https://doi.org/10.31925/farmacia.2022.3.16 **ORIGINAL ARTICLE**

IN VITRO AND *IN VIVO* APPROACH FOR THE DETERMINATION OF THE ANTI-ARTHRITIC POTENTIAL OF PSEUDOEPHEDRINE

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Abstract

Based on the traditional and scientific value of *Ephedra* against rheumatoid arthritis, the current study was projected to evaluate the therapeutic value of the main constituent of this plant. Pseudoephedrine, the active ingredient of *Ephedra*, has been tested in this study by using *in vitro* and *in vivo* techniques. For the *in vitro* study, various concentrations (50 - 6400 µg/mL) of pseudoephedrine were used to evaluate the effect on human red blood cells membrane and to establish the inhibitory effect on protein destruction. *In vivo* studies were carried out using egg albumin-induced paw oedema and formaldehyde-induced arthritis model. The results demonstrated a dose dependent protection on egg and bovine serum albumin denaturation. The tested drug showed a maximum membrane stabilizing effect at a dose of 6400 µg/mL and a pharmacologic effect at a dose of 40 mg/kg bw.

Rezumat

Ținând cont de utilizarea tradițională și de profilul științific al speciei *Efedra* în artrita reumatoidă, studiul actual explică valoarea terapeutică a principalului constituent al acestei plante. Pseudoefedrina, principiul activ din *Ephedra*, a fost testată în acest studiu prin utilizarea tehnicilor *in vitro* și *in vivo*. Pentru studiul *in vitro*, au fost utilizate diferite concentrații (50 - 6400 µg/mL) de pseudoefedrină în vederea evaluării efectului de stabilizare a membranei eritrocitelor umane și pentru a stabili efectul inhibitor asupra distrugerii proteinelor. Studiile *in vivo* au fost efectuate utilizând un model experimental de edem al labei indus cu albumină din ou și un model de artrită indusă cu formaldehidă. Rezultatele au demonstrat un efect doză-dependent asupra albuminei. Pseudoefedrina a prezentat efect de stabilizare a membranei la o concentrație de 6400 µg/mL și efecte farmacocologice la doza de 40 mg/kg c în modelele experimentale utilizate.

Keywords: rheumatoid arthritis, pseudoephedrine, formaldehyde, plethysmometer

Introduction

Rheumatoid Arthritis (RA) is an autoimmune disease where mitotic division of cells cause chronic inflammation that led to tissue destruction. As a result, physical inability, disorders of other body systems and organs including heart, kidney, etc. occur. An alarming situation is that rheumatoid arthritis occurs in 1% of population globally as reported by previous epidemiological study. Currently approved drugs for treatment of RA are non-steroidal anti-inflammatory drugs and disease modifying anti-rheumatic drugs (DMARDs). These available drugs have economic burden and severe side effects. Due to such limitations of therapeutics of RA, there is a need for alternative therapies [1].

In the last few years, reduced toxicity of herbal medicines has increased their importance in comparison to synthetic drugs. These herbal medicines are economical and devoid of toxic effects [1]. Pakistan has rich flora of medicinal plants, locally employed for treating various conditions of inflammation. Scientifically validation of these plants might be a reason for drug discovery [2]. The *Ephedraceae* family comprises 45 species of genus *Ephedra*. In Gilgit Baltistan, the traditional uses of *Ephedra gerardiana* (local name *asmani booti*) are analgesic, anti-inflammatory, for pulmonary disorders and oncological dysfunctions. The previous studies confirmed the scientific significance of this plant in rheumatoid arthritis (RA) [3]. The active phyto-constituent of *Ephedra* is pseudoephedrine (PE) [4, 5]. PE has inhibitory potential on prostaglandins in acute inflammatory model as well as suppresses the expression of reactive oxygen species (ROS) [6, 7]. Therefore, due to therapeutic effectiveness of *Ephedra* and its inhibitory effect on prostaglandins, the current study was designed to find the therapeutic value of pseudo-ephedrine against RA.

