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Analysis of antifertility activity and phytochemical studies of *Pergularia daemia* leaves in male albino rats

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ABSTRACT

Petroleum ether, benzene and ethanol extracts of *Pergularia daemia* leaves were administered intraperitoneally at the dose level of 100 and 200mg/kg body weight to male albino rats for 30 days. The results shows decrease in the number of spermatogonia, spermatocytes and spermatids in testis along with sperm count in caudal epididymis. Biochemical observations indicate increased levels of cholesterol and significant reduction in protein and glycogen content. The increased cholesterol content along with degeneration of Leydig cells indicates that inhibited steroidogenesis. The decrease in the weight of a testis and accessory reproductive organs further attributes low steroidogenesis. Out of three extracts tested, ethanol extract was more potent and seems to be antispermatogenic and antisteroidogenic activities. When ethanol extract was tested in immature rats for androgenic activity, it showed its antiandrogenic potency as the weight of accessory sex organs were reduced.

KEYWORDS

Pergularia daemia;
Testis;
Accessory organs;
Spermatogenesis;
Steroidogenesis;
Rat.

INTRODUCTION

Search for male antifertility agents in natural products remains a potential area of investigation[1-2]. Henshaw listed many plants used by primitive people in different countries to control fertility[3]. Though many indigenous plants have so far been investigated for antispermatogenic activity[4-10]. But effective drug, which is free from side effects, has come out till today. No reports are available on male antifertility activities of plant *Pergularia daemia.* However, attempts have been made to bring out safe and effective plant preparations as novel contraceptive for males.

Pergularia daemia belongs to the family Asclepidaceae and it is commonly called as utran. It exhibits several medicinal properties like diarrhoea, asthama, piles, and joint pains. In reference literature related to different system of medicine in India is mentioned that its use in gynecological troubles is described as good uterine tonic and it also acts as a sedative. Its susceptible use in treatment of snakebites is established hence it is considered as a promising herb among traditional healers[11]. Sadik et al., have reported a steroidal and alkaloidal fraction of *P.daemia* having significant antifertility activity in female mice[12-13]. Therefore, the present investigation on the three extract of the plant *P. daemia* at different dose level were used to evaluate their effect on the reproductive function in male rats is undertaken.

EXPERIMENTAL

The healthy and disease free fresh leaves of *P.daemia* were collected from in and around the Gulbarga University campus during the month of June and July 2006. A voucher specimen was deposited at the herbarium of the Department of Botany, Gulbarga University, Gulbarga, Karnataka, India. The leaves were shade-dried, powdered and subjected to Soxhlet extraction successively and separately non-polar to polar solvents i.e., petroleum ether (B. P. 60-80^{0}C), benzene and ethanol (95 %). The decoctions obtained each time were evaporated under reduced pressure below 45^{0}C. The dried mass was considered as the extract and individually screened for antifertility activity in albino rats. For administration to test animals the extracts were macerated in Tween-80 (1%) and resuspended in distilled water for their complete dissolution. The presence of various chemical constituents in plant extracts were determined by preliminary phytochemical screening as described by Kokate and Harnborne[14-15].

Adult, healthy and virgin Wistar strain male albino rats of 60-70 days old and 100-120g-body weight, were selected from the inbred animal colony for experimental use. The animals were maintained under uniform husbandary conditions of light and temperature and were given pellet diet as prescribed by Central Food and Technological Research Institute, Mysore, India (CFTRI) and tap water *ad libitum*.

After preliminary trials, 100mg and 200mg/kg body weight dose level were selected for evaluating the effects of the crude drugs. The animals were divided into seven groups consisting of six animals in each group and treated with plant extract intraperitoneally every day for 30 days as shown below.

