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Therapeutic appraisal of ephedrine against rheumatoid arthritis: A new indication

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Abstract: *Ephedra,* natural flora has been used traditionally to treat rheumatism since decades. The scientific evidence of anti-rheumatic effect of this plant has also been reported. But the anti-rheumatic activity of major constituent of this plant (ephedrine) has not been evaluated. Based on this, the current study was aimed to assess anti-arthritic activity of ephedrine by using *in vitro* and *in vivo* approaches. Correspondingly, enzyme linked immunosorbent assay was performed for the estimation of prostaglandins E2 (PGE2) and tumor necrosis factor-α (TNF-α) in serum of formaldehyde-induced arthritic animals. The results elaborated significant reduction in albumin denaturation and remarkable progress on stabilization of red blood cells outer membrane at higher concentration during *in vitro* experiments. The ephedrine (40mg/kg) revealed noteworthy ($p<0.001$) inhibition in paw swelling in animals intoxicated with albumin as well as formaldehyde as compared to animals of control group by *in vivo* results. In this assay, ephedrine (20 & 40 mg/kg orally) significantly suppressed the level of these inflammatory markers (PGE2 & TNF-α). Ephedrine exhibited anti-arthritic effect by decreasing pro-inflammatory cytokines (PGE2 & TNF-α). This experimental work pharmacologically supports the use of ephedrine as anti-rheumatic drug but limited to evaluate in immunological arthritic model.

Keywords: Ephedrine, rheumatoid arthritis, bovine serum albumin, PGE2, NSAIDs.

INTRODUCTION

Rheumatoid arthritis (RA), a multifactorial autoimmune disease, characterized with pain, redness, stiffness, and swelling of joints. Macrophage activation is a key point in disease progression that leads to raised levels of chemicals particularly tumor necrosis factor- alpha (TNF-α) and Prostaglandins E2 (PGE2) (Qasim, Alamgeer *et al*., 2021).

The underlying causes of this disease in developing countries are underprivileged socio-economic circumstances, frequent use of high doses of corticosteroids and recurrent incidence of severe infections (Naqvi *et al*., 2019).

Existing treatment strategies for RA include use of symptomatic pain relievers and disease modifying agents. These serious health issues posed by currently utilized therapeutic agents to treat RA put an emphasis to search for alternative therapy of inflammation with better efficacy (Drosos, Pelechas *et al*., 2020).

Drugs identified from herbal medication are being used to cure many diseases from a long time. A survey of WHO depicts that about 80% of the total population of world uses the herbal drugs for their basic health and care demand. Herbal medications prove to be a safety wall against the arising diseases of modern era (Choudhary *et al*., 2015).

Ephedra commonly known as "Asmani botti" is most widely distributed in northern areas of Pakistan. This plant has been traditionally used for ailment of rheumatoid arthritis for many years ago and approved scientifically for treatment of rheumatic disorder (Uttra, 2017). By considering recently reported anti-arthritic effects of ephedra, current study was aimed to use active constituents of ephedra (ephedrine) rather than whole herbal extracts.

MATERIALS AND METHODS

Anti-arthritic effect by in vitro study

Albumin denaturation protection method

Ephedrine was investigated for anti-arthritic potential by egg albumin destruction experiment through *in vitro* study. The preparation of reaction mixture (5mL) was carried out by using 2.8mL (phosphate buffered pH 6.4) and 0.2mL (egg albumin). After that ephedrine in

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different concentrations such as 50, 100, 200, 400,800, 1600, 3200 and 6400µg/mL was added in quantity of 2mL. Correspondingly, distilled water was used as control solution. Then prepared solutions were incubated at 37±2 °C for 15 min and then placed in oven (70°C & 5 min. After cooling the reaction solution, absorbance was notes at wavelength of 660 nm by UV spectrophotometer. The given formula was applied for calculation of percentage inhibition (Akhtar *et al*., 2021):

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

Where: V_t is absorbance of test sample, V_c is absorbance of control.

