Anti-inflammatory activity of whole plant of *Pergularia daemia* linn.

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ABSTRACT

Short lived and self limiting disorders are not normally treated with phytomedicines, but recently the use of some botanical preparations for chronic inflammatory conditions has become increasingly wide spread. Pergularia daemia Linn. belonging to family Asclepiadaeceae. Widely distributed in the old World tropics and sub-tropics from southern and tropical Africa through Arabia to Afghanistan, India and Shri-lanka. It consists of Coroglaucigenin, calactin, oleanolic acid, putranjivadione, calacin, calotropin and β-sitosterol etc. The plant is pungent, cooling, anthelmintic, laxative, antipyretic, cures biliousness, "kapha", asthma, "tridosha" ulcers, useful in eye troubles. The present studied has shown that ethanol extract is good significant ($p<0.001$) reduction in paw oedema to the extent of 44.18% and reduced the granuloma formation to the extent of 19.87% at 200mg/kg concentration, when compared to control group by Carrageenan induced rat paw oedema method and cotton pellet granuloma method in rats respectively.

Keywords: Pergularia daemia, Diclofenac sodium, Cotton pellet granuloma, Sterol, Column chromatography, Phytomedicines.

INTRODUCTION

Ayurveda an ancient system of Indian medicine has recommended in number of drugs from indigenous plant/animal sources for the treatment of several diseases or disorders [1]. Herbal remedies are unpurified plant extracts containing several constituents, which often work together synergistically [2]. The use of medicinal parts is accepted as the most common form of traditional medicine. In developing countries, herbal medicines continue to play important role in primary health care, especially where coverage of health service is limited [3].

Pergularia daemia Linn. (Asclepiadaeceae) A foetid –smelling lactioferous twinner found in the plains throughout the hotters parts of the India, ascending to an altitude of 1,000 m in the Himalayas [4]. Widely distributed in the old World tropics and sub-tropics from southern and tropical Africa through Arabia to Afghanistan, India and Shri-lanka [5]. It consists of Coroglaucigenin, calotropin, corotoxigenin, protouscharin, uscharidin (seeds); 3 β-hydroxyfriedelan, oleanolic acid, β-sitosterol (leaves); α-amyrin. Lupeol and their acetates, calacin, (Root); hentriacontane; β -amyrin, betaine, from the plant; sugar residues of the hydrolysaters of cardiac glycosides occurring in the plant afforded D-glucose, L-oleandrose, D-sermentose [6], Calotropagenin was the comman a glycon of all toxic glycosides [7]. The plant is pungent, cooling; anthelmintic, laxative, antipyretic; cures biliousness, "kapha", asthma, "tridosha". ulcers, useful in eye troubles, urinary discharges, leucoderma, strangury, uterine complaints, inflammations; facilitales parturition(Ayurveda) [8].

MATERIAL & METHODS

Procurement of drug

About 4kg of whole plant of *Pergularia daemia* Linn. were collected from Nandurbar, Dist-Nandurbar, (Maharashtra).

Drying & size reduction

The freshly collected whole plant of *Pergularia daemia* Linn. were shade dried and then powdered to required particle size.

Extraction

The dried material was reduced to course powder in a mechanical grinder and pass through sieve No. 40 to obtain about 1.5kg powder of desired particle size. About 700gms powder material was subjected to successive extraction with petroleum ether ($60\text{-}80^0$), benzene, chloroform, ethyl acetate, n-butanol and ethanol for 50 cycles per batch for 2 batches. The extraction was continued until the solvent in the thimble becomes clear indicating the completion of the extraction. After each extraction, the solvent was distilled off and the extract was concentrated at low temperature [9].

The percentage yield of petroleum ether (60-80^0), benzene, chloroform, ethyl acetate, n-butanol and ethanol extract was shown in Table 1.

Qualitative chemical investigation of extract

The extracts were subjected to preliminary qualitative chemical analysis [10], shown in Table 2.

Identification of active principle by thin layer chromatography

Adsorbent :Silica gel G Activated.
Plate size :20 cm x 8 cm
Plate thickness :0.2 mm
Solvent : Chloroform: methanol (85:15)
Spraying reagent : Anisaldehyde Sulphuric acid
Developing time : At 110^{0}c for 10 min [11]

The R_f Values of n-butanol and Ethanolic extracts shown in Table 3.

