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Effect of *Pedalium murex* Dry Seed Extract on Testicular Dysfunction in Streptozocin Induced Diabetes Mellitus in Wistar Albino Rats

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ABSTRACT

Pedalium murex plant an Indian seasonal herb has been found to be effective against gonorrhoea, dysuria, reproductive disorders etc by some researchers. Studies also reported its hypoglycemic, antioxidant effect in rodents. One study proved the efficacy of petroleum ether extracts of P.murex dry seed (PEPM) against ethanol induced testicular dysfunction in albino rats. Testicular dysfunction with STZ induced diabetes mellitus is caused by uncontrolled hyperglycemia and oxidative stress. The present study aimed to evaluate the effect of petroleum ether extracts of P.murex dry seed (PEPM) on testicular dysfunction in STZ induced T2DM. The parameters evaluated are FBG level , tissue MDA and SOD level, seminal analysis and histopathological study of testis by Johnsen's scoring. Thirty (30) wistar albino rats were randomly divided into 5 groups (n=6). Group1 (control), group-2 to group-5 were treated with single dose injection (i.p) of streptozotocin (40mg/kg) for induction of diabetes mellitus. All the rats received differentdrugs for 60 days as follows: Group 1(Tween 80) Group2: Disease Control (Tween 80), Group3- metformin (50 mg/kg), Group 4-PEPM (400 mg/kg) and Group 5-(PEPM)+ metformin (25mg/kg). On day 61, rats were sacrificed and FBG estimated, semen collected from caudal epididymis. Sperm count, motility, morphological abnormalities were noted by microscopic examination. Oxidative stress markers like MDA and SOD were estimated from testicular tissue homogenate. Histopathological examination of testis was done and scored by Johnsen's score. All the data were analysed by One way ANOVA except Johnsen's score which was analysed by Kruskal-Wallis test .$P< 0.05$ was considered significant. The normal rats received PEPM did not show any significant change in FBG. Diabetic control rats showed significant increase in FBG, alteration in testicular MDA level and SOD activity, decrease in sperm parameters and Johnsen's score. Metformin, PEPM (400 mg/kg) and combination of PEPM+ metformin treated groups significantly reversed the above parameters. Petroleum ether extract of P.murex dry seed possess anti-hyperglycemic, antioxidant and germ-cell protective properties in STZ – T2DM rats.

Keywords: PEPM, Oxidative stress, Sperm analysis, Johnsen's score.

INTRODUCTION

Diabetes mellitus is a common metabolic disorder, characterized by hyperglycemia. T2DM which contributes major share of total diabetic population occurs due to diminished insulin secretion or insulin resistance. There is an increasing trend of diabetic population encompassing 108 million in 1980 to 422 million in 2014.The prevalence of diabetes among adults e"18 years of age has been increased from 4.7% in 1980 to 8.5% in 2014. An estimated 1.5 million deaths in 2012 were directly caused by diabetes whereas another 2.2 million deaths contributed by high level of blood glucose. WHO projects that in 2030 diabetes will be the 7th leading cause of death[1]. It is usually associated with

severe functional and physiological complications if remained untreated/ sub optimally treated for a longer period. Poor glycemic control leads to structural and functional changes in various organs[2]. Male reproductive function alterations have been widely reported in animal models of diabetes[3, 4] comprising of decrease in weight of testis, increase abnormal spermatogenesis and diminution in sperm count[5] along with low levels in plasma testosterone[6] in diabetic rats.

It had been demonstrated that oxidative stress plays a major role for loss of male germ cells as induction of testicular apoptotic cell death by diabetes and was combated by antioxidant[7,8]. It is not fully understood whether increased blood sugar affects oxidative defense mechanism or increasing oxidative stress lead to pathogenesis of diabetes melitus[8, 9]. More recently, Arikawe *et al*. 2012 reported that streptozotocin induced and insulin resistance models of diabetes reduced testicular proliferating cell nuclear antigen(PCNA) index and mean seminiferous tubular diameter and testicular diameter[10].