Bovine serum albumin denaturation inhibition method

This experiment was also done for *in vitro* study. The composition of reaction solution in this experiment included 0.45mL (bovine serum albumin) and 0.05ml ephedrine/naproxen (50-6400µg/mL). Similarly the control mixture was distilled water, whereas product control has no bovine serum albumin. Spectrophotometer (660 nm) was used for calculating the absorbance. Then, percentage protection on protein destruction was determined by formula (Elisha *et al*., 2016, Hasan *et al*., 2015):

$$\text{Inhibition}\,(\%) = \frac{100 - (\text{Abs of Ts} - \text{Abs of Pc})}{\text{Abs of Ts}} \times 100$$

Whereas Ts represents test solution, Pc shows product control

Membrane stabilization method

Human red blood cells (RBCs) were used for this experiment. The blood sample and sterilized Alsever**'s** solution was added in same quantity. Reaction mixture was contained phosphate buffer (1mL), hypotonic solution (2mL) and ephedrine/naproxen (0.5mL) in different concentrations (50-6400µg/mL). After that 0.5mL from prepared suspension was inserted. The whole mixture was incubated at 37ºC for 30min. Then it was centrifuged at 3000 rpm. After centrifugation, the upper liquid was removed and absorbance was measured. For that purpose, UV spectrophotometer (560nm) was used. The percentage protection was noted in opposition to hypo tonicity prompted destruction by given formula. (Hasan *et al*., 2015):

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

In vivo anti-arthritic effect

Sprague-Dawley rats of both sexes were placed in cages at animal house of University of Sargodha, Pakistan according to national institute of health (NIH) guidelines (Care and Animals, 1986). All procedures were adapted after the approval by committee named "biosafety and ethical committee, UOS" (SU/ORIC/19).

Egg albumin induced inflammation

The procedure for egg albumin induced inflammation was performed by protocol given by Ren (Ren *et al*., 2017). There were 5 animals in each group. First group (Control) was treated with distilled water. Second, third and fourth groups animals were considered as test animals and were treated with ephedrine (test drug) 10, 20 and 40 mg/kg orally. Whereas, fifth group (standard) was given naproxen in oral dose of 10mg/kg. Then inflammation was developed by injecting 0.1mL of egg albumin into right hind paw off all group's animals post 1hr of treatment. On this calculated paw volume, percentage inhibition was measured by given equation (Ren *et al*., 2017):

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

Arthritis induced by formaldehyde

Formaldehyde was used for provoking arthritis in hind paw of experimental animals. The treatment of group I (arthritic control) animals with normal saline, group II (standard animals with naproxen 10mg/kg and group III, IV and IV with ephedrine (10, 20 and 40mg/k) respectively for 10 days. After 30min on same day, acute arthritis was provoked by injection of formaldehyde (2%) to paw surface of all groups. The same protocols were done on 3rd day. Anti-arthritic activity was evaluated by noting the paw volume with of Plethysmometer. This anti-arthritic potential was recorded as of percentage protection by given formula as mentioned above (Uttra, 2017)

Prostaglandin E2 and tumor necrosis factor-alpha measurement

Level of prostaglandin E2 and TNF-alpha in serum of arthritic animals were estimated by protocols given in kit provided by kit manufacturers (Abcam). Optical density (OD) was noted at 450 nm (Uttra *et al*., 2018).

STATISTICAL ANALYSIS

All values related to anti-arthritic effect were expressed as mean ± SEM. Two-Way ANOVA proceeded by Bonferroni test and One-Way ANOVA followed by Dunnet's test were performed by using Graph Pad Prism (version 8.00).Value ($p<0.005$) was considered as significant.