Column chromatography of ethanolic extract

Selection of mobile phase

The solvent system developed for TLC was used as mobile phase for column chromatography. Alteration in the composition of eluting solvent gradually to a reservoir of the first line with efficient mixing.

Preparation of column

The slurry of silica gel G (60-120 mesh size) was prepared by mixing the adsorbent with mobile solvent.

Adsorbent	: Silica gel G for column chromatography activated at 105^{0}c. For 1 hour
Length of column	: 41cm
Diameter of column	
Outer	: 3 cm
Inner	: 2.8 cm
Rate of elution	: 10-15 drops/min.
Volume of each Eluent collected	: 25 ml each
Total Volume of Eluent collected	: 100 ml
Elution	: Chloroform: methanol

Totally around 44 eluent were collected and each eluent was subjected to thin layer chromatography as described above for identification of sterols. The R_f value has been calculated.

Assessment of anti-inflammatory activity

Animals selection

Female mice weighing between 20-25 gm were used for acute toxicity study of various extracts. The animals were fasted overnight prior to the acute experimental procedures. Albino rats, wistar strain, of weighing 100-150 gm were used for acute model. Rats were kept in polypropylene cages and fed on standard laboratory diet with ad libitum. The animals were exposed to 12 hours of darkness and light each. The bedding materials of cages were changed every day. Rats were divided into fourteen groups of six each.

Acute toxicity study

Acute toxicity study was carried out according to OECD guidelines (Organization for economic co-operation and development) [12].

Selection of animals housing

The temperature of the experimental animal room was maintained at 22 ^{0}C ($\pm$ 3 ^{0}C) and lighting was kept artificial. The sequence being 12 hr light, 12 hr dark. Conventional laboratory diets and free access to water was given to the mice.

Preparation and administration of Doses

The test compounds i.e. petroleum ether (60-80^0) extract, benzene extract, chloroform extract, ethyl acetate, n-butanol extract, ethanol extract were administered orally. Animals were fasted prior to dosing with free access to water. The doses of 1 ml / 100 gm. b.w. of all extracts were given to the mice in stepwise procedure using fixed does of 1000mg/kg, 2000mg/kg. b.w. Food was given to the mice 3 to 4 hours after administering the test materials.

Signs and symptoms of toxicity were observed at 2000mg/kg in single animal for all extracts in sighting study. The same dose was given to three animals for main toxicity study.

Anti-inflammatory activity

Carrageenan induced rat paw oedema

The n-butanol and ethanol extracts was use. Rats were divided into fourteen groups of six each. They were starved overnight with water prior to the day of experiment.

Group I	: Served as control and received 1ml, water. p.o.
Group II	: Standard group Diclofenac sodium 15mg/kg b.w .
Group III & IV	:Petroleum ether (60-80^0) extract 100 mg/kg and 200 mg/kg
Group V & VI	: Benzene extract 100 mg/kg and 200 mg/kg
Group VII & VIII	: Chloroform extract 100 mg/kg and 200 mg/kg
Group IX & X	: Ethyl acetate extract 100 mg/kg and 200 mg/kg
Group XI & XII	: n-Butanol extract 100 mg/kg and 200 mg/kg
Group XIII & XIV	: Ethanol extract 100 mg/kg and 200 mg/kg

Thirty minutes after drug or test compound administration, 0.1 ml. of 1% carrageenan in distilled water was injected into the subplantar region of right hind paws of all groups. A mark was put on the leg at the malleolus to facilitate uniform dipping at subsequent readings. The paw oedema volume was measured with the help of plethysmograph by mercury displacement method, at zero hr. (Immediately after injecting carrageenan). The same procedure was repeated at 30 mins. 1, 2, 3 hours [13]. The difference between 1 hours and subsequent hours reading was taken as actual oedema volume. The percentage inhibition of paw oedema in the various treated groups was then calculated by using the formula;

Percentage inhibition = (1 – Vt/Vc) X 100

Where Vt = is the oedema volume in the drug treated group.

Vc = is the oedema volume in the control group.

