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Antifertility activity of *Cassia siamea* (stem bark) in male albino rats and phytochemical analysis by GC-MS technique

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Abstract
This study was conducted aiming to evaluate the chemical composition of the plant by GC-MS analysis and investigate its antifertility activity. The contraceptive activity was studied in male rats by oral administration of methanolic extract of *Cassia siamea* (stem bark) of two doses (50 and 100 mg/kg b. wt.) over a period of 60 days. After sacrificing the animal blood was collected for hormonal analysis and reproductive organs are dissected for histopathological examination. There was no significant changes were observed in the body weight whereas the weight of the reproductive organs reduced significantly (p<0.01). Sperm motility and sperm density (p<0.01) in epididymis decreased significantly that affected the process of spermatogenesis. The levels of androgens and gonadotropins (FSH, LH and testosterone) decreased significantly (p<0.01) interfering with the spermatogenic activity. The levels of glycogen, sialic acid and protein (p<0.01) also reduced significantly when administrated with the drug. The histopathological findings supported the suppression of fertility capacity in male rats. These results suggested that the *Cassia siamea* (stem bark) methanolic extract possess antifertility activity by suppressing the levels of hormones and other biochemical involved in markers spermatogenesis.

Keywords: Antifertility, androgens, gonadotropins, GC-MS, spermatogenesis

1. Introduction
Population explosion throughout the world is a major problem and its control is an issue of global concern. Hence antifertility agents are seeing introduced at a global level to hamper the reproductive process. Plants are used as therapeutic agents since ancient times which are reported to reduce the fertility rate in male [1]. These agents have many modes of actions to affect fertility like spermicidal activity, sperm motility and density and decrease the hormone levels (GnRH, FSH & LH) etc. [2].
Cassia siamea of belongs to the family Fabaceae and is widely cultivated in Southeast Asia. It is commonly used for the treatment of fever, skin disease, hypertension, insomnia, diabetes and asthma [3-5]. This plant possesses many compounds such as β-sitosterol, Flavonoid, glycosides, anthraquinones, bianthraquinones, alkaloids, kaempferol that are responsible for showing pharmacological properties [6, 7]. The phytochemicals such as β-sitosterol, kaempferol etc posses antifertility activity [8-11]. A survey of the literature revealed that no male and female antifertility activity has so far been carried out on of the stem bark of *Cassia siamea*. The current study deals with the antifertility screening of methanolic extract of stem bark of *Cassia siamea* in male albino rats.

2. Materials and Methods
2.1. Plant collection and preparation of extract
The stem bark of *Cassia siamea* was collected from the campus of University of Rajasthan, Jaipur during whole year. The plant was authenticated by Department of Botany, University of Rajasthan Jaipur. Herbaria are made and their voucher specimen retained in the Department for future references. The shade dried stem bark of the plant was coarsely powdered (100g) and was extracted with methanol, in a soxhlet extractor for 60-72 hr. The extract was concentrated to dryness under reduced pressure and controlled temperature (50-60 ˚C) to yield a brown solid (21g).

2.2. GC-MS Analysis
The methanolic extract of *Cassia siamea* was analysed using GC-MS analyser (TRACE 1300/1310GC with TSQ 8000 Evo MS). The analysis was performed in EI mode (70eV) equipped with in sample injection with splitless mode. The injection volume was 2µL with injection temperature at 250 ˚C with constant flow (0.90 ml/min.) of helium as the carrier gas.

The oven temperature of analyser started at 50 ˚C to 250 ˚C holding for 5 minutes at a rate of 10˚C/minute. Ion source temperature was maintained at 250˚C. The mass spectrum of compounds in sample was obtained by EI mode and detector was set to scan the mass ranging from 50 to 300 amu (atomic mass unit). The resulting data was processed by GC-MS post run analysis software.

The interpretation of data was performed using commercial mass spectral library options including NIST and Wiley libraries. The relative apparent percentage of each compound was calculated by comprising its peak area to the total area and then the spectrum of unknown component was compared with the spectrum of the component in the data base library.

