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EVALUATION OF *IN VIVO* ANTI-INFLAMMATORY ACTIVITY OF PERGULARIA DAEMIA

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ABSTRACT

Pergularia Daemia (PD) is a medicinal herb and used as folk medicine by the villagers. In our earlier study, the anti-inflammatory activity of PD extract was evaluated by in vitro method through membrane stabilization test and protein denaturation test and the results were encouragingly positive. Hence, in the present study, the anti inflammatory activity of the ethanol extract of PD was evaluated by in vivo methods viz., formalin induced inflammation and acetic acid induced writhing test in albino rats. The results showed that the formalin induced paw licking movements were decreased (non-significant) in the early phase (0-10 mins) but significantly decreased in the late phase (10-20 mins). The number of acetic acid induced abdominal constrictions significantly reduced after PD extract administration at the doses of 200 mg.kg and 300 mg/kg. The effect of the ethanol extract of PD was comparable with standard non-steroidal anti inflammatory drug viz. diclofenac sodium. Thus our results encourage to recommend and justify the use of the PD extract for the treatment of painful and

inflammatory conditions.

KEYWORDS: Anti-inflammatory, abdominal writhing, paw licking, In-vivo, Pergularia daemia.

INTRODUCTION

The thirst for the novel methods of treating the diseases has increased globally mainly because of the cost-effect and the secondary complications developed due to the modern medications. Many of them are turning towards the herbal medicine because of its safety, lesser cost and the hope of total cure of the disease. One such emerging herb is Pergularia Daemia (PD) which is already in use as folk medicine for so many ailments.[1, 2] But, the documentation of the same is lacking. Because of its curative popularity among the folks, but not among researchers, we were interested to go through various aspects of this plant. In our earlier study, we subjected the ethanol extract of the aerial part of the plant for phytochemical analysis and quantified the organic compounds of the extract by Gas Chromatogram Mass Spectrometric method (GCMS). It confirmed the presence of flavonoids, tannins, alkaloids, glycosides, terpenoids, steroids and carbohydrates along with five bioactive constituents viz. 2-hydroxy- methyl ester 2-Methoxy-4-vinylphenol, Phthalic acid di-(1-hexen-5-yl) ester, Ascorbic acid 2,6-dihexadecanoate and Methyl (Z)-5,11,14,17-eicosatetraenoate.[2]

As these bioactive compounds are concerned with many pharmacological and therapeutic properties, it encouraged us to go in to further details of the plant. Thus, we ventured into the evaluation of anti-inflammatory effect of the plant by *in-vitro* method and found that the PD extract was capable of rendering membrane stabilization of human red blood cells by inhibiting the hypotonically induced hemolysis and were capable of protecting the cells from protein denaturation. Both the anti-inflammatory effects were dose-dependent and the efficacy was comparable to that of diclofenac sodium, a standard non-steroidal anti-inflammatory drug (NSAID). We have already reported these in the earlier volume of this journal. [3] As these results were encouraging, we attempted to evaluate the anti-inflammatory effect of the extract by *in-vivo* method in the present study.

MATERIALS AND METHODS

The study was conducted on male albino rats of Wistar strain with the weight ranging from 180 to 200 gm. The Institutional Ethical Committee approved the study (03/007/2014). The animals were carefully maintained at a temperature of 25º C ± 1º C with a 12:12 h light/dark

cycle by housing them in polypropylene cages. They received the standard commercial food pellets and water *ad libitum*. The experimental proceedings were carried out between 9 AM and 2 PM. The ethanol extract of PD was dissolved in the vehicle viz. 5% tween 80 and it was administered to the animal orally. The efficacy of the anti-inflammatory effect of PD extract was evaluated by comparing it with the action of the non-steroidal anti-inflammatory drug (NSAID) named Diclofenac sodium.

Grouping of the animals

30 rats were divided into 5 groups, each consisting of 6 animals; Group1- control group treated with saline, Group 2 – test group treated with PD extract at 100 mg/kg body weight (bwt), Group 3 – test group treated with PD extract at 200 mg/kg bwt, Group 4 – test group treated with PD extract at 300 mg/kg bwt, Group 5 – standard group treated with diclofenac sodium at 100 mg/kg bwt.