Cotton pellet granuloma method

Foreign body granulomas were provoked in rats by subcutaneous implantation of pellets of compressed cotton. After several days, histological giant cells and undifferentiated connective tissue can be observed besides the fluid infiltration. The amount of newly formed connective tissue can be measured by weighing the dried pellets after removal.

Group I	: Served as control and received 1ml, water p.o.
Group II	: Standard group Diclofenac sodium 15mg/kg b.w.
Group III & IV	: Petroleum ether (60-80^0) extract 100 mg/kg and 200mg/kg
Group V & VI	: Benzene extract 100 mg/kg and 200 mg/kg
Group VII & VIII	: Chloroform extract 100 mg/kg and 200 mg/kg
Group IX & X	: Ethyl acetate extract 100 mg/kg and 200 mg/kg
Group XI & XII	: n-Butanol extract 100 mg/kg and 200 mg/kg
Group XIII & XIV	: Ethanol extract 100 mg/kg and 200 mg/kg

Male Westar rats with an average weight of 200 g are anaesthetized with ether. The back skin is shaved and disinfected with 70% ethanol. An incision is made in the lumbar region. By a blunted forceps subcutaneous tunnels are formed and a sterilized cotton pellet is placed on both sides in the scapular region. The animals are treated for 7 days subcutaneously or orally [13].

Then, the animals are sacrificed, the pellets prepared and dried until the weight remains constant. The net dry weight, i.e. after subtracting the weight of the cotton pellet is determined.

The average weight of the pellets of the control groups as well as of the test group is calculated. The percent change of granuloma weight relative to vehicle control group is determined.

Percentage inhibition =(WD-WC/WD) X 100

Where WD= Difference in pellet of drug treated group

WC = Difference in pellet of drug control group.

RESULTS AND DISCUSSION

Results of Extraction [Table 1] and qualitative chemical investigation [Table 2] of whole plant of Pergularia daemia Linn. has indicated the presence of following active principles for various extracts.

Petroleum ether (60-80^0) extract	: Glycosides
Benzene extract	: Glycosides
Chloroform extract	: Carbohydrates
n-Butanol extract	: Sterol, Carbohydrates.
Ethanol extract	: Sterol, Carbohydrates, Flavonoids

Table 1: Table showing the percentage yield of extracts of whole plant of *Pergularia daemia* Linn.

Extract	Weight of residue	% Yield
Petroleum-ether ($60\text{-}80^0$)	35 gm	5%
Benzene	10 gm	1.5%
Chloroform	8 gm	1.27%
Ethyl acetate	7 gm	1.12%
n-Butanol	15 gm	2.45%
Ethanol	66 gm	11 %

Table 2: Table showing the qualitative chemical investigation of extracts of whole plant *Pergularia daemia* linn.

Test	Petroleum ether ($60\text{-}80^0$) extract	Benzene extract	Chloroform extract	Ethyl-acetate extract	n-butanol extract	Ethanol extract
Test for Sterols						
a) test solution + Conc.H_2SO_4	-	-	-	-	+	+
b) Salkowski's Test	-	-	-	-	+	+
c) Test solution + Sulphur	-	-	-	-	+	+
d) Liebemann Burchardt's Test	-	-	-	-	+	+
Tests for triterpenoids						
a) Salkowski's Test	-	-	-	-	-	+
b)Liebermann Burchardt's Test	-	-	-	-	-	+
Test for Glycosides						
a) Baljet's Test	+	+	-	-	-	-
b) Keller-Kiliani Test	+	+	-	-	-	-
c) Raymond's Test	+	+	-	-	-	-
Tests for Saponins						
a) Foam Test	-	-	-	-	-	-
b) Haemolysis Test	-	-	-	-	-	-
Test for Carbohydrates						
a) Molisch's Test	-	-	+	+	+	+
b) fehlings Test	-	-	+	+	+	+
c) Barfoed's Test	-	-	+	+	+	+
d) Benedict's Test	-	-	+	+	+	+