Pedalium murex L. is a diffuse, more or less succulent herb found near the sea coast of south India, Mexico, and tropical Africa[11]. It is a sand dune herb in the coastal districts of Odisha, India and traditionally used by local people for various medicinal purposes like treatment of gonorrhoea and dysurea. The mucilage from leaves and young shoots is used as an aphrodisiac in seminal debility[12]. Extensive phytochemical analysis of this plant showed the presence of pedalitin and pedalin (major flavonoids) along with diosmetin, dinatin, dinatin-7-glucoronide, quercetin, quercimeritin, and quercetin-7-glucorhamnoside[13]. The presence of phytochemicals like triterpenoids such as ±-amyrin acetate, rubusicacid, ursolicacid,and lupeol acetate and steroids like ²-sitosterol , sapogenins and diosgenin are also reported[14]. Some studies had reported its hypoglycemic and antioxidant properties[15.] One literature evaluated its aphrodisiac activity on ethanol induced testicular dysfunction[16.]

On this context the present study was conducted to evaluate theeffect of petroleum ether extracts of *P.murex* dry seeds on testicular dysfunction in streptozotocin induced T2 DM in rats.

Aims and Objectives

To evaluate the effect of PEPM on FBS, on oxidative stress by estimating testicular MDA and SOD activity, on testicular function by semen analysis (sperm count, motility and abnormality) and histopathological study of testis by Johnsen's scoring in STZ induced T2DM.

MATERIALS AND METHODS

Thirty young adult male wistar albino rats of 150-200gm B.W were procured from a registered breeder (no. 526/02/bc/CPCSEA) and maintained in 12:12 hour light:dark cycle under standard laboratory conditions at a temperature of 24-28°C with a relative humidity of 60-70%. The rats were provided with standard pellet diet and water ad libitum. Experiments were carried out as per the CPCSEA guidelines at 10-11AM morning. The protocol was approved by the Institutional Animal Ethical Committee (Registration No. 472).

Preparation of drug

Pedalium murex dry seeds were purchased from the local market and authenticated by Prof A. K. Panigrahy, Dept. of Botany, Berhampur University, Odisha. Petroleum ether extract of the fine powder of *P. murex* dry seed was prepared using the sohxlet apparatus in our laboratory.

Toxicity study

Acute oral toxicity study was performed as per OECD-423 guidelines[17].

Male adult albino wistar rats were administered step wise from 5, 50, 300, and 2000 mg/kg b.w. of petroleum ether extract of *P.murex* dry seed per orally. The rats were observed for 24 hours. There was no mortality or behavioural changes found during the study period.

Induction of Diabetes

STZ induced Model

Diabetes was induced by administration of a single i.p. injection of streptozotocin in 0.1M citrate buffer at a dose of 40mg/kg in overnight fasted rats by dissolving in freshly prepared 5 mmol/L citrate buffer, pH 4.5[18]. Fasting Blood glucose levels was measured on day 2, and day 7 by using strips on

glucometer and individual fasting blood glucose level above 250mg/dl was considered diabetic.

Experimental design

Group1 (Normal Control): Received 5%v/v tween 80
Group 2: (Disease Control): Diabetic rats received 5% v/v tween 80
Group 3 (Standard) – Diabetic rats received metformin (50 mg/kg)
Group 4- Diabetic rats receive petroleum ether extract of *Pedalium murex* dry seed (PEPM) (400 mg/kg)
Group 5- Received *Pedalium murex* dry seed extract (PEPM) + metformin (25mg/kg)

All the drugs and vehicles were given through oral route for 60 days. Vehicles used for this study were tween 80 and distilled water for PEPM and metformin respectively.

Collection of blood, semen and testicular tissue for biochemical estimations

On day 61 after overnight fasting, blood was collected from the tip of the tail of the rats by needle prick for FBG test. Then the animals were sacrificed by cervical dislocation under light ether anaesthesia. The laprotomy was done by midline incision and the testis-epididymis removed. The semen was collected by making cuts on the caudae epididymis and vas deferens. The testis was preserved with neutral 10% formaldehyde solution.

