, anxiety and depression (Saleem *et al*., 2020a).

RA is an autoimmune and systemic inflammatory syndrome of joints. The exact etiopathogenesis of this morbid disease is still unknown. Different studies have revealed that immune regulatory factors are responsible for the disease, such as proinflammatory (tumor necrosis factor alpha (TNF (-α, interleukin (IL)-1β, IL-6) and anti-inflammatory (IL-4, IL10) cytokines balance and free radicals is worthwhile for arthritis. Clinical symptoms include swelling and pain in articular joints, fever, and fatigue etc (Mustafa Kiyani *et al*., 2019).

In present age, available therapy of RA includes non-steroidal anti-inflammatory drugs (NSAIDs), disease modifying anti-rheumatoid drugs (DMARDs), corticosteroids and biological agents such as etanercept (TNF- α inhibitor) that provide symptomatic relief as well as slow down the disease progression. The therapy of RA is lifelong and expensive. These medicines are accountable for many of adverse effects such as nephropathy, gastrointestinal tract disturbances, cardiovascular and hepatotoxicity (Akhtar *et al*., 2019; Sabir *et al*., 2018). Due to which use of herbal medicines are growing these days because of having less adverse effects and cost effectiveness (O Elansary *et al*., 2019).

Quercus dilatata (QD) Lindle.ex Royle (family: Fagaceae) is commonly known as Green oak or Holy oak (Shrestha *et al*., 2014; Youn *et al*. , 2017). It is distributed in Pakistan, Afghanistan, and temperate Himalayas from Kashmir to Nepal. The plant is used for the treatment of foul sores, diarrhea and as an astringent (Yoon *et al*., 2017). The seeds of the plants are used for

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the treatment of gastrointestinal hypertrophy, inflammation, menorrhagia, and diarrhea. The galls of Quercus are used for the treatment of wounds, infections and asthma (Kayani *et al*., 2014). The main objective of this study is to evaluate anti-inflammatory, anti-arthritic and anti-nociceptive activity of QD aqueous-methanolic extarct (QDME) and phytochemicals responsible for such activities.

MATERIALS AND METHODS

Animals

Sprague Dawley rats weighing between 100 to 150g were obtained from University of Agriculture Faisalabad and housed at temperature 25±2°C and humidity 44-56% with 12hr light dark cycle in the animal house, Faculty of Pharmaceutical Sciences, GCUF, Faisalabad. The animals were provided with standard rodent pellet diet and water *ad libitum*. All the animals were treated humanely along with the approval and guidelines of Institutional Ethical Committee (Ref No. GCUF-ERC-14352).

Plant collection and extraction

Fresh plant was collected from Peshawar and identified from Department of Botany, University of Peshawar, KPK, Pakistan and sample of plant was submitted under the voucher number Bot. 20153 (PUP) to the departmental herbarium.

Whole plant (3Kg) was washed, shade dried and grinded into coarse powder. The powder was extracted with aqueous methanolic solution (30:70 ratio) for 7 days by triple maceration method with occasional shaking. After 7 days, the soaked powder was filtered with whatman filter paper and pooled the filtrate The it was dried using rotary evaporator (Model: RE300, Stuart®, UK) at 40°C under reduced pressure. Finally stored in refrigerator at 8°C (Fatima *et al*., 2019).

Preliminary phytochemical analysis

The presence of alkaloids, flavonoids, glycosides, triterpenoids, saponins in the QDME was determined by following the standard procedures (Peerzada *et al*., 2020).

Total flavonoid contents (TFC)

The TFC content was determined by Aluminum trichloride method by following earlier procedure. Catechin was used as a standard and absorbance was measured at 510 nm. The test performed thrice (Saleem *et al*., 2020b; Yoon *et al*., 2016).

Total phenolic contents (TPC)

The TPC was determined by using the Folin- Ciocalteu reagent method by following earlier procedure. Gallic acid was used as standard and absorbance was measured at 765 nm. The test performed thrice and averaged was taken (Saleem *et al*., 2020b).