- Group-I: Control, received 0.2ml Tween-80 (1%) intraperitoneally.
- Group-II: Received 100mg of *P.daemia* leaves petroleum ether extract /kg body weight in 0.2ml Tween-80 (1%) intraperitoneally.
- Group-III: Received 200mg of *P.daemia* leaves petroleum ether extract /kg body weight in 0.2ml Tween-80 (1%) intraperitoneally.
- Group-IV: Received 100mg of *P.daemia* leaves benzene extract /kg body weight in 0.2ml Tween-80 (1%) intraperitoneally.
- Group-V: Received 200mg of *P.daemia* leaves benzene extract /kg body weight in 0.2ml Tween-80 (1%) intraperitoneally.
- Group-VI: Received 100mg of *P.daemia* leaves ethanol extract /kg body weight in 0.2ml Tween-80 (1%) intraperitoneally.
- Group-VII: Received 200mg of *P.daemia* leaves ethanol extract /kg body weight in 0.2ml Tween-80 (1%) intraperitoneally.

Spermatogenic/Antispermatogenic activity

The control and treated animals were sacrificed 24 hour after the last treatment. The testes, epididymis, seminal vesicles, vas deferens were excised, blotted free of blood, carefully made free from the surrounding fat and connective tissue and weighed up to the nearest milligram on an electronic balance. Fresh tissues from testis, epididymis and vas deferens were processed for the estimation of protein, glycogen and cholesterol [16-18]. Besides, they were fixed in Bouin's fluid, embedded in paraffin, sectioned at 5 μm and stained with haematoxylin-eosin for histological examination[19]. The micrometric measurements such as testicular and seminiferous tubular diameters were made and calculated by the method described by Deb et al.[20]. Spermatogenic element count was made from randomly chosen twenty round cross-sections taken from the middle part of the testis[21]. The cauda epididymal sperm suspension was prepared in normal saline and epididymal sperm count was estimated by the method of Kempinas and Lamano Carvalho[22].

Androgenic/antiandrogenic activity

Among the three extracts of *P.daemia* leaves the ethanolic extract showed maximum antispermatogenic activity. Therefore the ethanolic extract of *P. daemia* at the dose level of 200mg/kg body weight was used alone to test androgenic/antiandrogenic activity. Wistar strain immature rats of 25 days old weighing between 35-40gm were administered intraperitoneally for 7 days as follows.

- Group-I: Control, received 0.2ml Tween-80 (1%) intraperitoneally.
- Group-II: Received 20μg/animal of testosterone in 0.1ml olive oil intraperitoneally
- Group-III: Received 200mg ethanol extract /kg body

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weight in 0.2ml Tween-80 (1%) intraperitoneally.

All the three groups of animals were sacrificed on day 8 by cervical dislocation and the testes, epididymis, seminal vesicles, vas deferens were excised, blotted free of blood, carefully freed from surrounding fat and connective tissue and weighed up to the nearest milligram and adjudged for androgenic/antiandrogenic activity. The data were statistically analyzed by Student's *t* test 'p' values < 0.05 were considered significant[23].

RESULTS

Phytochemical screening (TABLE 1)

The petroleum ether, benzene and ethanol extracts of *P.daemia* leaves were used for the preliminary phytochemical testis (TABLE 1). The petroleum ether extract showed positive test for alkaloids, steroids and carbohydrates, benzene extract showed positive test for steroids, carbohydrates, glycosides and flavones and in ethanol extract showed positive tests for alkaloids, steroids, carbohydrates, glycosides and flavones.

Changes in testis

3.1.1. Gravimetric and histometric changes

Changes in the body weight (TABLE 2)

The body weight of the petroleum ether extract of both the dose level treated animals was significantly ($p<0.001$) reduced, when compared to control. But benzene and ethanol extracts of both the dose level treated animals showed no comparable changes with control.