Materials and Methods

In vitro study

Protection on denaturation of protein (egg albumin): 0.2 mL of egg albumin, 2.8 mL phosphate buffered saline (pH 6.4), 2 mL of already prepared solution of different concentrations of pseudoephedrine (50 - 6400 µg/mL) and standard drug naproxen were mixed. The reaction mixture was kept in incubator for 15 min at a temperature of 37 ± 2°C. After incubation it was heated in oven at 70°C for 5 minutes. The test tubes of reaction mixture were cooled for 5 minutes and were scanned on UV-visible spectrophotometer (λ = 660 nm). By analysing the absorbance of the reaction mixture and control solution, the percentage inhibition was calculated, using the below formula [8]:

$$\text{Inhibition (\%)} = \frac{\text{Abs of Test control} - \text{Abs of Test sample}}{\text{Abs of Test sample}} \text{ x } 100.$$

Protective effect on denaturation of protein (bovine albumin): 0.5 mL of different concentrations of pseudo-ephedrine in distilled water and 0.45 mL of 5% aqueous solution of bovine albumin were mixed and the mixture was used for the spectrophotometric analysis. The pH mixture was regulated at 6.3 and the reaction mixture was put in incubator for 20 min at 37°C and then allowed to cool. 2.5 mL phosphate buffer saline (pH 6.3), in cooled mixture were added and then the absorbance was measured by using an UV-visible spectrophotometer. Purified water was kept as control. Naproxen served as a standard drug. Percentage inhibition was determined by using the below formula [9]:

$$\text{Inhibition [\%]} = \frac{\text{100 - (Abs of Test solution - Abs of Product control)}}{\text{Abs of Test control}} \text{ x } 100.$$

Human RBCs membrane stabilization method. Individuals of experiment were prohibited to use of NSAIDs 2 weeks before the commencement of procedure. Alsever's solution was prepared and mixed with an equal volume of blood. The mixture was centrifuged at 3000 rpm to separate cells. 10% w/v suspension was obtained. Test solution consisting of 1 mL of phosphate buffer, 2 mL of hypotonic saline, 1 mL of pseudoephedrine (50 - 6400 µg/mL) and 0.5 mL of 10% w/v human RBCs was formerly used. Following the above, control and standard solution were also obtained by normal saline and naproxen, respectively. All of them were incubated at 37°C for 30 min and were put in a centrifuge machine at 300 rpm for separation. Clear liquid above was separated and the haemoglobin content was measured using spectro-photometer at 660 nm. Considering 100% haemolysis in the control, percentage haemolysis was determined and then percentage of human RBCs membrane stabilization was determined [9].

$$\text{Protection [\%]} = \frac{\text{100 - (Optical density of sample)}}{\text{Optical density of control}} \text{ x } 100.$$

In vivo study

Experimental animals. Young Sprague Dawley (SD) of both sexes were kept at 23 ± 2ºC and in 12 hours light and dark cycle, at animal house, College of Pharmacy, University of Sargodha, Pakistan. Animals were fed with standard pellet diet and water *ad libitum* according to the standards of INH.

Egg-albumin induced inflammation. Inflammation was induced by egg albumin following the protocol given by Ahsan *et al*. [2]. Firstly, animals were fastened for 24 hours and then divided into 5 groups, each group containing 5 animals. First group was considered as control and it was given distilled water. Second group was the standard group and it was administered naproxen orally 10 mg/kg bw [10]. Third, fourth and fifth group are the experimental or test groups and received test drug (pseudoephedrine). The dose was administered orally, with 10, 20 and 40 mg/kg respectively. Animals were placed for 1 hour and then inflammation was induced by 0.1 mL injection of egg albumin into the right hind paw of all animals. The swollen paw was measured by using a plethysmometer at 0, 1, 2, 3 and 4 hours after inflammation. Percentage inhibition was calculated by using calculated paw volume [2].

$$\text{\% inhibition} = \frac{\text{paw volume of control group - paw volume of treated group}}{\text{paw volume of control group}} \text{ x } 100.$$