Test for Alkaloids						
a) Mayer's Test	-	-	-	-	-	-
b) Wagner's Test	-	-	-	-	-	-
c) Hager's Test	-	-	-	-	-	-
Tests for Flavonoids						
a) Ferric Chloride Test	-	-	-	-	-	+
b) Shinoda Test	-	-	-	-	-	+
c) Zn-HCL reduction Test	-	-	-	-	-	+
d) Alkaline reagent Test	-	-	-	-	-	+
e) Lead acetate Test	-	-	-	-	-	+
Test for Tannins						
a) Ferric Chloride Test	-	-	-	-	-	-
b) Gelatin Test	-	-	-	-	-	-
Test for Proteins						
a) Millon's Test	-	-	-	-	-	-
b) Xanthoprodteic Test	-	-	-	-	-	-
c) Biuret Test	-	-	-	-	-	-
d) Ninhydrin Test	-	-	-	-	-	-
Test for aminoacids						
a) Ninhydrin Test	-	-	-	-	-	-
b) Test for tyrosine	-	-	-	-	-	-
c) Test for tryptophan	-	-	-	-	-	-
Tests for Flavonoids						
a) Ferric Chloride Test	-	-	-	-	-	+
b) Shinoda Test	-	-	-	-	-	+
c) Zn-HCL reduction Test	-	-	-	-	-	+
d) Alkaline reagent Test	-	-	-	-	-	+
e) Lead acetate Test	-	-	-	-	-	+

(+) Present and (-) Absent

Presence of sterols in n-Butanol and Ethanol extract were identified by TLC profile. The n-Butanol and Ethanol extract on TLC revealed the presence of 2 sports respectively in Chloroform: Methanol (85:15) solvent system.

Table 3: Table showing the R_f Values of n-butanol and Ethanolic extracts for presence of Sterol

Extract	Colour	R_f Values
n-Butanol	Pink	0.91
	Blue	0.53
Ethanol	Pink	0.92
	Blue	0.55

Table 4: Table showing the isolation of active principle of ethanolic extract of whole plant of *Pergularia daemia* linn. by column chromatography

Solvents	Concentration	No. of Eluent	No. of spot	Colour	Avg. R_f value
Chloroform	100	1 to 4	One spot	Pink	0.90
Chloroform: Methanol	90 : 10	5 to 8	Two spot	Pink Pink	0.91 0.90
Chloroform: Methanol	80 : 20	8 to 12	One spot	Pink	0.91
Chloroform: Methanol	70 : 30	12 to 16	One spot	Pink	0.88
Chloroform: Methanol	60 : 40	16 to 20	One spot	Blue	0.60
Chloroform: Methanol	50:50	20 to 24	One spot	Blue	0.56
Chloroform: Methanol	40 : 60	24 to 28	One spot	Green	0.58
Chloroform: Methanol	30 : 70	28 to 32	One spot	Green	0.62
Chloroform: Methanol	20 : 80	32 to 36	No spot	-	-
Chloroform: Methanol	10 : 90	36 to 40	No spot	-	-
Methanol	100	41 to 44	No spot	-	-

Acute toxicity study was carried out according to OEDC guidelines in albino mice. The acute toxicity study of various extracts of *Pergularia daemia* Linn whole plant was showed signs of toxicity like tremour, convulsion and deep breathing at 2000 mg/kg bw. $1/10^{th}$ of the same dose for all these extract were taken as therapeutic dose i.e. 200 mg/kg. b.w.

The results obtained from the carrageenan- induced rat paw oedema indicated that, ethanol extract has shown good significant ($p<0.001$) reduction in paw oedema to the extent of 44.18% at 200mg/kg concentration, respectively from 1^{st} to 3^{rd} hours when compared to control group. The n-butanol extract has reduced the paw oedma ($p<0.001$), to the extent of 40.05% at 200mg/kg concentration when compared to control group.

However, petroleum ether ($60\text{-}80^0$c) extract, benzene extract, chloroform extract, ethyl acetate extract has reduced ($p<0.01$) the paw oedema to the extent of 20.52%, 21.63%, 11.51% and 10.52% respectively at 200mg/kg concentration when compared with control group. Whereas diclofenac sodium also significantly ($P<0.001$) reduced paw oedema form 1^{st} to 3^{rd} hr when compared to control group.

It appears from the study that n-butanol and ethanol extract of treated group showed good significant anti-inflammatory activity as compared to standard group, where as petroleum ether extract, benzene extract, chloroform extract, ethyl acetate extract treated group showed anti-inflammatory activity. The Results of anti-inflammatory activity have been shown in Table 5 and in form of Figure 1.