Procedure for determining the anti-inflammatory activity by *in-vivo* study

The formalin induced paw licking test

This test was done according to the method of Hunskaar S and Hole K (1987).[4] The extract (100, 200, 300 mg/kg bwt) was administered 60 min before and diclofenac sodium (100mg/kg bwt) was administered 30 min before the experiment. Following this, 9 µl of 2% formalin was injected in the sub plantar region of the right hind paw to induce pain. Injection of formalin produced a time-based biphasic response i.e., an early phase within 10 min and late phase within 20 to 30 min of formalin injection. The response was in the form of paw-licking movements.

Acetic acid-induced writhing test

This test was done according to the method of Whittle BA (1964).[5] The grouping of the animals, dosage of the extract and administration of the extract and the drug were similar to that of formalin induced paw licking test. Then, each animal was given intraperitoneal injection of 1% aqueous solution of acetic acid at the dose of 10 ml/kg bwt. Immediately after the injection, each animal was observed for the appearance of writhing movements. One writhing movement was considered as a contraction of the abdominal muscles together with a stretching of the hind limbs. Anti-inflammatory activity was considered as the reduction in the number of abdominal constrictions in the test groups compared to that of control group. It was calculated by using the formula

$$\text{Percent inhibition} = \left(\frac{\begin{array}{l}\text{Number of writhing movements in control animals} - \\ \text{Number of writhing movements in treated animals}\end{array}}{\text{Number of writhing movements in controls}} \right) \text{X } 100$$

Histological examination

Thin paraffin sections of right hind paw were taken using a microtome and stained with haematoxylin and eosin. Staining technique with haematoxylin and eosin with formalin fixed specimen was done by the method of C.F.A Cullings, (1974).[6]

Statistical analysis

The data were analyzed in SPSS 17th version. The statistical significance between the groups was determined by one-way analysis of variance (ANOVA) and the significance within the groups was obtained by Tukeys multi comparison test.

RESULTS

In the Tables

Number in the parenthesis indicate the number of animals in each group

Values are the number of paw licking movements expressed as mean (Table 1) and mean difference (Table 2 & 3) ± SEM.

Drug is diclofenac sodium

Dosage of PD extract and the drug is in mg/kg bwt.

Phase I: Paw licking movements observed in 0 - 10 minutes after formalin injection

Phase II: Paw licking movements observed in 20 minutes after formalin injection

Significance level is fixed at $p < 0.05$

The formalin-induced paw licking test

In the early phase (0-10 min) of formalin injection, PD extract-treated animals showed lesser number of paw licking movements in dose dependent manner (100 mg - 13.50 ± 0.17, 200 mg - 12.00 ± 0.24, 300 mg - 12.83 ± 0.20) when compared to that of controls (14.17 ± 0.22). But, the reduction was not statistically significant. However, in the late phase of formalin injection (20-30 min), the PD extract-treated animals showed statistically significant reduction in the number of paw licking movements in dose dependent manner (100 mg - 11.33 ± 0.18, 200 mg - 9.17 ± 0.18, 300 mg - 9.33 ± 0.18) compared to that of control (15.17 ± 0.22) (Table 1, 2, 3).

Histological evidence for anti-inflammatory effect of PD extract

Histological sections from control animals showed smooth tissues without inflammation, destruction, and swelling in the paws (Fig 1). Animals injected with formalin in right hind paw showed enlarged cavities in the paw tissue indicating intense edema (shown in red arrow) and infiltration of inflammatory polymorphs, mainly neutrophils (shown in blue arrow) (Fig 2). The oral administration of PD treated at 100, 200 and 300 mg/kg (Fig 4, 5, and 6) showed a decrease in edema as well as infiltration of inflammatory cells .These findings were almost similar to that of diclofenac sodium treated group (Fig 3).

Table 2. Effect of PD extract between Phase I and II on paw licking movements after formalin injection.