HPLC analysis

After sample preparation, the solution was run at HPLC using 280nm UV visible detector. Pump of specification LC-10AT and SPD-10AV and column Shim-pack CLC-ODS (C-18) having 25cm×4.6mm dimensions were used. Mobile phase composed of two solutions i.e. A: (H2O: AA (acetic acid) -94:6, of pH=2.27 and B: ACN (100%) were used. Flow rate of mobile phase was 1 mL/min, for 10 mins through column. The sample peak was observed at UV detector and compared with standard for evaluation of peak as well as retention time (Yoon *et al*., 2016).

In-vitro and in-vivo analysis of anti-inflammatory and anti-arthritic activity

Inhibition of protein (Egg albumin) denaturation method

Egg albumin (EA) protein denaturation method was used to evaluate the anti-arthritic activity of QD. Three solutions, each consisting of 5mL reaction mixture, were prepared i.e. test solution (0.2mL EA, 2.8mL of phosphate buffer saline (PBS, pH 6.4) and 2mL of different concentrations of QDME (50-600 µg/mL)), test control solution (2mL DW in place of extract), and standard solution (2mL of different concentrations of diclofenac sodium (DS) in place of extract). The reaction mixtures were kept in an incubator at 37°C±2 for 15min. They were cooled and heated at 70°C for 5min and then cooled again at room temperature. After this, absorbance was measured at 660nm. Percentage inhibition of protein denaturation was calculated (Saleem *et al*., 2019).

HRBC membrane stabilization method

Blood (3mL) was collected from healthy volunteer who had not taken NSAID for at least 2 weeks. Fresh blood was mixed with equal volume of sterile Alsever solution. This mixture was centrifuged for 15min at 3000rpm in centrifuge machine and then supernatant layer was removed. The packed cells were washed three times with isotonic saline solution (0.85%w/v NaCl, pH 7.2). Final suspension (10% v/v) was prepared by adding isotonic saline solution in packed cells.

Three solutions were prepared i.e. test solution (0.5mL of HRBC suspension, 2mL of hypotonic saline (0.36%w/v NaCl), 1mL PBS (0.15M, pH 7.4) and 0.5mL of different concentrations of QDME (50-600 µg/mL), test control solution (o.5mL of DW in place of extract), standard solution (0.5mL of different concentrations of DS). The reaction mixture was kept in incubator at 37°C±2 for 30min and centrifuged at 3000rpm. The supernatant was removed and absorbance was measured at 560nm. Percentage protection against hemolysis induced by hypotonicity was calculated (Saleem *et al*., 2019). The test was performed in triplicate.

Carrageenan induced paw edema

The rats weighing 150-200g were divided into 4 groups each consisting of 6 rats as disease control (DC) group

received normal saline (2mL), standard group received DS and test groups received QDME (250 mg/Kg and 500 mg/Kg) with the help of gavage needle. Carrageenan 0.1mL of 1% w/v solution was injected into sub-planter region of left hind paw of all rats 1hr of post administration. Paw diameter was measured with the help of digital vernier caliper at 1, 2, 3, 4 hr post induction and percentage inhibition was calculated (Peerzada *et al*., 2020).

Formaldehyde induced arthritis

The rats were randomly divided into 4 groups. The therapy was administered following previous protocol for 10 days. The 0.1mL of 2% formaldehyde solution was injected into sub-planter region of left hind paw in rats after 30min of administration of therapy and repeated on 3rd day. Paw edema was measured at even day till the end of therapy. Percentage inhibition was calculated (Saleem *et al*., 2019).

Determination of analgesic activity

Acetic acid induced writhing test in rats

Group arrangement and therapy was same as above except ibuprofen (20 mg/Kg) was used as a positive control**.** After 30min of administration of extract and standard by oral rout, Acetic acid (10 mL/Kg) was injected intraperitoneally to all rats. After that numbers of writhing were counted between 5-20 min for each rat. Percentage inhibition was calculated (Ismail *et al*., 2017).

