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Assessment of antiarthritic potential of *Ephedra gerardiana* by *in vitro* and *in vivo* methods

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Abstract

The present study determines the antiarthritic potential of *Ephedra gerardiana* ethanolic extract and its ethyl acetate, *n*-butanol and aqueous fractions adopting *in vitro* and *in vivo* tests. *In vitro* tests included thermally induced bovine serum albumin denaturation and egg albumin denaturation, also membrane stabilizing assay at concentration of 50-6400 µg/mL, whereas *in vivo* study comprised formaldehyde-induced arthritis at 50, 100 and 200 mg/kg doses. The crude extract and fractions inhibited protein denaturation and stabilized red blood cells membrane in concentration-dependent fashion, with maximal effect achieved at 6,400 µg/mL (p<0.001). Similarly, in formaldehyde model, the extract and fractions dose-dependently reduced injected paw volume and diameter, with maximum reduction at 200 mg/kg (p<0.001). However, results of aqueous fraction were on a par with hydroalcoholic extract in each test. These results suggest that *E. gerardiana* provides protection against arthritis that might be owing to the existence of phytoconstituents thus, supporting folkloric claim.

Introduction

Rheumatoid arthritis is a systemic autoimmune ailment and most imperative inflammatory arthritis in humans. The cause of rheumatoid arthritis is unknown.

Synthetic drugs employed to treat rheumatoid arthritis include NSAIDs, IL-1 receptor antagonist, DMARDs, anti-TNF-α drugs and glucocorticoids. Nevertheless, their use is accompanied with surplus adverse effects comprising immunosuppression and advent of opportunistic infections. Thus, the need of hour is to develop novel, long acting antiarthritic agents with least adverse effects.

Patients with rheumatoid arthritis are among highest consumers of complementary and alternative medications and they pursue alternative methods for symptomatic relief. Natural herbs offer better results to mankind in dealing with this looming disease and plant

derived drugs have attained admiration in last few years both in developing and developed countries owing to their natural origin and fewer adverse effects (Nair et al., 2011).

The Ephedraceae family contains 45 species of Ephedra genus that are native to sub-tropical and temperate areas of Europe, Asia, Central and North America (Ratsch, 1998). In Pakistan, *Ephedra gerardiana* Wall [Local name: Asmani Booti, Sopat, Soopt (Urdu), Sopt, Soom (Shina)], is found mostly in Northern areas of Pakistan, where local people commonly use whole plant, roots and stem for treating rheumatism and painful joints (Qureshi et al., 2006; Gorsi and Miraj, 2002). It is also used in respiratory tract infections, bone fractures, heart diseases and syphilis (Qureshi et al., 2006). Since, no pharmacological data is available on *E. gerardiana* to the best of our knowledge, a study was piloted to appraise its potential antiarthritic effect

employing both *in vitro* and *in vivo* procedures.

Materials and Methods

Plant collection and identification

E. gerardiana aerial parts were attained from Northern areas of Pakistan and were identified by Dr. Shair Wali Khan, Department of Botany, Karakoram International University of Gilgit Baltistan Pakistan. For future reference, voucher sample (No. EG-15-18) was placed in herbarium of Faculty of Pharmacy, University of Sargodha.

Preparation of extract and fractions

Dried and coarsely grounded aerial parts (stems and leaves) of *E. gerardiana* was extracted by cold maceration process using 10 L (70:30, v/v ratio of ethanol to distilled water) solvent and was soaked at room temperature for 3 days. After 72 hours of intermittent stirring on daily basis, entire substance was strained first using cotton cloth and thereafter via filter paper (Whatman No. 1). This technique was done again three times and collective filtrate on a rotary evaporator (40-50°C) was evaporated under reduced pressure (-760 mm Hg). The plant extract was further air-dried to acquire thick, semi-solid material, providing 21.3% yield. Activity directed fractionation of crude extract (130 g) was achieved using various organic solvents (dichloromethane, *n*-hexane, ethyl acetate and *n*-butanol). The yield of dichloromethane (1.5%) and *n*-hexane (2.3%) was very low so, only aqueous (67.7%), *n*-butanol (16.9%) and ethyl acetate (11.5%) fractions were used to perform activities (Alamgeer et al., 2016). The solutions of test substances were made in distilled water to carry out all the experiments.