Group (6)	Duration of paw licking		Paired difference (mean ± SEM)	Significance p <
	Phase 1	Phase 2		
Control	15.83 ± 2.07	15.17 ± 0.70	0.67 ± 2.42	0.794
PD 100	13.50 ± 0.43	11.33 ± 0.49	2.17 ± 0.83	0.048*
PD 200	12.00 ± 0.86	9.17 ± 0.48	2.83 ± 0.54	0.003*
PD 300	12.83 ± 0.60	9.33 ± 0.49	3.50 ± 0.87	0.019*
Drug 100	8.83 ± 0.60	8.67 ± 0.42	0.17 ± 0.83	0.849

Table 2.Effect of PD extract on paw licking movements after formalin injection - Significance between groups in Phase I.

Formalin Test	Control	PD extract 100	PD extract 200	PD extract 300	Drug 100
Control	------	0.67 ± 0.92	2.17 ± 0.92	1.33 ± 0.92	5.33 ± 0.92
		p < 0.949	p < 0.164	p < 0.607	p < 0.000*
PD extract 100	−0.67 ± 0.92	-------	1.50 ± 0.92	0.67 ± 0.92	4.67 ± 0.92
	p < 0.949		p < 0.497	p < 0.949	p < 0.000*
PD extract 200	−2.17 ± 0.92	−1.50 ± 0.92	--------	0.83 ± 0.92	3.17 ± 0.92
	p < 0.167	p < 0.497		p < 0.893	p < 0.016*
PD extract 300	−1.33 ± 0.92	−0.67 ± 0.92	0.83 ± 0.92	--------	4.00 ± 0.92
	p < 0.924	p < 0.949	p < 0.893		p < 0.002*
Drug 100	−5.33 ± 0.92	−4.67 ± 0.92	−3.17 ± 0.92	−4.00 ± 0.92	-------
	p < 0.016*	p < 0.000*	p < 0.016*	p < 0.002*	

Table 3. Effect of PD extract on paw licking movements after formalin injection - Significance between groups in Phase II

Formalin Test	Control	PD extract 100	PD extract 200	PD extract 300	Drug 100
Control	------	3.83 ± 0.75	6.00 ± 0.75	5.83 ± 0.75	6.50 ± 0.75
		p < 0.000*	p < 0.000*	p < 0.000*	p < 0.000*
PD extract 100	−3.83 ± 0.75	-------	2.17 ± 0.75	2.00 ± 0.75	2.67 ± 0.75
	p < 0.000*		p < 0.053	p < 0.085	p < 0.012*
PD extract 200	−6.00 ± 0.75	−2.17 ± 0.75	--------	−0.17 ± 0.75	−0.50 ± 0.75
	p < 0.000*	p < 0.053		p < 0.999	p < 0.961
PD extract 300	−5.83 ± 0.75	−2.00 ± 0.75	0.17 ± 0.75	--------	0.67 ± 0.75
	p < 0.000*	p < 0.850	p < 0.999		p < 0.896
Drug 100	−6.50 ± 0.75	−2.67 ± 0.75	−0.50 ± 0.75	−0.67 ± 0.75	-------
	p < 0.000*	p < 0.012*	p < 0.961	p < 0.896	

