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 Ephedra în artrita reumatoidă, studiul actual explică valoarea terapeutică a principalului constituant 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 membranelor eritrocitare 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 membranelor la o concentrație de 6400 µg/mL și efecte farmacologice 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 pseudoephedrine 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]:

\[
\text{Inhibition (\%)} = \frac{\text{Abs of Test control} - \text{Abs of Test sample}}{\text{Abs of Test control}} \times 100.
\]

Protection on denaturation of protein (bovine albumin): 0.5 mL of different concentrations of pseudoephedrine 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]:

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

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 were put in a centrifuge machine at 300 rpm for 30 minutes and then percentage of human RBCs membrane stabilization was determined [9].

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

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]:

\[
\text{Inhibition (\%)} = \frac{V_e - V_t}{V_e} \times 100,
\]

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>
<thead>
<tr>
<th>Concentrations (μg/mL)</th>
<th>% inhibition of egg albumin protein denaturation</th>
<th>% inhibition of bovine serum albumin protein denaturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>6400</td>
<td>75.15</td>
<td>78.36</td>
</tr>
<tr>
<td>3200</td>
<td>68.82</td>
<td>73.68</td>
</tr>
<tr>
<td>1600</td>
<td>67.74</td>
<td>69.59</td>
</tr>
<tr>
<td>800</td>
<td>67</td>
<td>57.89</td>
</tr>
<tr>
<td>400</td>
<td>65.40</td>
<td>50.87</td>
</tr>
<tr>
<td>200</td>
<td>63.96</td>
<td>45.61</td>
</tr>
<tr>
<td>100</td>
<td>49.54</td>
<td>36.25</td>
</tr>
<tr>
<td>50</td>
<td>49.54</td>
<td>31.57</td>
</tr>
</tbody>
</table>

Table I

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

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>
<thead>
<tr>
<th>Concentrations (μg/mL)</th>
<th>% inhibition of RBCs membrane stabilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>6400</td>
<td>50.06</td>
</tr>
<tr>
<td>3200</td>
<td>48.95</td>
</tr>
<tr>
<td>1600</td>
<td>47.86</td>
</tr>
<tr>
<td>800</td>
<td>47.40</td>
</tr>
<tr>
<td>400</td>
<td>46.71</td>
</tr>
<tr>
<td>200</td>
<td>46.71</td>
</tr>
<tr>
<td>100</td>
<td>43.66</td>
</tr>
<tr>
<td>50</td>
<td>40.93</td>
</tr>
</tbody>
</table>

Table II

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

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, 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 pseudoephedrine 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.
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 joints 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* 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

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

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

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

**Table IV**  
Effect of different doses of pseudoephedrine against formaldehyde induced arthritic model

<table>
<thead>
<tr>
<th>Time</th>
<th>Control</th>
<th>Arthritic control</th>
<th>Pseudoephedrine 10 mg/kg bw</th>
<th>Pseudoephedrine 20 mg/kg bw</th>
<th>Pseudoephedrine 40 mg/kg bw</th>
<th>Naproxen 10 mg/kg bw</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Day</td>
<td>0.71 ± 0.028</td>
<td>0.70 ± 0.041</td>
<td>0.77 ± 0.025</td>
<td>0.71 ± 0.037</td>
<td>0.69 ± 0.039</td>
<td>0.72 ± 0.040</td>
</tr>
<tr>
<td>2nd Day</td>
<td>0.70 ± 0.027</td>
<td>1.35 ± 0.034</td>
<td>1.26 ± 0.004* (6.66%)</td>
<td>1.23 ± 0.029* (8.88%)</td>
<td>1.14 ± 0.026a (15.55%)</td>
<td>1.32 ± 0.040b (2.22%)</td>
</tr>
<tr>
<td>4th Day</td>
<td>0.70 ± 0.029</td>
<td>1.56 ± 0.034</td>
<td>1.42 ± 0.026* (8.97%)</td>
<td>1.41 ± 0.032b (9.61%)</td>
<td>1.19 ± 0.025a (23.71%)</td>
<td>1.41 ± 0.037c (9.61%)</td>
</tr>
<tr>
<td>6th Day</td>
<td>0.69 ± 0.030</td>
<td>1.54 ± 0.036</td>
<td>1.34 ± 0.033* (12.98%)</td>
<td>1.20 ± 0.024a (22.07%)</td>
<td>0.99 ± 0.023a (35.71%)</td>
<td>1.32 ± 0.035d (14.28%)</td>
</tr>
<tr>
<td>8th Day</td>
<td>0.72 ± 0.028</td>
<td>1.52 ± 0.032</td>
<td>1.24 ± 0.024* (18.42%)</td>
<td>1.07 ± 0.026a (29.60%)</td>
<td>0.81 ± 0.025a (46.71%)</td>
<td>1.20 ± 0.033b (21.05%)</td>
</tr>
<tr>
<td>10th Day</td>
<td>0.70 ± 0.026</td>
<td>1.50 ± 0.031</td>
<td>1.10 ± 0.016a (26.6%)</td>
<td>0.96 ± 0.022a (36.1%)</td>
<td>0.71 ± 0.031a (52.66%)</td>
<td>1.10 ± 0.035b (26.66%)</td>
</tr>
</tbody>
</table>

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

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 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 compared 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.
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.

References