Hot plate method

Eddy's Hot plate method was used to determine analgesic activity of QDME. Group arrangement and therapy was same as carrageenan induced paw edema model. After 1hr of therapy administration, individual animal was placed on a hot plate (Aibote®, China) that was set earlier to 50-55□C. The time at which rats showed response by paw licking or jumping was noted and 15sec was taken as cut off time (Peerzada *et al*., 2020).

STATISTICAL ANALYSIS

Data were analyzed by Graph pad prism 5 software and expressed as mean ±SEM. Two-way ANOVA followed by Bonferroni multiple comparison post-hoc test was used for all results except acetic acid induced writhing test for which one-way ANOVA followed by Dunnet's test were used. The level of significant was considered $P< 0.05$.

RESULTS

The percentage yield of dry extract of the plant was 13.65%. Phytochemical analysis of the plant extract confirmed the presence of alkaloids, flavonoids, phenols, saponins, tannins, tri-terpinoids, steroids and glycosides. The TPC and TFC of QDME was 18.06 mg of gallic acid/g of extract and 23.45 mg of catechin/g of extract respectively.

HPLC analysis

HPLC analysis of QDME detected different compounds as shown in table 1 and fig. 1.

Table 1: HPLC analysis of aqueous methanolic extract of *Q. dilatata*

Sr. No.	Compound	Retention time (min)	Area (mV.s)	Peak (no.)	Quantity (ppm)
1	Quercetin	3.147	23.583	2	1.24
2	Gallic acid	4.580	87.033	5	3.13
3	Syringic acid	16.880	95.018	11	2.37
4	m-Coumeric acid	20.160	88.716	13	1.06
5	Sinapic acid	26.327	199.441	15	6.18

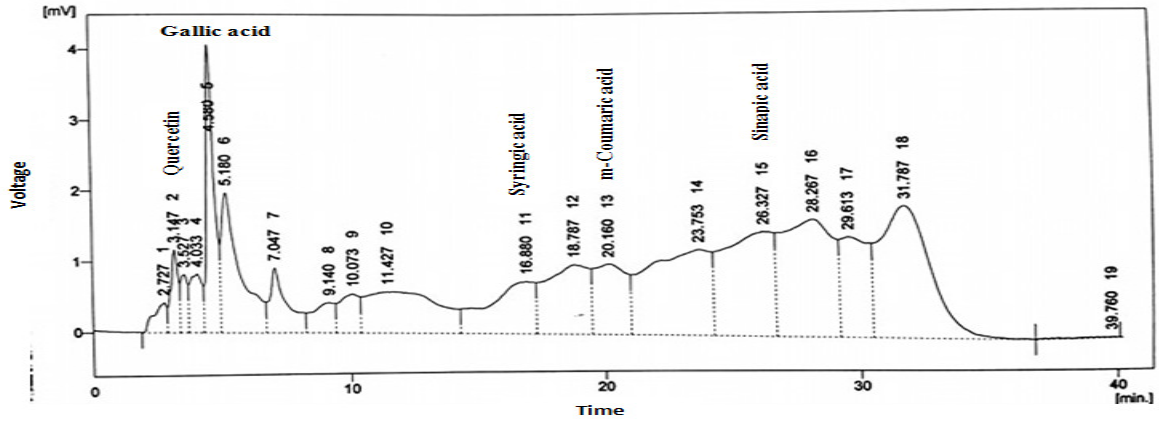

Fig. 1: HPLC chromatogram of aqueous methanolic extract of *Quercus dilatata.*

Effect of QD on inhibition of EA protein denaturation

The QDME showed remarkable %age inhibition of protein denaturation dose dependently that was significantly ($p<0.001$) different from DS at respective concentration as shown in table 2. The highest inhibition (81.867%) was exhibited by 600µg/mL in comparison to DS (86.600%).

Effect of QD on HRBC membrane stabilization method

The QDME showed noteworthy protection of HRBC membrane against hypotonicity at all tested concentration that was significantly ($p<0.001$) different from DS at respective concentration as illustrated in table 3. The plant extract stabilized HRBC membrane in concentration dependent manner. The highest protection was presented by QDME (93.400±0.058%) that was notably greater than DS (80.100%) at 600 µg/mL.