Table 5: Table showing the effect of extracts of whole plant *Pergularia daemia* linn. on carrageenan induced rat paw oedema method

Group	Test Material (dose)	Mean increase in paw volume and % inhibition		
		1 hr.	2 hr.	3 hr.
1.	Control	1.29 ± 0.152	1.73 ± 0.200	1.90 ± 0.116
2.	Standard (Diclofenac sod.) 15mg/kg	0.95 ± 0.158 (26.35%)	1.09 ± 0.178 (36.99%)	1.03 ± 0.163 (45.78%)
3.	Petroleum ether extract (100mg/kg)	1.06 ± 0.116 (17.82%)	1.38 ± 0.019 (20.23%)	1.66 ± 0.168 (12.63%)
4.	Petroleum ether extract (200mg/kg)	1.13 ± 0.212 (12.40%)	1.38 ± 0.200 (20.23%)	1.51 ± 0.292 (20.52%)
5.	Benzene extract (100mg/kg)	1.19 ± 0.364 (7.75%)	1.24 ± 0.167 (14.45%)	1.50 ± 0.342 (21.05%)
6.	Benzene extract (200mg/kg)	1.99 ± 0.361 (15.00%)	1.28 ± 0.254 (26.01%)	1.47 ± 0.285 (21.63%)
7.	Chloroform extract (100mg/kg)	1.28 ± 0.0941 (0.77%)	1.52 ± 0.178 (12.13%)	1.78 ± 0.253 (6.31%)
8.	Chloroform extract (200mg/kg)	1.22 ± 0.0740 (5.42%)	2.58 ± 0.081 (15.45%)	1.68 ± 0.223 (11.57%)
9.	Ethyl acetate extract (100mg/kg)	1.28 ± 0.98 (0.77%)	1.48 ± 0.305 (14.45%)	1.50 ± 0.340 (21.05%)
10.	Ethyl acetate extract (200mg/kg)	1.23 ± 0.191 (4.65%)	1.59 ± 0.204 (8.09%)	1.70 ± 0.159 (10.52%)
11.	n-Butanol extract (100mg/kg)	1.09 ± 0.0659 (15.50%)	1.35 ± 0.100 (21.96%)	1.22 ± 0.189 (30.78%)
12.	n-Butanol extract (200mg/kg)	1.47 ± 0.285 (21.63%)	0.57 ± 0.158 (26.35%)	1.07 ± 0.328 (40.05%)
13.	Ethanol extract (100mg/kg)	1.10 ± 0.0815 (14.72%)	1.22 ± 0.169 (35.78%)	1.00 ± 0.145 (42.19%)
14.	Ethanol extract (200mg/kg)	1.09 ± 0.879 (15.00%)	1.05 ± 0.110 (39.50%)	0.72 ± 0.486 (44.18%)