Formaldehyde-induced arthritis. Animals of both genders were studied and were divided into six groups. First group was the control group and the animals received distilled water. Second group of animals was called the arthritic control group and also they were administered distilled water. Third group of animals was the standard group and received naproxen 10 mg/kg bw orally [10]. Test animals of first drug were in the fourth, fifth and six group and were given 10, 20 and 40 mg/kg bw of pseudoephedrine orally, respectively. After an hour, 2% formaldehyde was administered by injection to all groups except the first group, on the first day of the experiment. On the third day, again 0.1 mL of formaldehyde was injected to all animals. On day 0, 2nd, 4th, 6th, 8th and 10th day of experiment, volume of paw was measured with the plethysmometer. The percentage inhibition was calculated according to methods explained by Uttra AM [9]:

$$\text{Inhibition (\%)} = \frac{V_c - V_t}{V_c} \text{ x } 100,$$

where: V_c = paw volume of control, V_t = paw volume of treated.

Statistical analysis

The data related to the anti-arthritic effect were expressed as mean ± SEM. Two-way ANOVA followed by Bonferroni multiple comparison Post hoc test was adopted using the Graph-Pad-Prism version 8.00. A difference in the mean values of $p < 0.05$ was considered statistically significant.

Results and Discussion

Protective effect of pseudoephedrine on protein denaturation (egg albumin and bovine serum albumin)

Pseudoephedrine in various concentrations had been employed in order to evaluate its protective effect against denaturation of egg and bovine serum albumin. The results disclosed that pseudoephedrine produced 75.15% and 78.36% protection against denaturation of egg as well as bovine serum albumin at concentrations of 6400 µg/mL compared to 70.27% and 49.28% naproxen inhibition as shown in Table I. The percentage protection decreased, by reducing the concentration of treated solution and standard drug solution.

Table I

Effect of different concentrations of pseudoephedrine on inhibition of egg and bovine serum albumin denaturation

Concentrations µg/mL	Pseudoephedrine		Naproxen	
	% inhibition of egg albumin protein denaturation	%inhibition of bovine serum albumin protein denaturation	% inhibition of egg albumin protein denaturation	%inhibition of bovine serum albumin protein denaturation
6400	75.15	78.36	70.27	49.28
3200	68.82	73.68	60.26	41.35
1600	67.74	69.59	59.37	36.41
800	67	57.89	49.54	31.71
400	65.40	50.87	48.24	23.34
200	63.96	45.61	45.44	9.71
100	49.54	36.25	40.27	8.50
50	49.54	31.57	36.51	7.16

Effect of Pseudoephedrine membrane stabilization of human RBCs

This study was performed to develop new therapy for arthritis. The pseudoephedrine caused stabilization of human red blood cells membrane against its destruction due to heat and hypotonic saline solution. This stability of pseudoephedrine was expressed in the form of percentage. The maximum stabilization effect was observed at concentration of 6400 µg/mL. This effect decreased towards the lowest concentration of 50 µg/mL.

Table II

Effect of different concentrations of pseudoephedrine on human red blood cells membrane stabilization

Concentrations µg/mL	% inhibition	
	Pseudoephedrine	Naproxen
6400	50.06	51.27
3200	48.95	49.36
1600	47.86	48.21
800	47.40	48.10
400	46.71	47.37
200	46.71	46.21
100	43.66	43.21
50	40.93	33.25

The percentage stabilization showed in Table II was about 50.06% at the highest concentration of 6400 µg/mL and 40.93% at the lowest concentration of 50 µg/mL. These results were comparable to the standard drug. This protection of pseudoephedrine against destruction by hypotonic solution confirmed its anti-arthritic effect.

In vivo results

Effect of pseudoephedrine against egg albumin induced inflammation

Pseudoephedrine anti-inflammatory effect has been measured at various doses of about 10 mg/kg bw, 20 mg/kg bw and 40 mg/kg bw. At the low dose of about 10 mg/kg, pseudoephedrine, during the early phase of inflammation, on about 0 h to 1 h, revealed less significant effect. But at the late phase the low dose inhibitory effect of pseudoephedrine has been increased significantly. Then pseudoephedrine dose was increased to 20 mg/kg bw. This dose of pseudo-ephedrine exhibited significant effect ($p < 0.001$) at the initial as well as at the final phase of inflammation. To confirm this significant effect of pseudoephedrine against egg albumin-induced inflammation, the dose has been doubled to 40 mg/kg bw. This dose has highly significant inhibition of inflammation as compared to 40 mg/kg bw. This significant effect has also been observed at all hours such as 0 h, 1 h, 2 h, 3 h and 4 h. The standard drug naproxen also produced anti-arthritic effect at significance level at late phase of inflammation.