Chemicals

The chemicals used in this study were: ethanol, dichloromethane (Sigma-Aldrich, USA), *n*-hexane (Sigma-Aldrich, USA), *n*-butanol (Sigma-Aldrich, USA), ethyl acetate (Sigma-Aldrich, USA), bovine serum albumin (Sigma-Aldrich, USA), egg albumin from fresh hen's egg, disodium hydrogen phosphate (Merck, Germany), sodium chloride (Sigma-Aldrich, USA), formaldehyde (VWR, England), aspirin (UNI-CHEM, Germany), sodium hydroxide (Sigma-Aldrich, USA), potassium dihydrogen phosphate (Riedel-de-Haën, USA), diclofenac sodium (Sigma-Aldrich, USA) and piroxicam (Sigma-Aldrich, USA).

Experimental animals

Young and healthy male and female Sprague Dawley rats were used. The animals were kept in controlled temperature (23 to 25°C) and in 12 hours light and dark cycle at animal house, Faculty of Pharmacy, University of Sargodha. Animals were fed with standard pellet diet and water *ad libitum*.

Egg albumin denaturation inhibition

The 5 mL of test solution contained 0.2 mL of fresh hen's egg albumin, phosphate buffer of pH 6.4 (2.8 mL) and 2 mL of various concentrations (50-6400 µg/mL) of crude extract, aqueous, *n*-butanol and ethyl acetate fractions and standard drug (diclofenac sodium), respectively. Double-distilled water in similar quantity worked as control. The mixtures for 15 min were incubated at 37 ± 2°C and next heated for 5 min at 70°C. The absorbance was assessed at 660 nm, after cooling. The inhibition percentage of egg albumin denaturation was figured out as under (Alamgeer et al., 2015).

Inhibition percentage = [(Absorbance control – Absorbance test sample) / Absorbance control] × 100

BSA denaturation inhibition

In order to estimate *in vitro* antiarthritic efficacy, protein denaturation inhibition technique was adopted (Narayani et al., 2014). Different concentrations (50, 100, 200, 400, 800, 1600, 3200, 6400 µg/mL) of hydroalcoholic extract, fractions (aqueous, *n*-butanol, ethyl acetate) and reference drug (aspirin) were prepared, respectively. 0.5 mL of test control solution comprised 5% w/v aqueous solution of bovine serum albumin, BSA (0.45 mL) and 0.05 mL distilled water. Similarly, 0.5 mL product control solution contained test solution (0.05 mL) and distilled water (0.45 mL). Moreover, 0.5 mL test solution consisted of BSA (0.45 mL) and test solution (0.05 mL). The 0.5 mL standard solution included aspirin (0.05 mL) and BSA (0.45 mL). The pH of aforementioned solutions was altered to 6.3 via 1N HCl. Later, for 20 min, the solutions were incubated at 37°C and then for 3 min heated at 57°C. Thereafter, preceding mixtures were supplemented with phosphate buffer (2.5 mL) after cooling. The absorbance at 660 nm was evaluated through UV-Visible spectrophotometer and inhibitory percentage of BSA denaturation was calculated as under:

Inhibition percentage = 100 – [(Abs TS – Abs PC)/ Abs TC] × 100

Where,

Abs=Absorbance, TS=Test solution, PC=Product control, TC=Test control

Human red blood cell (HRBC) membrane stabilization assay

Healthy humans who did not take NSAID'S atleast 2 weeks before conducting experiment donated the blood, which was combined with similar quantity of Alsever's solution. The centrifugation of this solution at 3,000 rpm was then done. Packed cells were removed, rinsed with normal saline and a suspension of 10% v/v human red blood cell was made with normal saline.