Changes in testes and accessory organs (TABLE 2)

The weight of the testis was decreased slightly in both the dose level of all extract treated groups and which is significant ($p<0.001$). The weight of accessory organs like epididymis was reduced in all the treated groups at both the dose level and which is significant. The weight of vas deferens was reduced highly significantly with the administration of both the dose level of petroleum ether extract. But in benzene and ethanol treated groups at both the dose level showed slight reduction of vas deferens weight and which is significant ($p<0.01$). The weight of seminal vesicle was reduced in significantly in both the dose level of petroleum ether and benzene administration. But it was highly significant reduction only in the ethanol extract treated group at both the dose level when compared to control group.

Biochemical changes in testis and accessory organs (TABLES 3 and 4)

The decrease in the level of protein and glycogen content in testes was slightly observed in petroleum ether and benzene extracts at both the dose level administration. But, it was significantly decrease and in ethanol extract at both the dose level of administration. The cholesterol content of the testes was increased highly significantly ($p<0.001$) in both the dose level of ethanol

TABLE 1: Phytochemical screening of various extracts of *P.daemia* leaves

Plant constituents	Petroleum ether	Benzene	Ethanol
Alkaloids	+ve	-ve	+ve
Steroids	+ve	+ve	+ve
Carbohydrates	+ve	+ve	+ve
Glycosides	-ve	+ve	+ve
Amino acids & Proteins	-ve	-ve	-ve
Saponins	-ve	-ve	-ve
Flavones	-ve	+ve	+ve
Oils & Fats	-ve	-ve	-ve
Phenols and Tannins	-ve	-ve	-ve

+ = positive, - = negative

TABLE 2: Gravimetric changes in the testis and accessory organs due to administration of various extracts of *P.daemia* leaves

Group	Treatment	Body weight (mg)	Testis	Epididymis	Vas deferens	Seminal vesicle
I	Control (Tween-80 (1%))	160.50±2.12	1.41±0.08	0.51±0.08	0.10±0.01	0.83±0.02
II	Petroleum ether (100 mg)	107.50±3.50**	1.38±0.02	0.39±0.01	0.06±0.00*	0.49±0.06*
III	Petroleum ether (200 mg)	100.00±0.00**	0.97±0.07**	0.38±0.01*	0.05±0.00*	0.38±0.10*
IV	Benzene (100 mg)	133.33±11.5*	1.26±0.26*	0.41±0.06	0.07±0.01	0.55±0.20*
V	Benzene (200 mg)	146.66±23.0	1.17±0.15*	0.39±0.04	0.06±0.00*	0.60±0.50
VI	Ethanol (100 mg)	146.33±1.52	1.33±0.07	0.44±0.03	0.06±0.00*	0.43±0.06*
VII	Ethanol (200 mg)	150.00±5.00	1.18±0.13*	0.34±0.08*	0.06±0.00*	0.31±0.06**

Duration: 30 days; organ weight: mg/100 gm body weight, Values are mean ± S.E., Six animals were maintained each group, *p<0.01, **p<0.001, when compared to control

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TABLE 3: Biochemical changes in the testis due to administration of various extracts of *P. daemia* leaves

Group	Treatment	Protein (µg/mg)	Cholesterol (µg/mg)	Glycogen (µg/mg)
I	Control (Tween-80 (1%))	3.84±0.05	16.80±1.13	8.5±0.09
II	Petroleum ether (100 mg)	3.22±0.08	20.70±2.12	7.79±0.10
III	Petroleum ether (200 mg)	3.10±0.02*	26.40±3.39*	7.59±0.19
IV	Benzene (100 mg)	3.28±0.00	19.60±0.50	7.99±0.19
V	Benzene (200 mg)	3.30±0.02	24.70±3.50*	7.86±0.90
VI	Ethanol (100 mg)	3.00±0.16*	23.10±1.27*	7.00±0.28*
VII	Ethanol (200 mg)	2.88±0.00**	34.00±3.90**	6.59±0.09*

Duration: 30 days; organ weight: mg/100 gm body weight, Values are mean ± S.E., Six animals were maintained each group, *p<0.01, **p<0.001, when compared to control

TABLE 4: Biochemical changes in the epididymis and vas deferens due to administration of various extracts of *P.daemia* leaves