Evaluation of sperm motility, count, and abnormalities

The collected seminal fluid placed in 1 ml of modified Kreb's ringer bicarbonate buffer (PH 7.4). This sperm suspension was evaluated for sperm count and percent motility. The count was done in a Neubauer haemocytometer. The percentage motility was observed by the progressive and non-progressive movements of sperms under compound microscope. For identification of the abnormal sperm the suspension was stained with eosin and smeared on glass slides, air dried and examined by bright field microscope under an oil immersion lens. The percentages of abnormal sperms were calculated.

Estimation of Antioxidant Parameters

Testicular tissues was washed in cold isotonic solution (0.9% v/w), decapsulated and homogenized in ice-cold tris- HCl buffer solution(ph-7.4, 0.2mmol/L) by glass-teflon homogenizer for 2 mins at 11200*g. The homogenate was centrifuged at 3500*g fr 60 min and the supernatant was obtained. Both MDA (nmol/mg of protein) and SOD levels were measured in the homogenate as described in literatures[19,20].

Histopathological study

The tissues were processed by embedding in paraffin and 5¼m thick sections were obtained and stained with hematoxylin and eosin stain for light microscopic analysis. The pathologist who was

Table 1: Effect of PEPM on FBG and testicular tissue MDA level and SOD activity in STZ- diabetic rats

	Drug/dose	FBG (mg/dl)	MDA (nmol/ mg of tissue)	SOD (Units/ mg of tissue)
Normal control(no disease)	Tween 80	107.33±12.68	3.02±0.08	40±13
Disease control	Tween 80	285.33±61.12$^{\$\$\$}$	9.04±0.77$^{\$\$\$}$	15±1.5$^{\$\$\$}$
PEPM	400mg/kg	222±14.64***	5.93±0.37***	28 ±6.1
Metformin	50mg/kg	122.83±13.04***	4.31±1.09***	27±3.8
PEPM + Metformin	400mg/kg+ 25 mg /kg	127.83±16.67***	4.89±0.17***	37 ±13***

PEPM (Petroleum ether extract of Pedalium murex dry seed), n=6, Data expressed in Mean ± SD, one way ANOVA with Bonferroni's test, $$$ indicates p value <0.001(normal control vs diabetic control) and *** indicates p value <0.001(diabetic control Vs drug treated groups)

unaware of the experimental procedures examined the samples histopathologically and Johnsen's score was calculated according to the modified Johnsen Scoring system[21].

Histological criteria and the modified Johnsen's score system for assessment of spermatogenesis: 10 = complete spermatogenesis and perfect tubule; 9 = many spermatozoa present and disorganized spermatogenesis; 8 = only a few spermatozoa present;7 = no spermatozoa but many spermatids present; 6 = only a few spermatids present; 5 = no spermatozoa or spermatids but many spermatocytes present; 4 = only a few spermatocytes present; 3 =only spermatogonia present; 2 = no germ cells but only Sertolicells present; 1 = no germ cells and no Sertoli cells present.

Table 2: Effect PEPM on parameters of semen analysis in STZ induced diabetic rats

Group	Sperm count (x10^6/ml)	Motility (%)
Normal Control	87±9.695	47±12.99
Diabetic control	17.17 ±4.215$	28.50 ±5.282$
Metformin	42.00 ±12.84*	46.67 ±3.445
P. murex	49.50 ±13.81*	57.00 ±7.014*
P.murex+ metformin	47.67 ±12.99*	65.33 ±4.676* €

N=6; values expressed as Mean ± SD , Data analyzed by one way ANOVA test with Bonferroni's test,$ indicates p value <0.05(diabetic control Vs normal control) * indicates p value <0.05 (drug treated Vs diabetic control);€ p<0.05 (metformin treated Vs combination group)

Statistical analysis

Parametric data such as FBG, sperm count, motility, abnormal morphology, MDA levels and SOD activity were analysed by one way ANOVA followed by Bonferroni's post hoc test. The non-parametric data i.e Johnsen's score was analysed by Kruskal-Wallis test with Dunn's multiple comparison test. P< 0.05 was considered significant.