Test solution contained 2 mL hypotonic saline, phosphate buffer (1 mL), 0.5 mL test samples at several concentrations (50, 100, 200, 400, 800, 1600, 3200, 6400 μg/mL), correspondingly and 0.5 mL of 10% v/v HRBCs. Similarly, test control comprised 2 mL distilled water, phosphate buffer (1 mL) and 10% v/v HRBCs (0.5 mL). Further, standard solution comprised 0.5 mL of 10% v/v HRBCs, 2 mL of hypotonic saline, phosphate buffer (1 mL) and 0.5 mL of diclofenac sodium at same concentrations. The test solutions for 30 min were incubated at 37°C and centrifuged at 3000 rpm. The spectrophotometer at 560 nm was used to estimate hemoglobin content in supernatant. The percentage membrane stabilization was worked out by under mentioned formula (Alamgeer et al., 2015):

Percentage membrane stabilization = 100- [(Sample optical density / Control optical density) × 100]

Formaldehyde-induced arthritis

Animals were alienated into various groups of n=5. Group A received distilled water (3 mL/kg) only, Group B received 10 mg/kg piroxicam, Groups C, D, E were given 50, 100, 200 mg/kg of *E. gerardiana* crude extract, separately, Groups F, G, H were treated with 50, 100, 200 mg/kg of aqueous fraction, correspondingly, Groups I, J, K received 50, 100, 200 mg/kg of *n*-butanol fraction, accordingly and Groups L, M, N were administered 50, 100, 200 mg/kg of ethyl acetate fraction, respectively. The test solutions were administered orally to rats. On the day first, 30 min subsequent to oral administration of test substance, acute non-immunological arthritis was prompted via sub planter

injection of formaldehyde 2% (0.1 mL), which was repeated on third day. Drug treatment continued for 10 days. Arthritis was appraised by determining paw volume using digital plethysmometer (Alamgeer et al., 2017). The percentage inhibition of paw volume by plant extract, fractions and piroxicam was equated with arthritic control.

$$\%\text{inhibition} = \frac{Vc - Vt}{Vc} \times 100$$

Vc = Paw volume of control, Vt = Paw volume of treated

Statistical analysis

The results were described as mean ± standard error of mean (S.E.M). Statistical analysis was accomplished making use of two-way analysis of variance (ANOVA) test, followed by Bonferroni posttest, using GraphPad Prism 5. The value of significant difference was considered at p<0.05.