Fig 1

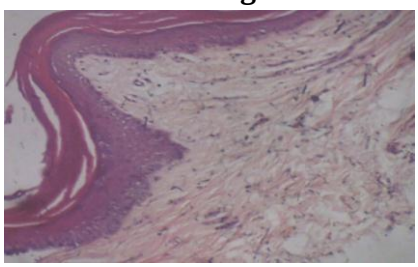

Fig 2

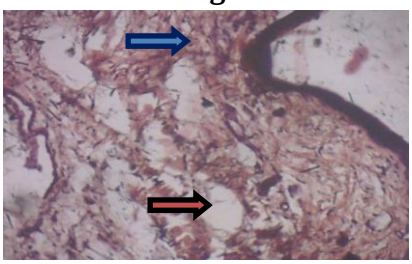

Fig 3

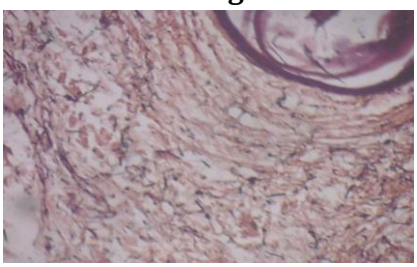

Fig 4

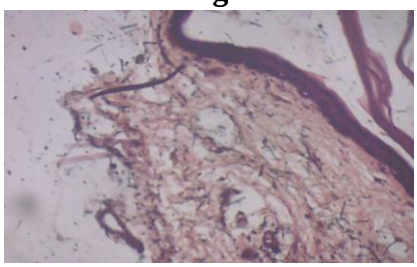

Fig 5

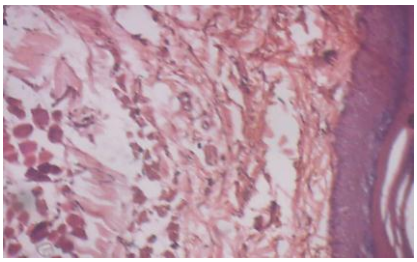

Fig 6

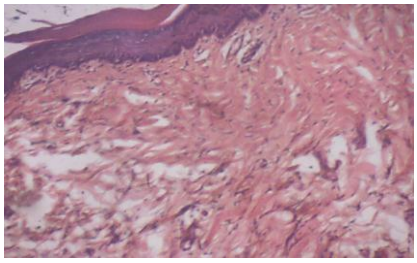

Fig 1 to 6: Sections of paws from rats treated with PD extract and the drug in the model of formalin induced edema.

Fig 1: Normal appearance of the right hind limb paw in control animal

Fig 2: Affected appearance of the right hind limb paw in control animal after formalin injection

Fig 3: Appearance of the right hind limb paw after diclofenac sodium (100 mg/kg bwt) with formalin injection

Fig 4: Appearance of the right hind limb paw after PD (100 mg/kg bwr) with formalin injection.

Fig 5: Appearance of the right hind limb paw after PD (200 mg/kg bwt) with formalin injection.

Fig 6: Appearance of the right hind limb paw after PD (300 mg/kg bwt) with formalin injection.

Red arrow – Enlarged cavities with intense edema.

Blue arrow - Infiltration of inflammatory polymorphs.

Acetic acid induced abdominal writhing test

The PD extract significantly reduced the number of abdominal writhing movements induced by acetic acid injection and the effect was dose-dependent: in control animals, the numbers of writhing movements were maximum of 14.33 ± 1.17. After treating with PD extract, the numbers of writhing movements were reduced depending upon the dose: with 100 mg/kg bwt, the numbers of writhing movements were reduced to 0.17 ± 0.83, with 200 mg, to 9.17 ± 0.79 and with 300 mg, to 8.17 ± 0.68. Accordingly, the percentage inhibition was also increased as per the dose of PD extract - 29.07%, 36.05% and 43.02%. These effects were comparable to the effect of diclofenac sodium at 100mg/kg bwt, 7.00 ± 0.73 with 51.16% inhibition (Table 4 and 5)

Table 4. Effect of PD extract on number of abdominal writhing movements induced by acetic acid and the percentage inhibition of the writhing movements

Group	Mean ± SEM of writhing	% inhibition
Control	14.33 ± 0 .74	------
PD 100	10.17 ± 0.83	29.07
PD 200	9.17 ± 0.79	36.05
PD 300	8.17 ± 0.68	43.02
Drug 100	7.00 ± 0.73	51.16

Table 5. Effect of PD extract on number of abdominal writhing movements induced by acetic acid and the percentage inhibition of the pain - Significance between the groups