Group	Treatment	Epididymis		Vas deferens	
		Protein (µg/mg)	Cholesterol (µg/mg)	Protein (µg/mg)	Glycogen (µg/mg)
I	Control(Tween-80 (1%))	4.28±0.05	9.60±2.26	4.12±0.04	4.19±0.10
II	Petroleum ether (100 mg)	3.90±0.14	15.20±1.13*	3.92±0.14	3.86±0.19
III	Petroleum ether (200 mg)	3.95±0.07	16.80±1.13*	3.84±0.05	3.59±0.37
IV	Benzene (100 mg)	4.08±0.00	12.80±2.26*	4.04±0.05	3.99±0.20
V	Benzene (200 mg)	4.12±0.05	17.60±2.28*	4.00±0.00	3.93±0.09
VI	Ethanol (100 mg)	3.68±0.05*	22.40±2.20**	3.76±0.07*	3.94±0.14
VII	Ethanol (200 mg)	3.64±0.00*	29.60±1.18**	3.68±0.06*	3.38±0.02*

Duration: 30 days; organ weight: mg/100 gm body weight, Values are mean ± S.E., Six animals were maintained each group, *p<0.01, **p<0.001, when compared to control

TABLE 5: Micrometric and spermatogenic changes in the testis due to administration of various extracts of *P.daemia* leaves

Group	Treatment	Diameter of testis (µm)	Diameter of seminiferous tubule (µm)	Spermatogonia	Spermatocytes	Spermatids	Sperm count (millions/ cauda)
I	Control (Tween-80(1%))	6150.00±20.00	308.62±3.91	103.60±5.30	162.70±6.30	102.80±2.50	2.80±0.28
II	Petroleum ether (100 mg)	6137.00±12.20	305.32±2.21	103.20±2.30	161.90±8.20	102.10±1.25	2.10±0.14
III	Petroleum ether (200 mg)	6147.10±02.20	304.92±8.81	102.90±3.10	161.20±5.80	102.50±1.90	1.60±0.23*
IV	Benzene(100 mg)	6097.00±10.50*	302.21±5.62	92.21±2.18	158.21±4.29	92.91±5.21*	2.20±0.28
V	Benzene(200 mg)	5992.00±21.80**	296.95±0.73*	87.16±0.79*	146.00±1.39*	80.80±4.55**	2.00±0.36
VI	Ethanol(100 mg)	5965.00±34.00**	280.26±6.31**	82.29±0.54*	132.18±2.41**	72.86±6.91**	1.80±0.29*
VII	Ethanol(200 mg)	5750.08±77.39**	260.64±2.08**	67.38±5.51**	91.92±1.11**	51.64±3.42**	1.30±0.14**

Duration: 30 days; organ weight: mg/100 gm body weight, Values are mean ± S.E., Six animals were maintained each group, *p<0.01, **p<0.001, when compared to control

extract. But, it was slightly increase and significant ($p<0.01$) in treated with both the dose level of benzene and petroleum ether extract administration. The epididymis protein content were decreased and cholesterol content was increased in all the three extract treated groups, but, it was only significant in the group of ethanol extract administered at both the dose level. In vas deferens the protein and glycogen content was decreased due to administration of all the three extracts at both the dose level. But, in ethanol extract administrated group it was significantly decreased respectively.

Micrometric changes of testis

A micrometric measurement like diameter of testis is decreased non-significantly with the treatment of both the doses of petroleum ether and benzene extracts of *P.daemia*. Significant ($p<0.01$) reduction is obtained with the treatment of both the doses of ethanol extract. Similarly the diameter of seminiferous tubule is non-significantly decreased in all the treated extract groups, except the high dose of ethanol extract treated group and which is decreased significantly ($p<0.01$).

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TABLE 6: Gravimetric changes of the testis and accessory organs due to administration of ethanol extract of *P.daemia* leaves.