Results

The antiarthritic effect of *E. gerardiana* aqueous ethanolic extract and its fractions was appraised against egg albumin denaturation (Figure 1). Plant extract and fractions displayed noteworthy (p<0.001) egg albumin denaturation inhibition in a concentration-dependent manner (50-6400 μg/mL). The hydro-alcoholic extract at 6400 μg/mL offered 3219.4% inhibition and among the fractions, aqueous fraction proved to be most efficacious with 2899.3% egg albumin denaturation inhibition at 6400 μg/mL. Though, diclofenac sodium
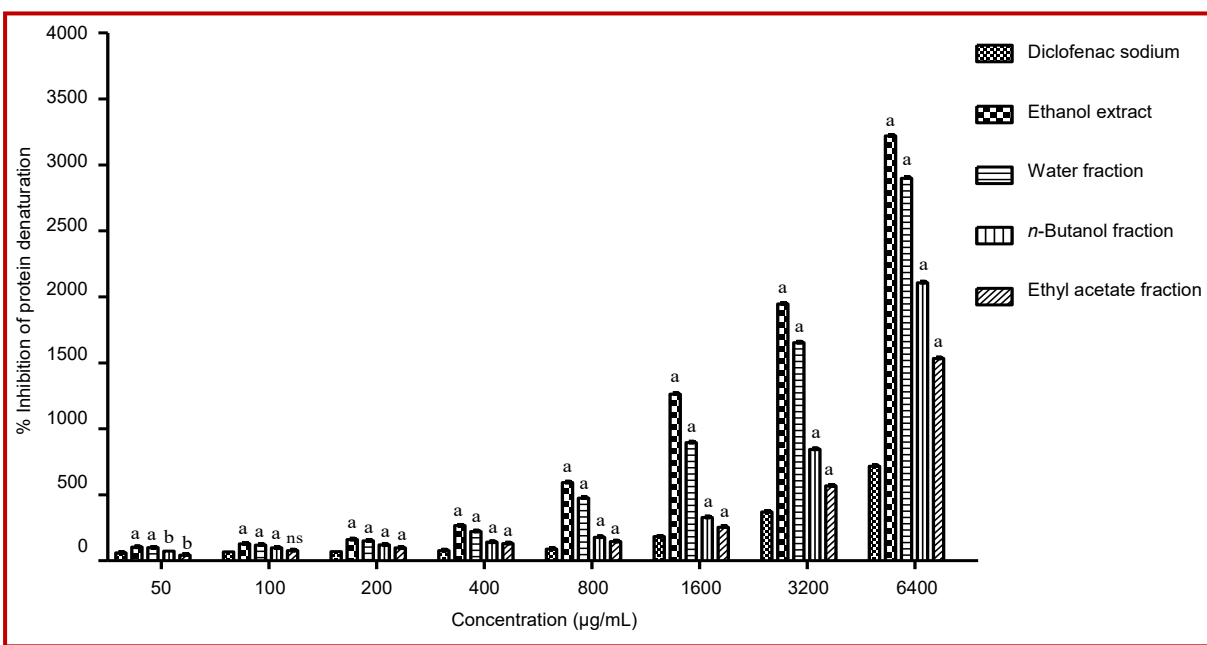