RESULTS

Inhibitory effect of ephedrine on heat induced egg albumin protein denaturation

Various concentrations of ephedrine showed dose dependent inhibition on protein denaturation. According to results shown in table 1, ephedrine produced 75.45% protection against albumin destruction at 6400µg/mL whereas naproxen showed 72.97% protection against denaturation of egg albumin at same concentration. On

Table 1: Effect of different concentrations of ephedrine on inhibition of albumin denaturation

Concentrations µg/mL	% inhibition	
	Ephedrine	Naproxen
6400	75.45	72.97
3200	60	62.16
1600	57.65	57.27
800	47.74	45.94
400	45.94	44.14
200	44.14	42.34
100	40.54	38.37
50	33.33	31.53

Table 2: Effect of different concentrations of ephedrine on inhibition of bovine serum albumin denaturation

Concentrations (µg/mL)	% inhibition	
	Ephedrine	Naproxen
6400	69	47.36
3200	62.57	39.76
1600	57.89	32.74
800	42.1	28.65
400	38.01	18.71
200	33.33	7.6
100	28.65	7.01
50	25.73	5.26

Table 3: Effect of different concentrations of ephedrine and naproxen on human red blood cells membrane stabilization

Concentrations	% inhibition	
(µg/mL)	Ephedrine	Naproxen
6400	67.6	52.57
3200	63.09	52.24
1600	53.47	51.24
800	52.83	50.97
400	51.8	48.41
200	35.07	46.77
100	21.67	42.13
50	15.8	32.69

Results were expressed in the form of % inhibition.

the other side the lowest concentration of ephedrine (50µg/mL) has 33.33% inhibition on protein destruction. Likewise this inhibitory effect was also decreased for minimum concentration of naproxen (standard drug).

Consequences of ephedrine on bovine serum albumin protein denaturation

Similar to inhibitory effect on egg albumin destruction, these results also claimed dose related protection on destruction. These findings indicated the maximum inhibition (69%) of ephedrine at 6400µg/mL. likewise the standard drug inhibition was 47.36% at same concentration as mentioned in table 2.

Effect of ephedrine on membrane stabilization of human RBCs

The ephedrine protected the membrane of RBCs after exposure to hypotonicity. This stabilizing effect of ephedrine was recorded as percentage protection in table 3. The maximum effect (67.60%) was showed by highest concentration (6400µg/mL) of test drug. This stabilizing effect of ephedrine against hemolysis by hypotonicity supported its anti-arthritic claim.

Effect of ephedrine against egg albumin induced inflammation

The anti- inflammatory effect of ephedrine at various doses has evaluated by *in vivo* study through the induction

Table 4: Effect of different doses of ephedrine at 10, 20 and 40 mg/kg body weight against egg albumin induced paw edema.

Paw volume (mL) against egg albumin induced inflammation					
Time (Hr)	Control	Naproxen (10 mg/kg)	Ephedrine (10 mg/kg)	Ephedrine (20 mg/kg)	Ephedrine (40 mg/kg)
0	0.774±0.008	0.788± 0.014	0.803± 0.024	0.818± 0.033	0.634± 0.014
1	1.32± 0.006	1.278± 0.014[d]	1.2800±0.016[d]	1.214± 0.025[a]	0.684± 0.014[a]
2	1.244±0.004	1.182± 0.010[b]	1.176± 0.009[b]	1.124± 0.007[a]	0.522± 0.015[a]
3	1.144±0.005	1.078± 0.004[b]	1.078± 0.004[b]	1.030± 0.009[a]	0.458± 0.007[a]
4	1.146±0.005	1.018± 0.012[a]	1.006± 0.009[a]	0.952± 0.010[a]	0.408± 0.004[a]

Results were described in form of Mean ±SEM where [a] $p<0.001$ and [b] $p<0.01$ and [d]=$p>0.05$. Results were analyzed by Two-way ANOVA proceeded by Bonferroni test.

Table 5: Effect of ephedrine (at 10, 20 and 40 mg/kg dose) and naproxen on paw volume in formaldehyde induced arthritic rats.