Effect of Q D extract on formaldehyde induced arthritis

There was a visible inflammation noticed on 2nd day and onwards in DC rats. After 4th day of therapy, there was a gradual reduction in paw edema in QDME and standard groups in contrary to DCG. Maximum inhibition of paw edema was noticed with QDME 250 (59.05%) and 500mg/Kg (63.22%) that was significantly varied from DC rats as shown in table 4.

Effect of Q D on carrageenan induced paw edema

The rats of all groups showed maximum inflammation at 3rd hour. The inflammation was notably attenuated by the treatment with plant extract 250 (17.14%) and 500 mg/Kg (19.43%) and DS (25.33%) at 4th hr that was significantly different from the DCG as shown in table 5.

Table 2: Effect of *Q. dilatata* on %age inhibition of egg albumin protein denaturation

Treatment groups	50 µg/mL	100 µg/mL	200 µg/mL	400 µg/mL	600 µg/mL
DS	67.200±0.058	75.367±0.033	80.567±0.033	83.100±0.058	86.600±0.058
QDME	58.733±0.033*	64.833±0.033*	75.100±0.058*	80.567±0.033*	81.867±0.033*

Values are expressed as mean ±SEM. Where *= $p<0.001$. DS: Diclofenac sodium

Table 3: Effect of *Q. dilatata* on %age of HRBC membrane stabilization

Treatment groups	50 µg/mL	100 µg/mL	200 µg/mL	400 µg/mL	600 µg/mL
DS	86.500±0.058	88.800±0.058	84.567±0.285	83.000±0.000	80.100±0.058
QDME	58.700±0.058*	64.100±0.058*	90.100±0.058*	91.100±0.058*	93.400±0.058*

Values are expressed as mean ±SEM. Where *= $p<0.001$

Table 4: Effect of *Q. dilatata* on formaldehyde induced arthritis

Treatment groups	Paw size (mm)				
	2nd day	4th day	6th day	8th day	10th day
DC	7.498±0.169	8.584±0.150	9.626±0.120	10.306±0.145	9.428±0.167
Standard (20 mg/Kg)	5.460±0.191* (27.18%)	6.688±0.182* (22.08%)	3.308±0.052* (65.65%)	3.304±0.056* (67.94%)	3.308±0.052* (64.91%)
QDME 250 mg/Kg	7.028±0.344* (6.2%)	6.204±0.082* (27.72%)	4.602±0.064* (52.19%)	4.220±0.267* (59.05%)	4.220±0.267* (55.23%)
QEME 500 mg/Kg	7.354±0.322* (1.92%)	5.944±0.364* (30.75%)	4.010±0.132* (58.34%)	3.790±0.189* (63.22%)	3.754±0.174* (60.18%)

Values are expressed as mean ±SEM. Where *= $p<0.001$

Table 5: Effect of *Q. dilatata* on Carrageenan induced paw edema

Treatment groups	Paw size (mm)				
	0 hr	1hr	2hr	3hr	4hr
Disease control	3.674±0.127	5.700±0.179	6.414±0.185	6.652±0.152	7.034±0.082
Standard (20 mg/Kg)	3.420±0.094	4.658±0.092*	5.392±0.207*	5.588±0.166*	5.250±0.109* (25.33%)
Extract of *Q. dilatata* (250 mg/Kg)	3.732±0.097	4.852±0.087*	5.636±0.135*	6.002±0.117*	5.828±0.081* (17.14%)
Extract of *Q. dilatata* (500 mg/Kg)	3.498±0.251	4.806±0.067*	5.484±0.109*	6.070±0.109*	5.948±0.157* (19.43%)

Values are expressed as mean ±SEM. Where *= $p<0.001$

Table 6: Analgesic activity of *Q. dilatata* extract on hot plate and acetic acid induced writhings test