Figure 1: Effect of *E. gerardiana* on inhibition of protein (egg albumin) denaturation. Results are expressed as mean ± SEM (n=3), using two way ANOVA followed by Bonferroni posttest. [a]p<0.001, [b]p< 0.05 when compared to standard
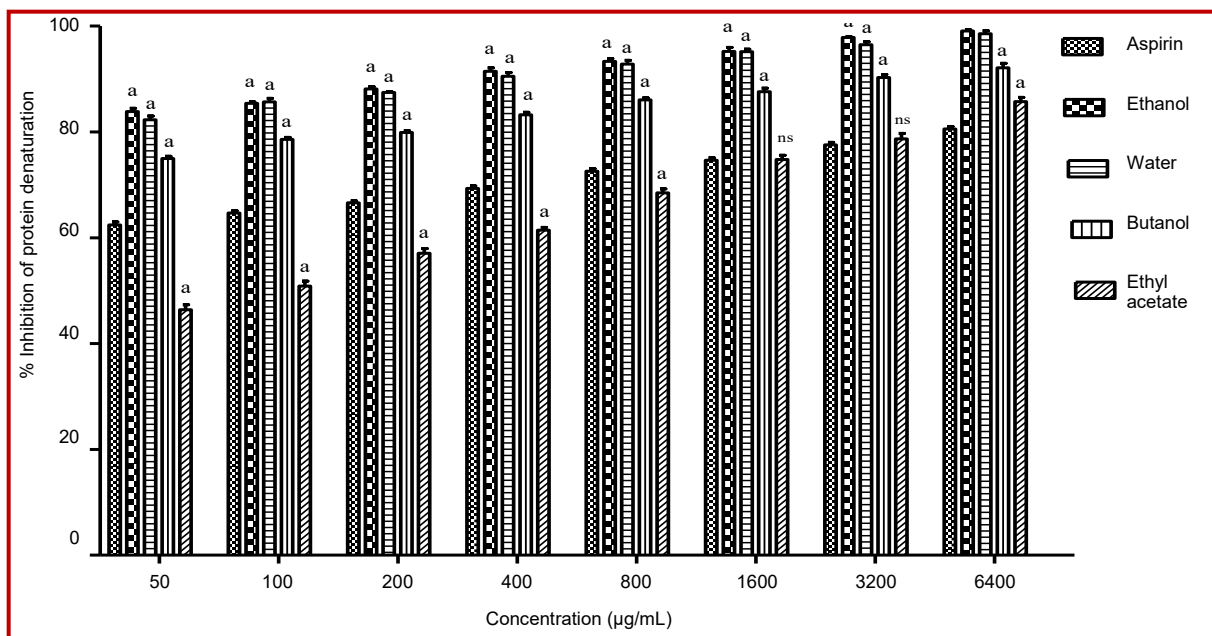
Figure 2: Effect of EG on inhibition of protein (BSA) denaturation. Results are expressed as mean ± SEM (n=3), using two way ANOVA followed by Bonferroni posttest. [a]$p<0.001$, [ns]non-significant when compared to standard
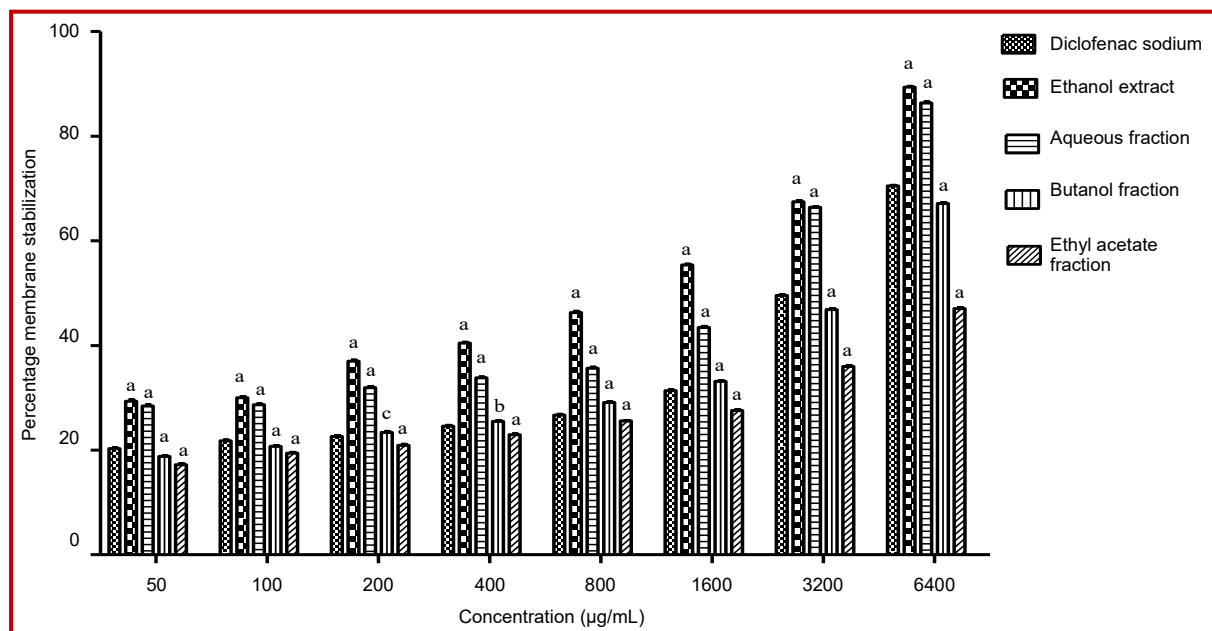

Figure 3: Effect of *Ephedra gerardiana* on human red blood cell membrane stabilization. Results are expressed as mean ± SEM (n=3), using two way ANOVA followed by Bonferroni posttest. [a]$p<0.001$, [b]$p<0.01$, [c]$p<0.05$ when compared to standard.

exhibited concentration-dependent protein denaturation inhibition but it showed less effect compared to crude extract and fractions of *E. gerardiana* at similar concentrations.