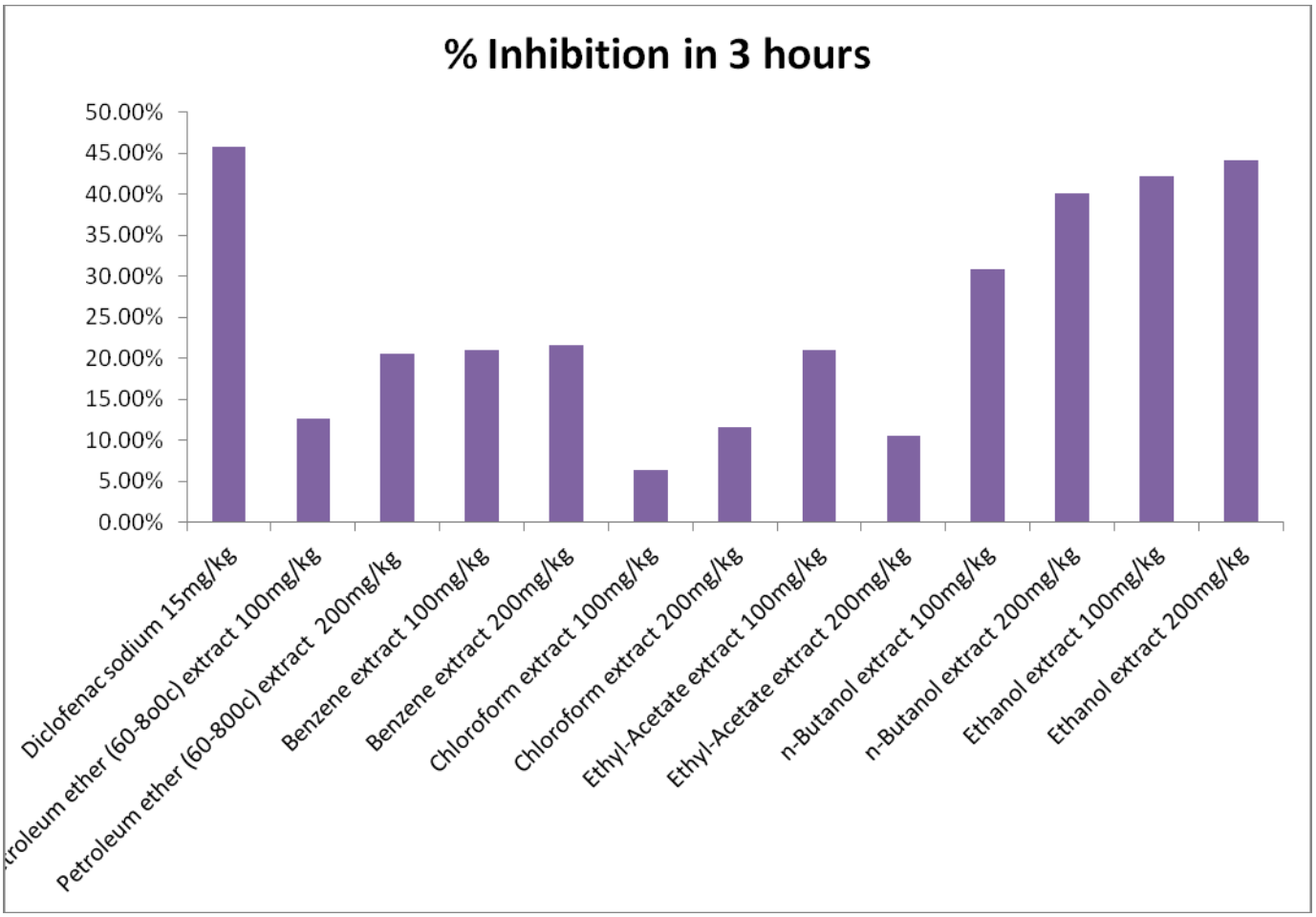

Figure 1: Figure showing the anti-inflammatory activity of whole plant of *Pergularia daemia* Linn. by using carrageenan induced rat paw edema method.

The results obtained indicated that ethanol extract has shown good significant activity at concentration of 200mg/kg and reduced the granuloma formation to the extent of 19.87% respectively when compared to control group. The n-butanol extract has shown good activity at concentration of 200mg/kg and reduced the granuloma formation to the extent of 16.83% when compared to control group. The benzene extract and chloroform extract have reduced the granuloma formation to the extent of 13.96% and 15.08% respectively at 200mg/kg concentration when compared to control group.

However, the petroleum ether (60-80^{0}c) extract and ethyl acetate extract have fail to reduced the granuloma formation to the extent of 7.38% and 8.90% respectively at 200mg/kg concentration when compared to control group.

Whereas Diclofenac sodium has shown significant reduction in granuloma formation as compared to control group.

The Results of this anti-inflammatory activity and percent inhibition is shown in Table 6 and Figure 2

Table 6: Table showing the effect of extracts of whole of *Pergularia daemia* linn. on cotton pellet granuloma method