RESULTS

The *P.murex* dry seed extract at 400mg/kg dose as well as its combination with half effective dose of metformin (25 mg/kg) produced significant decrease in FBG in comparison with diabetic control which were similar to normal control and standard drug metformin (50mg/kg). This study showed that PEPM alone as well as in combination with metformin, decreased the testicular tissue MDA level and increase in SOD activity significantly against disease control and similar to that of normal control / metformin (vide Table no.1).

In the table no. 2, there is a significant decrease in sperm count (17.17 ±4.215) and motility (28.50 ±5.282) in the diabetic control compared with normal control. PEPM treated group as well as its combination with metformin showed significant increase in both the parameters (count-49.50±13.81, motility- 57.00 ±7.014) compared to diabetic control.

Table 3: Effect of PEPM on Johnsen's Scoring of Testicular Histopathology in STZ DM rats

Group	Drug	Johnsen score	95% CI	Significance
Control	Tween 80	9±0.63	8.34-9.66	-
Streptozocin	Tween 80	5.16±0.75***	4.37-5.95	* p value <0.0001 (vs normal control)
induced	Metformin (50 mg/kg)	7.16±0.75##	6.37-7.95	##p<0.05 (vs diabetic control)
Diabetes	PEPM (400 mg/kg)	7±0.89##	6.06-7.93	## p<0.05 (vs diabetic control)
	Metformin(25 mg/kg)+ PEPM(400 mg/kg)	8.16±0.75##	7.37-8.95	# p < 0.05 (vs diabetic control)

n=6,Values expressed as Mean ± SD, Data analysed by Kruskal-wallis test with Dunn's multiple comparison test

As for the motility the combination of PEPM with half the effective dose of metformin (25 mg/kg) produced significant increase(65.33 ±4.676) in comparison to the group treated with metformin alone(46.67 ±3.445).

It is depicted in figure no.1 that structural abnormalities affecting tail, mid piece, head of sperms, significantly increased in diabetic group leaving only 52.66% normal compared to control. Tail abnormalities account for maximum percentage of morphological changes in the sperms with 25.66% in diabetic control which is reduced to 15.67% in metformin, 17% in PEPM treated and 11.83% in PEPM+Metformin treated groups. There is a significant decrease in morphologically abnormal sperms as a whole in drug treated groups compared to that of disease control.(figure no.2)

In diabetic control group the Johnsen's score was significantly lower i.e 5.16 compared to normal control(9.0). PEPM at 400 mg/kg produced significant increase in Johnsen's score compared with diabetic rats. But the combination of PEPM (400mg/ kg) with half dose of metformin (25 mg /kg bw) showed a significant increase in the score which was similar to normal control (Table No. 3,figure no.3)

DISCUSSION

Reports suggested that STZ-diabetic wistar albino rats have significantly higher fasting plasma glucose, higher area under curve of an oral glucose tolerance test, in comparison with other strains[22]. So wistar albino rats were used for the diabetic model in this study.

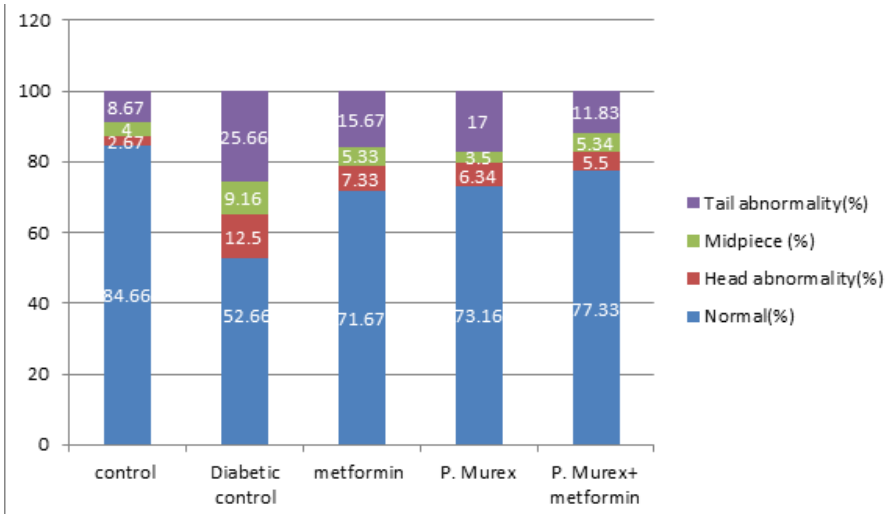

Fig. 1: Effect of drugs on morphological abnormalities of sperms in STZ induced diabetic rats