Acetic acid test (6)	Control	PD extract 100	PD extract 200	PD extract 300	Drug 100
Control	------	4.17 ± 1.44	5.17 ± 1.44	5.67 ± 1.44	7.33 ± 1.44
		p < 0.056	p < 0.012*	p < 0.005*	p < 0.000*
PD 100	−4.17 ± 1.44	-------	1.00 ± 1.44	1.50 ± 1.44	3.17 ± 1.44
	p < 0.056		p < 0.956	p < 0.835	p < 0.215
PD 200	−5.17 ± 1.44	−1.00 ± 1.44	--------	0.50 ± 1.44	2.17 ± 1.44
	p < 0.012*	p < 0.956		p < 0.997	p < 0.572
PD 300	−5.67 ± 1.44	−1.50 ± 1.44	0.50 ± 1.44	--------	1.67 ± 1.44
	p < 0.005*	p < 0.835	p < 0.997		p < 0.776
Drug 100	−7.33 ± 1.44	−3.17 ± 1.44	−2.17 ± 1.44	−1.67 ± 1.44	-------
	p < 0.000*	p < 0.215	p < 0.572	p < 0.776	

DISCUSSION

The results of the present study proved the anti-inflammatory activity of PD extract in vivo study. Formalin test and acetic acid tests are in practice for assessing analgesic and anti-inflammatory activities of a new drug. Especially the acetic acid test is used in analgesic drug development for visceral pain.[7] So we adopted them for our study also. The results revealed that PD extract was effective in reducing the formalin and acetic acid-induced pain. In formalin test, the effect in the early phase was lesser compared to the late phase in all the three doses (Table 1). Actually, the pain in the early phase was considered to be neurogenic pain and the pain in the late phase is supposed to be inflammatory pain. Accordingly, the drugs that inhibit pain in early phase are said to alleviate neurogenic pain and the drugs that reduce pain in the late phase are supposed to inhibit the inflammatory pain.[8] Our results showed that PD extract reduced the pain in the early phase (though to a lesser extent) and also in the late phase. So, it may be possible that this extract is capable of alleviating neurogenic pain as well as inflammatory pain. This is an additional fact for recommending PD extract as an anti-inflammatory agent. This was already proved by in vitro method in our earlier study.[3]

Similar studies in literature survey a confirmation to say the effectiveness of montelukast at a dose of (0.5-2 mg/kg) significantly decreased the formalin-induced increase in paw volume as compared to control rats in a dose-dependent manner, with histological studies showing an acute inflammation in the dermis and epidermis with extensive extravasations, mainly

polymorphonuclear (PMN) leucocytes and injection of montelukast (1 mg/kg) and indomethacin (5 mg/kg), reduced the tissue injuries induced by formalin in the paw skin.[9] So with a similar finding in our histological analysis, it may be revealed that PD has peripheral anti-inflammatory effect.

The probe into the probable mechanism of anti-inflammatory action of PD extract will be intersting. In fact, the acetic acid produces inflammation by activating phospholipase A2 and releasing several chemical mediators like arachidonic acid, eicosanoids and cytokines from cell membrane. These substances induce the production of prostaglandins and leukotrienes which finally cause inflammation. [10] The anti-inflammatory effect of PD extract may be due to inhibition of phospolipase A2 or even blocking cyclooxygenase (COX-1 and/or COX-2). [11] Cyclooxygenase I and II induce inflammation by increasing the production of prostaglandins from arachidonic acids. [12] If these are inhibited, inflammation can be reduced or cured. NSAIDs like aspirin functions by this mechanism. [12] According to Baskar and Balakrishnan, the anti-inflammatory and analgesic activity of PD extract also may be mediated by this mechanism.[13] Histological studies paw injected with formalin in formalin test were used to evidential prove the anti-inflammatory activity of plants. The presence of bioactive compounds like saponins, alkaloids, steriods and flavanoids present in the extract as reported in our earlier study may be the reason for these actions.[2]

CONCLUSION

PD is an herbal plant used widely as folk medicine by many people. But the authenticity of it is not documented much. We have reported earlier about the bioactive components, antioxidants and anti-inflammatory effect of the aerial parts of ethanol extract of the plant by in vitro method. In the present study, we confirmed the anti-inflammatory effect of the ethanol extract of the plant by in vivo method by comparing the effect with the effect of the standard anti-inflammatory drug viz. diclofenac sodium. It encourages us to recommend this plant extract as one of the herbal drug in quenching the thirst for the novel methods of treating the diseases.

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