Table III

Effect of different doses of pseudoephedrine against egg albumin - induced paw oedema

Time (Hour)		Paw volume (mL)			
	control	Pseudoephedrine 10 mg/kg bw	Pseudoephedrine 20 mg/kg bw	Pseudoephedrine 40 mg/kg bw	Naproxen 10 mg/kg bw
0	0.95 ± 0.004	1.21 ± 0.011	0.80 ± 0.013	0.81 ± 0.007	1.82 ± 0.014
1	1.52 ± 0.006	1.42 ± 0.007[c] (6.57%)	1.02 ± 0.018[a] (32.89%)	0.9 ± 0.004[a] (40.78%)	1.43 ± 0.014[d] (5.92%)
2	1.32 ± 0.004	1.21 ± 0.005[a] (7.23%)	0.96 ± 0.047[a] (27.27%)	0.82 ± 0.005[a] (69.87%)	1.26 ± 0.010[c] (4.54%)
3	1.21 ± 0.005	1.1 ± 0.006[a] (9.09%)	0.98 ± 0.45[a] (19.01%)	0.72 ± 0.004[a] (61.49%)	1.12 ± 0.004[a] (7.43%)
4	1.19 ± 0.005	0.99 ± 0.007[a] (16.80%)	0.68 ± 0.013[a] (42.85%)	0.62 ± 0.007[a] (52.1%)	0.97 ± 0.012[a] (18.48%)

Results are described in form of mean ± SEM where "a" is $p < 0.001$; "b" is $p < 0.01$; "c" is $p < 0.05$ and "d" is $p > 0.05$

Formaldehyde induced arthritis by pseudoephedrine

The efficacy of pseudoephedrine in the non-immunological arthritis induced by formaldehyde was appraised by measuring the paw volume at different days. Formaldehyde causes significant increase in paw volume in arthritic control groups. Therefore, pseudoephedrine significantly prevented the arthritic effect of formaldehyde. It caused highly significant inhibition of paw oedema on the 6th, 8th and 10th days at all doses such as 10, 20 and 40 mg/kg bw. But this protective effect was drug dose-dependent. These different oral doses of 40, 20 and 10 mg/kg bw, on day 10, caused 52.66%, 36.1% and 26.6% percentage inhibition of paw oedema respectively. At day 2, the 10 mg/kg bw dose cause less significant effect as compare to 20 mg/kg bw. The 40 mg/kg bw dose also caused highly ($p < 0.001$) inhibition at all days. Furthermore, the standard naproxen at dose of 10 mg/kg bw has less anti-arthritic as compared to the 10 mg/kg bw dose of pseudoephedrine, as mentioned in Table IV.

Table IV

Effect of different doses of pseudoephedrine against formaldehyde induced arthritic model

Time	Paw volume (mL)					
	Control	Arthritic control	Pseudoephedrine 10 mg/kg bw	Pseudoephedrine 20 mg/kg bw	Pseudoephedrine 40 mg/kg bw	Naproxen 10 mg/kg bw
0 Day	0.71 ± 0.028	0.70 ± 0.041	0.77 ± 0.025	0.71 ± 0.037	0.69 ± 0.039	0.72 ± 0.040
2nd Day	0.70 ± 0.027	1.35 ± 0.034	1.26 ± 0.00[d] (6.66%)	1.23 ± 0.029[c] (8.88%)	1.14 ± 0.026a (15.55%)	1.32 ± 0.040[d] (2.22%)
4th Day	0.70 ± 0.029	1.56 ± 0.034	1.42 ± 0.02[b] (8.97%)	1.41 ± 0.032[b] (9.61%)	1.19 ± 0.025[a] (23.71%)	1.41 ± 0.037[c] (9.61%)
6th Day	0.69 ± 0.030	1.54 ± 0.036	1.34 ± 0.03[a] (12.98%)	1.20 ± 0.024[a] (22.07%)	0.99 ± 0.023[a] (35.71%)	1.32 ± 0.035[a] (14.28%)
8th Day	0.72 ± 0.028	1.52 ± 0.032	1.24 ± 0.02[a] (18.42%)	1.07 ± 0.02[a] (29.60%)	0.81 ± 0.025[a] (46.71%)	1.20 ± 0.033[a] (21.05%)
10th Day	0.70 ± 0.026	1.50 ± 0.031	1.10 ± 0.018[a] (26.6%)	0.96 ± 0.022[a] (36.1%)	0.71 ± 0.031[a] (52.66%)	1.10 ± 0.035[a] (26.66%)