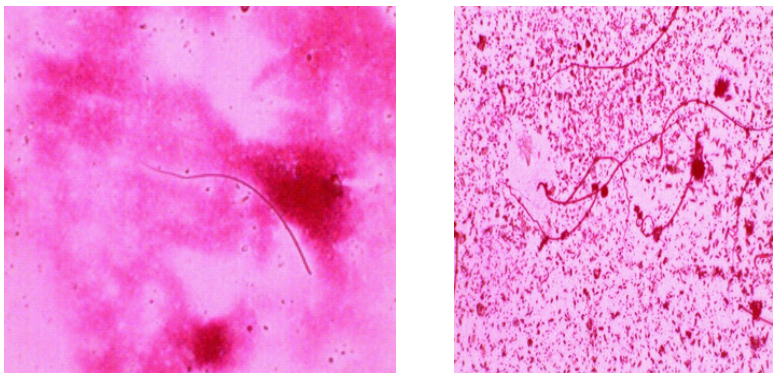

Fig. 2: Sperm Morphology in STZ- T2DM

In our study it was found that the diabetic rats who received PEPM showed significant decreased of the FBG comparing with disease control. This study result is similar to other study which reported the antihyperglycemic effect of *P.murex* plant extract in alloxan induced diabetic models[23] which is due to its free radical scavenging properties. In our study we found a significant decrease in sperm count and motility rate in diabetic rats. This corroborates with other study[18].Other studies explored the effects of hyperglycemia on epididymal sperm quantity, quality in STZ-diabetic male rats where diabetes resulted in diminished sperm counts within the testis and epididymis[5,6].

The percentage of abnormal sperm morphology in STZ induced diabetic rats is found to be significantly higher ($p<0.05$) compared to the normal group indicating germ cell line damage caused by diabetes mellitus (Fig. No. 2 A, B, C). Groups treated with PEPM showed a significant decrease in abnormal sperm percentage compared to diabetic control denoting the restoration of the damage caused in the germ cells. Similar results