Paw volume (mL)					
Time (Days)	Arthritic control	Naproxen (10 mg/kg)	Ephedrine (10 mg/kg)	Ephedrine (20 mg/kg)	Ephedrine (40 mg/kg)
0	0.632± 0.041	0.672± 0.040	0.712± 0.028	0.648± 0.035	0.646± 0.040
2nd	1.296± 0.034	1.272± 0.040[d]	1.258± 0.029[d]	1.146± 0.030[b]	1.066± 0.025[a]
4th	1.498± 0.034	1.364± 0.037[c]	1.366± 0.026[c]	1.354± 0.033[b]	1.108± 0.027[a]
6th	1.484± 0.036	1.278± 0.035[a]	1.314± 0.026[b]	1.112± 0.027[a]	0.908± 0.027[a]
8th	1.45± 0.032	1.14± 0.033[a]	1.178± 0.029[a]	0.93± 0.025[a]	0.71± 0.027[a]
10th	1.44± 0.031	1.036± 0.035[a]	1.122± 0.029[a]	0.87± 0.025[a]	0.604± 0.040[a]

Results were described in form of Mean and SEM where [A]=$p<0.001$, [B]=$p<0.01$, [C]=$P<0.05$and [D]=$p>0.05$. Analyses was done by Two-Way ANOVA proceeded by Bonferroni's test.

of inflammation by egg albumin. Ephedrine showed dose dependent inhibition of inflammation. Low dose (10mg/kg) of ephedrine exhibited non significant inhibition whereas higher dose (40mg/kg) remarkably ($P<0.001$) inhibited the edema induced by albumin. Therefore, ephedrine at 40mg/kg has percentage inhibition of 64.39% post 4h induction of inflammation. As given in table 4 the anti-inflammatory effect against albumin induced inflammatory model increases at late phase of inflammation.

Ephedrine impact on paw volume in formaldehyde induced arthritic animals

Effect of ephedrine against non-immunological arthritis induced formaldehyde was determined. The findings of this study claimed dose dependent reduction in increase in paw volume. At 20 and 40mg/kg remarkable ($P<0.001$) reduction in paw swelling was noted at day 6, 8 and 10 as depicted in table 5. On day 8, the reduction in paw volume by minimum dose of ephedrine (20mg/kg) was 36% and with 40mg/kg of ephedrine was 51. It has been concluded that the highest dose of ephedrine showed 58% inhibition on rise in paw volume at day 10.

Effect of ephedrine on PGE2 and TNF-α in serum of experimental animals

There was notable expression of PGE2 and TNF-α in arthritic animal at the end of study as depicted in fig. 1 (a & b). After administration of ephedrine (20 and 40mg/kg orally), the level of these markers were significantly ($p<0.001$) reduced. Where as low dose (10mg/kg P.O) of ephedrine has less significant ($p<0.005$) effect on expression of PGE2 and non-significant ($p>0.005$) effect on TNF-α in comparison to control group.

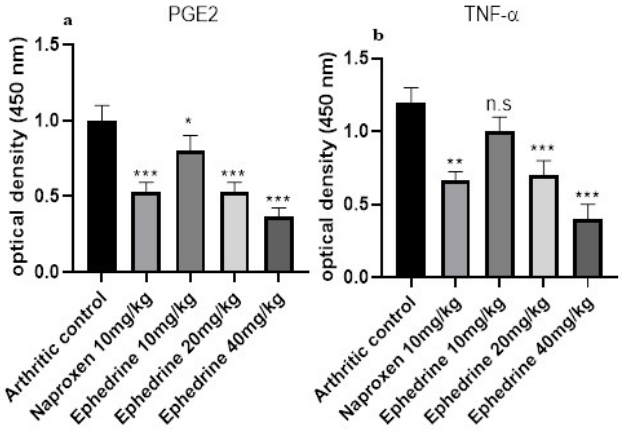

Fig. 1 (a-b): Effect of ephedrine at three doses (10, 20 & 40 mg/kg orally) on PGE2 (a) and TNF-α (b) in formaldehyde-induced arthritis. One-Way ANOVA followed by Dunnet's test was done for statistical evaluation whereas * represents $p<0.001$, ** indicate $p<0.01$ and * is $p<0.05$ and [n.s] is $p>0.05$