Results are described as mean ± SEM where "a" is $p < 0.001$; "b" is $p < 0.01$; "c" is $p < 0.05$ and "d" is $p > 0.05$

Rheumatoid arthritis is a disease caused by interaction of T-cells with pro-inflammatory cytokines that attack the body's own molecules, cells or tissues. Accumulation of inflammatory mediators and cytokines in the bone junctions lead to rheumatoid arthritis by activation of various inflammatory cascade that cause damage to bones and joints. Synovial inflammation is majorly due to cytokines [11].

For calculating the amount of inflammation and analysing the medicinal effects of drug, prevention of protein destruction and measuring the swelling on paw is an easy and time-saving practice.

Pseudoephedrine is the major constituent of *Ephedra*. The folklore use of pseudoephedrine is mostly for jaundice, rheumatism, leprosy and other body dysfunctions. Pharmacological suggestions for various traditional uses have been given, but scientific evidences need to be found [12]. The core objective of the current study was to estimate the effectiveness of pseudoephedrine in *in vitro*/*in vivo* models.

Our study showed the anti-rheumatic activity of pseudoephedrine. It has prevented the destruction of albumin proteins and also stabilized the human RBCs membrane. Destruction of proteins is due to the alteration in the protein binding and compounding, due to various chemical and physical factors [13]. Research demonstrated that inhibition of protein denaturation by using pseudoephedrine is the same as in the case of the standard drug and furthermore the inhibition is influenced by the constituent's concentration.

Any damage to the lysosomal membrane results in the release of the inflammatory mediators. Human RBCs

membrane stabilization method is used to evaluate the anti-rheumatic activity of pseudoephedrine as the RBCs membrane is similar to that of the lysosomal membrane [14]. The effect of pseudoephedrine is highlighted using this method as the results presented in Tables I and II emphasize this.

Inflammatory mediators like histamine are released by the inflammatory agent, for example albumin, to induce oedema. Histamine participates in the inflammatory pathway and also causes smooth muscles relaxation resulting in the leakage of exudates. This exudate is the cause of inflammation symptoms like swelling. As swelling of paw is minimized by pseudoephedrine (Table III), it may inhibit the release of histamine [15]. Formaldehyde causes oedema in the paw and this can be prevented by any anti-rheumatic agent. Inflammation is induced by formaldehyde in two phases (i) initial phase and (ii) inflammatory phase. At the initial stage, substance P is released which may cause pain in joints. Other inflammatory mediators like serotonin and prostaglandins are release in later phase and cause inflammation and increase vascular permeability [16]. Drugs effecting CNS can prevent both phases whereas later or second phase only can be influenced by peripherally acting drugs [17]. It has been mentioned in Table IV that pseudoephedrine significantly prevented the rise in paw volume after the injection of formaldehyde.

Results concluded that 40 mg/kg bw of pseudoephedrine showed significant effect over a dose of 10 mg/kg bw naproxen. Above mentioned experiments such as inhibition of protein denaturation and human RBCs stabilization confirmed the use of pseudoephedrine as anti-rheumatic agent.

Conclusions

It is concluded that the pseudoephedrine has positive effect against rheumatic fever that may be due to its effect on inflammatory mediators. This study scientifically supports the anti-rheumatic effect of *Ephedra* due to its active constituent pseudoephedrine. Further studies are needed in order to evaluate its effect on specific inflammatory markers such as IL-1 and TNF-alpha.

Conflict of interest

The authors declare no conflict of interest.

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