Similarly, concentrations ranging from 50-6400 µg/mL were tested to find out the inhibitory percentage of BSA denaturation using aspirin as a standard. A significant concentration dependent rise (p<0.001) in percentage inhibition was detected for all the concentrations tested,

with a dose-dependent upsurge in antiarthritic activity (Figure 2). Results depicted 99.1% impediment of BSA at 6400 µg/mL of crude extract. Nonetheless, aqueous fraction produced maximum inhibitory effect than *n*-butanol and ethyl acetate fractions i.e., 98.5, 92.1 and 85.7%, correspondingly at 6400 µg/mL. The effect of extract and fractions at 6400 µg/mL was more pronounced than aspirin (80.5%).

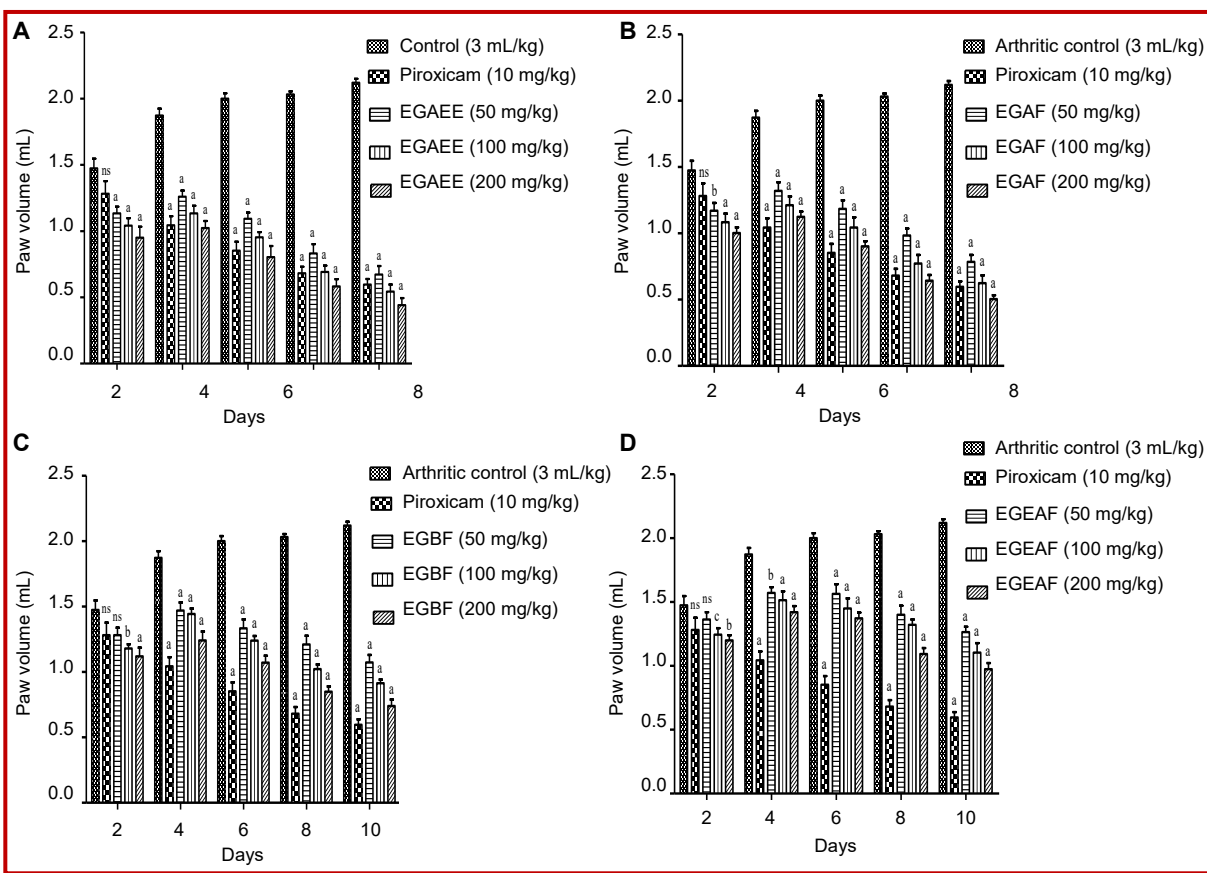In human red blood cell membrane stabilization test,