Groups	Reaction time (min)					No. of writhings (%age)
	0	30	60	120	180	
DC	3.780± 0.116	4.180± 0.097	4.740± 0.051	4.440± 0.154	4.040± 0.108	45.40± 2.619
Standard (20 mg/Kg)	4.800± 0.100*	8.160± 0.204*	10.960± 0.223*	14.040± 0.178*	15.858± 0.138*	14.400± 1.208*
QDME 250 mg/Kg	3.940± 0.256	6.140± 0.216*	7.860± 0.093*	11.440± 0.166*	11.900± 0.221*	24.600± 1.364*
QDME 500 mg/Kg	3.940± 0.093	7.020± 0.251*	9.420± 0.185*	13.040± 0.140*	15.462± 0.187*	19.200± 1.356*

Values are expressed as mean ±SEM. Where *= p<0.001.DC: Disease control

Effect of QD on acetic acid induced writhing and hot plate test

The plant extract (250 and 500 mg/Kg) and standard significantly reduced the number of abdominal contraction as compared with DC group as presented in table 6. Number of writhings of QDME 500 mg/Kg was insignificantly different from standard but significantly (p<0.001) different from DC group.

In hot plate method, with the passage of time there was increase in latency period of response for treated rats. There was significant (p<0.05) increase in latency period in extract treated group prominently at 500 mg/Kg as compared with disease control groups. Results are depicted in table 6.

DISCUSSION

Protein denaturation is a well-known cause of inflammation leading toward RA by producing auto-antibodies in vicinity. Protein denaturation occurs in case of strong external stimuli i.e. heat and proteins lose their secondary and tertiary structure (Saleem *et al*., 2019). In our study, the plant extract at all tested concentrations significantly inhibited protein denaturation and stabilized the HRBC membrane in contrast to DS dose dependently.

Further anti-inflammatory and anti-arthritic potential of the plant extract was evaluated by employing *in-vivo* assays. Carrageenan induces acute inflammation by acting on complement system or may involve mediators such as, histamine, prostaglandins (PGs) and 5-HT. In our study, the plant extract alleviated the paw edema by inhibiting the release of inflammatory mediators like PGs, histamine and serotonin from activated immune cells (Peerzada *et al*., 2020; Yin *et al*., 2019). Formaldehyde upon injection at sub-planter region induces inflammation by the release of mediators and cytokines. Initial response at first dose is initiated which after second dose of formaldehyde initiates late phase response of inflammation. Late phase response causes edema that leads towards arthritic condition. Results of formaldehyde study indicated that the plant extract exerted anti arthritic effect by blocking both of the phases as compared with DC and standard. The QDME (250 and 500 mg/Kg) has profound anti-inflammatory and antiarthritic activity might be due to presence of quercetin or phenolic compounds such as sinapic acid and gallic acid that are known for these activities. Previously, quercetin inhibited both early and late phase of inflammation by inhibiting TNF-α, PGE_2, phospholipase A2, lipoxygenase, and cyclo-oxygenases (Yin *et al*., 2019; Saleem *et al*., 2020b).

Hot plate method induces pain by thermal stimulus that elicit CNS in test animal. While acetic acid induced writhing method induces pain by chemical stimuli which is peripheral response. Writhings are basically contractions in abdominal muscles in response to irritant administered. Irritation causes pain which transmits signals to CNS which release mediators such as prostaglandins. Results revealed that test group exhibited significant anti-nociceptive activity when compared to disease and standard control groups. The plant extract has exceptional anti-nociceptive activity at 500 mg/Kg dose might be due to high flavonoids and phenols contents (Ihtisham *et al*., 2013). HPLC analysis explored the presence of various phenolic acids and flavonoids. Of which quercetin, coumaric, sinapic, gallic, syringic acids have remarkable anti-inflammatory, anti-nociceptive and antioxidant activity (O Elansary *et al*., 2019).

CONCLUSION

On the basis of above findings, it concluded that QDME could be used for the treatment of inflammation, pain and arthritis. The *in-vitro* and *in vivo* anti-inflammatory, anti-arthritic and analgesic activities might be due to presence of flavonoids, phenols and alkaloids detected in the plant extract. Activity guided fractionation and isolation of main constituent should be done in future.

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