Group	Treatment(Dose)	Testis	Epididymis	Vas deferens	Seminal vesicle
I	Control(Tween-80 (1%)	452.8±4.68	113.5±5.78	26.3±1.32	173.2±4.60
II	Testosterone(10 µg/rat/day)	483.3±2.56**	175.1±5.95**	42.2±2.33**	238.1±4.41**
III	Ethanol(200 mg/kg)	441.0±3.29	73.3±5.01**	14.2±2.73**	93.4±4.03**

Duration: 07 days; organ weight: mg/100 gm body weight, Values are mean ± S.E., Six animals were maintained each group, *p<0.01, **p<0.001, when compared to control

Spermatogenic changes of testis (TABLE 5)

The process of spermatogenesis is impaired in all the groups treated with *P.daemia* leaves extracts. The number of spermatogenic elements like spermatogonia, spermatocyte and spermatids were decreased; it is significant ($p<0.01$) with low dose and highly significant ($p<0.001$) with high dose of ethanol extract administration. Non-significant reduction was observed in the spermatogenic elements with the treatment of both the doses of petroleum ether and benzene extracts.

Sperm count (TABLE 5)

The cauda epididymal sperm count was decreased in all the three extracts treated groups, but it was significant due to ethanol extract administration at both the dose level.

Androgenic/antiandrogenic activity (TABLE 6)

The administration of testosterone to immature albino rats caused a highly significant ($p<0.001$) increase in the wet weights of epididymis, vas deferens and seminal vesicles, but non-significant weight increase in testes. Administration of ethanol extract of *P.daemia* leaves at the dose level of 200mg/kg body weight was decreased the wet weight of accessory organs like epididymis, vas deferens and seminal vesicle highly significantly ($p<0.001$), but non significant reduction in the testis wet weight, when compared to control.

DISCUSSION

Pergularia daemia leaves suppressed sperm production, as evidenced by the reduction in the number of spermatogenic elements and sperm count. Similar results were found by the administration of *Crotalaria juncea*[4-6] in rats and mice, *Momordica charantia*[7] in rats, *Hibiscus rosa sinensis*[8] in rats and *Melia azedarch*[9] in rats. The principal cells of epididymis synthesize protein, which have important role for maturation of spermatozoa[24]. In the present study petroleum ether, benzene and ethanol extracts of *P.daemia* leaves have reduced the weight of testes. The observed reduction of the testosterone weight may be due to the altered production of seminiferous tubular fluid[25], which is under the control of testosterone and FSH[26,27]. Testosterone is known to regulate the growth and secretory activity of accessory sex organs[28-30]. Therefore, the results observed in accessory sex organs weight in the present study may be due the non-availability of androgen. It is well established that the LH leutinises the cholesterol to produce pregnanalone which is subsequently metabolized to progesterone[31,32]. The increased level of cholesterol in the testes and accessory sex organs in the present study may be due to the altered steroidogenesis, leading to reduced conversion of cholesterol to androgens. Whether this reduction is mediated through decreased availability of pituitary LH or directly due to its antiandrogenic activity on the accessory organ has to be tested. The reduced protein content may also be another reason as the content and the androgen increase the protein anabolism and decrease the catabolism of amino acids so a significant reduction in the androgen deficiency[33]. While the reduction in glycogen content indicates the low energy source of carbohydrates for spermatogenesis in the testes, which is dependent on the availability of estrogen[34]. And it reflects decreased number of post-meiotic germ cells. Which are thought to be the sites of glucose metabolism[35]. As the administration of leaves extracts has caused reduction in the spermatogenesis, steroidogenesis and androgen production, it may alter the sexual behavior and may cause antifertility. Out of the three extracts tested, ethanol extract at 200mg/kg body weight dose level is more effective in causing antispermatogenic and antisterodogenic activities. The ethanol extract when tested in immature rats has shown antiandrogenic effects. This effect may also lead to the antifertility potency of the leaves extract of *P.daemia*.

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