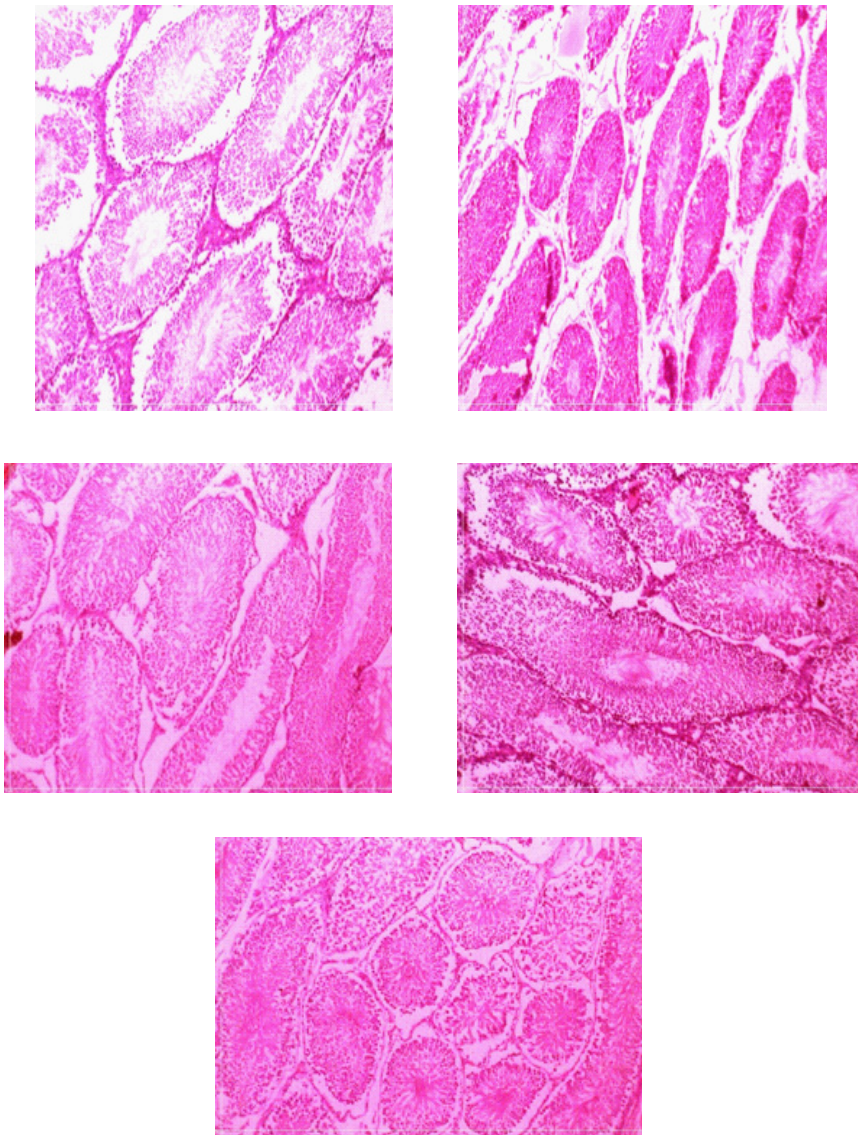

Fig. 3: Effects of Drugs on Histopathology of Testis

were noted by Balamurugan *et al* while studying the effects of petroleum ether extract of *P.murex* against ehanol induced infertility[16]. These morphological abnormalities can be attributed to increased lipid peroxidation, severe oxidative damage to the testis and epididymis in the pathogenesis of diabetes caused by an acute dose of streptozotocin. A study by Shrilatha *et al* has shown these oxidative stress induced morphological changes in the sperms to be persistent and progressive eventually resulting in increased DNA damage and higher number of abnormal sperms[24].

In the present study we observed that streptozotocin induced diabetes mellitus in rats resulted in changes in oxidative stress markers, causing an elevation of malonaldehyde and decrease in superoxide dismutase activity in testicular tissue showing a significant difference than the normal group. *P. murex* treated groups corrected the aberration in antioxidant enzyme levels bringing down MDA levels and increasing SOD activity closer to the normal. This can be attributed to the phytochemicals like tannins and flavonoids like pedaltin and pedalin. This study result is similar to other study, which proved antioxidant activity of methanolic extract of *P. murex* fruits by restoring decreased activity of enzymes superoxide dismutase, catalase etc in CCl4 induced hepatotoxic models in rats[25].

From the assessment of Johnsen's scoring in our study we found that the diabetic rats had a significantly lower score (< 6) compared to control which indicates the presence of testicular damage (Fig No. 3 (A,B) . Similar results were shown by other studies who stated that mean seminiferous tubule diameter and Johnsen's criteria values decreased in diabetic rats.[26,27] The thickness of the seminiferous tubule basement membrane plays an important role in spermatogenesis. PEPM given alone or metformin by itself did not cause a significant increase in Johnsen score. But in the combination group, histopathology of the testes showed protection from testicular injury induced by diabetes (Fig No.3 C,D,E) . There was a significant increase in the score which was similar to normal control.

CONCLUSION

It is concluded from this study, *Pedalium murex* dry seed extract significantly reduced the FBG in STZ induced T2DM and also reversed the altered oxidative stress parameters like testicular MDA level and SOD activity. The testicular damage in terms of low sperm count, low percentage of motile sperm, increase percentage of abnormal sperm and histopathological changes developed were significantly reversed by PEPM and or PEPM with metformin. The naturally derived phytochemicals present in PEPM posseses anti-hyperglycemic, germ-cell protective and antioxidant properties with minimal toxicity might give a direction in which further research can be done in this field.

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