Figure 4: Effect of *E. gerardiana* extract and fractions: (A) Aqueous ethanolic extract, (B) Aqueous fraction, (C) *n*-butanol fraction, (D) Ethyl acetate fraction) on paw volume of formaldehyde induced arthritic rats. [a]$p<0.001$, [b]$p<0.01$, [c]$p<0.05$, [ns]non-significant when compared to arthritic control. EGAEE= *E. gerardiana* aqueous ethanolic extract, EGAF= *E. gerardiana* aqueous fraction, EGBF=*E. gerardiana* butanol fraction, EGEAF= *E. gerardiana* ethyl acetate fraction

plant extract and its fractions notably protected erythrocyte membrane against lysis induced by heat and hypotonic solution in a concentration dependent manner (50-6400 µg/mL) (Figure 3). Plant extract, aqueous, *n*-butanol and ethyl acetate fractions at 6400 µg/mL concentration showed maximum protection of human red blood cell in hypotonic solution i.e., 89.3, 86.3, 67.1 and 47.0%, in that order. All the results were compared with diclofenac at same concentrations, which showed 70.4% protection of human red blood cell in hypotonic solution at 6400 µg/mL.

The anti-arthritic potential was also appraised *in vivo* using formaldehyde prompted arthritis in rats. Sub planter injection of formaldehyde in arthritic control group showed substantial increase in paw volume and diameter throughout the study period. The assessment made on 10th day presented that treatment with 200 mg/kg of plant extract, aqueous, *n*-butanol and ethyl acetate fractions considerably ($p<0.001$) decreased injected hind paw volume i.e., 79.1, 76.2, 65.0, 54.0 (Figure 4) and paw diameter i.e., 75.0, 73.6, 61.0 and 50.9% (data not shown), respectively as compared with arthritic control group. However, piroxicam (10 mg/kg)

treated animals exhibited 71.8 and 70.6% inhibition of paw volume and diameter, correspondingly on 10th day.

Discussion

The outcomes of existing study have discovered dose-dependent antiarthritic potential of aqueous ethanolic extract, aqueous, *n*-butanol and ethyl acetate fractions of *E. gerardiana* in both *in vitro* and *in vivo* techniques. The *in vitro* methods used to check antiarthritic activity included inhibition of BSA and fresh hen's egg albumin denaturation and membrane stabilization assay, while *in vivo* method involved formaldehyde induced arthritis in rats.

Preceding investigations have stated that protein denaturation is the principal cause of inflammatory and arthritic disorders that proceed to auto-antigens generation thus, resulting in rheumatic disorders. The denaturation possibly implicates variation in hydrogen, electrostatic, disulphide and hydrophobic bonds (Gautam et al., 2013). Numerous anti-inflammatory

drugs dose dependently deter thermally prompted protein denaturation (Menon et al., 2011). Therefore, substances which can avert denaturation of protein would be considered valuable for developing anti-arthritic drugs. In the current analysis, hydro-alcoholic extract and fractions of *E. gerardiana* markedly subdued protein denaturation (BSA and egg albumin) and results were more pronounced than standard drug. From the findings, it could be concluded that *E. gerardiana* is capable of subsiding auto-antigens production, which circuitously reduces protein denaturation and hence relieves arthritis.