DISCUSSION

The present study has been carried out to explore the anti-arthritic activity of ephedrine by using *in vitro* techniques and *in vivo* models. Different factors cause protein denaturation by modification in bonding include temperature variations stress and alteration in pH of the medium (Prasad, Yashwant *et al*., 2013) (Saleem, Saleem *et al*., 2020). The present study has confirmed the dose dependant inhibition of ephedrine on albumin (egg & bovine serum) protein as shown in tables 1 and 2.

In human red blood cell membrane (HRBCs) stabilizing assay, substance which stabilize RBCS membrane may also have protective effect on lysosomal membrane. However, this study proposed that stable RBCs membrane inhibit the release of lysosomal enzyme during inflammation (Manan, Saleem *et al*., 2020). Additionally, hemolysis by hypotonic solution result in leakage of protein rich fluid (Kumar, Bhat *et al*., 2012). According to another investigation, phospholipase A2 is released from ruptured membrane that involve in release of various markers such as PGE2 (Umukoro and Ashorobi, 2006). Our findings indicated stabilizing effect of ephedrine on human (RBCs) membrane as mentioned in table 3. Therefore, it can be supposed that this effect might be due to its anti-oxidant activity or due to inhibitory effect on PGE2.

As previous research work, it has been informed that histamine is released after injecting albumin. Albumin activates the mast cells to release histamine that cause edema. Histamine cause relaxation of endothelial smooth muscle cells and participate in vascular cascade of acute inflammation (Qasim, Alamgeer *et al*., 2021). Ephedrine showed maximum inhibition in rise of paw swelling (table 4). This significant inhibition test drug on paw swelling might be due to its preventive effect on the release of histamine.

One of the best suitable techniques to assess anti-proliferative activity is to reduce or prevent the edema caused by formaldehyde in animal paw joint. This method showed identical mechanism of RA in human (Thite, Patil *et al*., 2014). Formaldehyde provoked arthritis by destruction of tissue at injection site (Ben, Etim *et al*., 2016). Formaldehyde provoked the release of substance P in early phase which is majorly responsible for pain in joints. Bradykinin, prostaglandins, serotonin and histamine are discharged in second phase. These are the key mediators in developing swelling and inflammation as they cause induction and progression of marked vasodilatation and permeability. These both phases are inhibited uniformly by the drugs which act through CNS whereas those drugs which have peripheral effects only can inhibit the second or late phase only (Owoyele, Adenekan *et al*., 2011). It has been demonstrated in the results of this study that the proliferative edematous reaction was subdued by the ephedrine and pseudoephedrine at a dose of 20 & 40mg/kg to a greater extent than 10mg/kg naproxen. Furthermore, ephedrine expressed much better effects at day 8 and at day-10 as presented in table 5.

PGE2 and TNF-α are another potential targets for dug development of RA. These markers responsible for vasodilation during vascular events of inflammation (Uttra, Shahzad *et al*., 2018) and ultimately lead to bone and cartilage erosion mechanism of RA (Shabbir, Shahzad *et al*., 2014). In current experiment, ephedrine at 20 and 40mg/kg orally caused significant ($p<0.001$) suppression of PGE2 and TNF-α as represented by fig. 1 (a-b). This findings support the mechanism of action of ephedrine for treatment of arthritis induced by formaldehyde.

Both prevention of protein denaturation and stabilization of RBC's membrane as well as decrease in paw edema and pro-inflammatory cytokines (PGE2 and TNF-α) represented that ephedrine confirmed its traditional use in rheumatism.

CONCLUSION

This study concludes the anti-arthritic effect of ephedrine that may be due to its inhibitory effect on inflammatory markers like histamine, albumin and prostaglandins. However, further studies are mandatory to conduct its immune system effect in order to explore their precise modes of action.

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