Weight of Cotton pellet (mean ± S.D. mg)					
Group	**Test Material (dose)**	**Initial weight**	**After 7 days**	**Difference**	**% Inhibition**
1.	Control	50	80.83	29.64 ± 2.60	-
2.	Diclofenac sodium (15mg/kg)	50	72.60	22.60 ± 1.59	23.75%
3.	Petroleum ether (60-80^{0}c) extract (100mg/kg)	50	77.95	27.95 ± 1.59	5.70%
4.	Petroleum ether (60-80^{0}c) extract (200mg/kg)	50	79.47	29.47 ± 1.53	7.38%
5.	Benzene extract (100mg/kg)	50	76.00	26.00 ± 1.58	12.28%
6.	Benzene extract (200mg/kg)	50	75.50	25.50 ± 1.59	13.96%
7.	Chloroform extract (100mg/kg)	50	76.65	26.65 ± 1.56	13.46%
8.	Chloroform extract (200mg/kg)	50	75.17	25.17 ± 1.59	15.08%
9.	Ethyl-Acetate extract (100mg/kg)	50	77.50	27.50 ± 1.59	7.21%
10.	Ethyl-Acetate extract (200mg/kg)	50	77.00	27.00 ± 1.58	8.90%
11.	n-Butanol extract (100mg/kg)	50	74.90	24.90 ± 1.59	15.99%
12.	n-Butanol extract (200mg/kg)	50	74.65	24.65 ± 1.59	16.83%
13.	Ethanol extract (100mg/kg)	50	74.29	24.29 ± 1.58	18.04%
14.	Ethanol extract (200mg/kg)	50	73.75	23.75 ± 1.58	19.87%

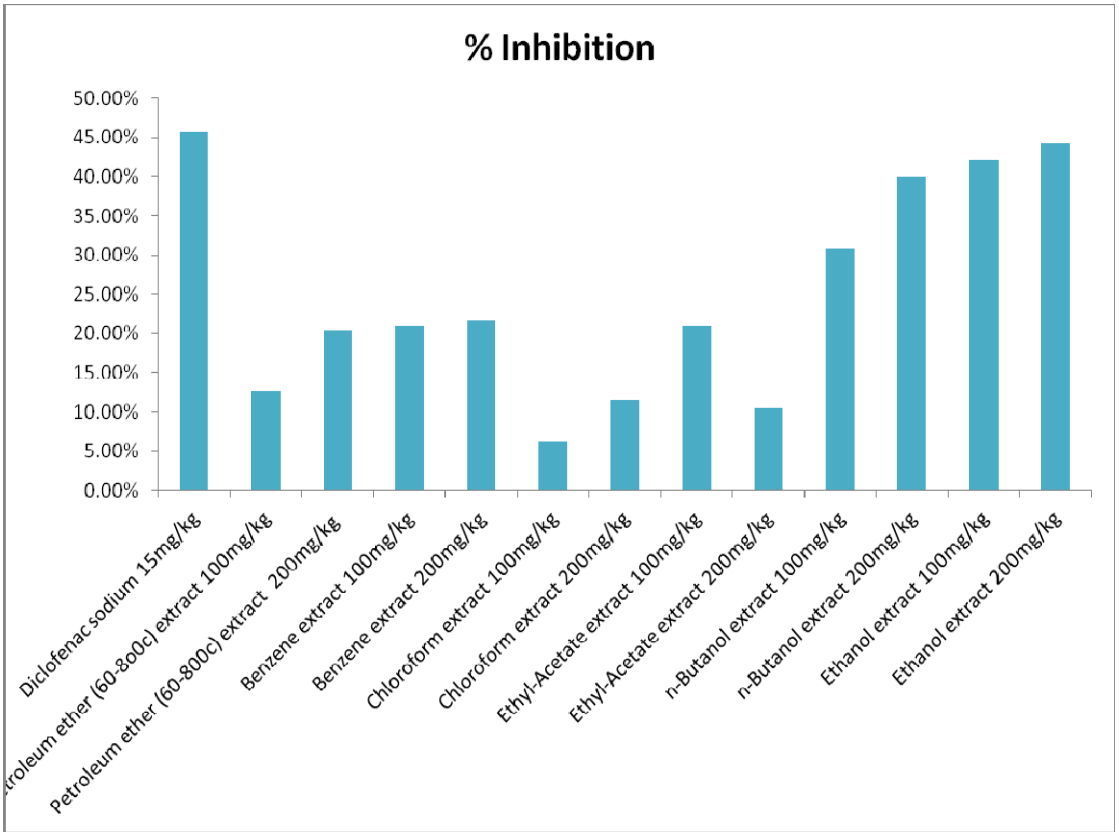

Figure 2: Figure showing the anti-inflammatory activity of whole plant of *Pergularia daemia* Linn. by using cotton pellet granuloma method

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