The membrane of erythrocytes is analogous to membrane of lysosomes and stabilization of human red blood cell from hypotonicity instigated lysis can be allied with antiarthritic property of drug. The hypotonic solution causes rupturing of lysosomal mem-brane, consequently bringing about release of lysosomal enzymes (bacterial enzymes and proteases) and phospholipase A2 that recruits the generation of inflammatory intermediaries. Hence, stabilization of red blood cell membrane obviates the liberation of inflammatory intermediaries, which diminishes cell rupture and tissue injury (Alamgeer et al., 2015). Some NSAIDS and glucocorticoids stabilize lysosomes in tissue cells and thereby preclude the discharge of lysosomal enzymes into cytoplasm of cells, thus avoiding deterioration (Udavant et al., 2012). *E. gerardiana* and its fractions notably repressed hypotonicity induced human red blood cell hemolysis in concentration-dependent manner, compared with diclofenac sodium. The findings of present work suggest membrane stabilization as an additional mechanism of anti-arthritic effect of *E. gerardiana*, which might be due to direct interaction of phytoconstituents with membrane components such as proteins, albeit, exact mechanism of membrane stabilization is not documented.

Formaldehyde-induced arthritis is a most apposite model for screening an agent with plausible anti-arthritic, anti-inflammatory and antiproliferative activity, as it resembles human arthritis (Ben et al., 2016). Formaldehyde tempts arthritis by denaturing proteins at the site of injection, which primes to development of an immunological response against degraded products (Nair et al., 2012). Moreover, formaldehyde persuaded arthritis is biphasic in nature i.e., it expresses neurogenic components, which influence brain activity and then lead to tissue-mediated reaction. Neurogenic phase directly excites paw engorgement together with centrally interceded effects of pain via the discharge of pain intermediaries in later phase (Owoyele et al., 2011). The primary stage of edema is attributable to liberation of histamine, serotonin and kinin-like substances while, second accelerating phase of edema formation may be owing to prostaglandin like substances release (Shaikh, 2011). Earlier studies assert that agents that act on CNS impede both stages equally, though peripherally

working substances obstruct later stage (Alamgeer et al., 2017). The reduction in paw volume and diameter detected in formaldehyde model may be due to the conceivable mechanism of *E. gerardiana* to inhibit these chemical mediators of inflammation. The marked decline in paw volume and diameter was presented by 200 mg/kg of crude extract and its aqueous fraction. Moreover, *E. gerardiana* extract and fractions inhibited both phases of inflammation, therefore it acts centrally.

Ephedra species contain alkaloids such as ephedrine, norephedrine, methylephedrine, pseudoephedrine, norpseudoephedrine, and methylpseudoephedrine. Besides, in some Eurasian *Ephedra* species ephedroxane and ephedradine A-D, have been found. *Ephedra* genus also comprises flavonoids (leucoanthocyanidin, leucopelargonine, leucodelphinidin, lucenine, vicenin-1 and 2), tannins and benzylmethylamine (Ibragic and Sofić, 2015). In addition, phytochemical investigations and review of literature shown the existence of flavonoids, alkaloids, terpenoids, saponins and glycosides in *E. gerardiana* stem (Jamil et al., 2012). Also, formerly in DPPH test, *E. gerardiana* leaf extract has shown antioxidant activity with 13.3 ± 0.6 µg/mL IC_{50} value (Kumar and Singh, 2011). Among the aforementioned constituents, saponins and alkaloids are recognized to reduce arthritic index, prevent articular swelling and control IL-1β and TNF-α levels in inflammatory tissues of arthritic rats. Flavonoids are often used for their antioxidant effect against free radicals and also possess anti-inflammatory properties (Fatima and Fatima, 2016). Hence, it can be proposed that antiarthritic activity of *E. gerardiana* hydroalcoholic extract and fractions could be due to combined effect of alkaloids, flavonoids, saponins and antioxidant property of *E. gerardiana*. The presence of these compounds in extract can strongly support anti-arthritic potential of *E. gerardiana* and its use in traditional alternative and complementary medicine.

Conclusion

E. gerardiana may serve as an effective antiarthritic drug and effect may be speculated owing to its phytoconstituents.

Financial Support

Ethical Issue

The study protocol was approved by Institutional Animal Ethics Committee, Faculty of Pharmacy, University of Sargodha (Approval No. 51A45 IEC UOS). The animals were treated according to standard procedures guided by National

Research Council (NRC, 1996).

Conflict of Interest

The authors declare no conflicts of interest.

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