2.3. Antifertility Activity

The male adult wistar rats weighing 150-250 g were used in the experiment and divided into 3 groups consisting of 6 animals in each and intial weights were recorded. The study was conducted after obtaining Institutional animal ethical committee clearance. Group I was the control group while II and III were the treatment groups of methanolic extract of C. siamea with the dose levels of 50 mg/kg body weight and 100 mg/kg body weight respectively. The animals were gavaged orally with the dose levels for 60 days and control animals received similar volume of vehicle (distilled water) 60 days. After the end of the treatment, their final body weights were recorded and were sacrificed under mild ether anaesthesia. Blood was collected through cardiac puncture and serum was obtained by centrifugation for performing various biochemical assays. The reproductive organs Viz., testes, Seminal vesicle, vas deferens were dissected out, weighed and used for tissue biochemistry; sperm motility and density were observed in semen squeezed out from the reproductive organs. The sperm motility was evaluated in percentage and visualised at 400x magnification for sperm count. The no. of sperm cells in semen was viewed under a magnification of 40x, counted and expressed as millions/mm. Testes were further fixed in 10% formalin and processed for paraffin embedding, sections were obtained with a microtome and stained with hematoxylin and eosin stain (H/E) and observed under a light microscope. All the observed data were expressed as mean ±SEM (n=6). The data was analysed by one way ANOVA followed by post hoc test.

3. Results

This study was carried out to test the MeOH extract of *C. siamea* (Stem Bark) in male albino rats. The GC-MS analysis of *C. siamea* methanolic extract (stem bark) was performed and revealed the presence of 17 compounds that could contribute the antifertility property of the plant. The identification of the phytoconstituents was confirmed on the basis of peak area, retention time, molecular weight and molecular formula. The first compound that was identified with retention time of 25.16 (min) was 1-hexyl-2-nitrocyclo hexane and the last compound with retention time of 34.27 (min) was dl-a-tocopherol (Table 1).

Table 1: GC-MS Analysis of *Cassia siamea* (stem bark) crude extract

S. No	Retention time	Compound Name	Molecular Formula	Molecular Weight	Peak Area %
1	26.01	6,11-Eicosadienoic acid, methyl ester	$C_{21}H_{38}O_2$	322	2.68
2	26.12	(9S,10R)-9,10-Epoxy-3Z,6Z-heneicosadiene	$C_{21}H_{38}O$	306	0.51
3	26.92	Diethylene glycol dibenzoate	$C_{18}H_{18}O_5$	314	0.41
4	27.5	Docosanoic acid, methyl ester	$C_{23}H_{46}O_2$	354	0.37
5	27.85	Phthalic acid, di(2-propylpentyl) ester	$C_{24}H_{38}O_4$	390	61.61
6	29.18	Sulfurous acid, cyclohexylmethyl octadecyl ester	$C_{25}H_{50}O_3S$	430	0.22
7	29.54	1,1':2',1":2",1"'-Quaterphenyl	$C_{24}H_{18}$	308	0.61
8	29.89	Tetracosanoic acid, methyl ester	$C_{25}H_{50}O_2$	382	0.4
9	30.96	Squalene	$C_{30}H_{50}$	410	0.59
10	31.67	Eicosane	$C_{20}H_{42}$	282	0.49
11	32.12	Tetradecanoic acid, 10,13-dimethyl-, methyl ester	$C_{17}H_{34}O_2$	270	0.27
12	32.23	Diethyl 1-diphenylphosphinamino-1-phenylmethanephosphonate	$C_{23}H_{27}NO_4P_2$	443	0.61
13	32.73	2-methylhexacosane	$C_{27}H_{56}$	380	0.37
14	32.98	Sulfurous acid, cyclohexylmethyl tetradecyl ester	$C_{21}H_{42}O_3S$	374	0.16
15	33.21	1-Hexyl-2-nitrocyclohexane	$C_{12}H_{23}NO_2$	213	0.13
16	33.33	1-Decene, 3,3,4-trimethyl-	$C_{13}H_{26}$	182	0.26
17	34.27	dl-à-Tocopherol	$C_{29}H_{50}O_2$	430	1.28

There were no significant changes in the final body weight of all groups when compared with initial body weight after 60 days of treatment (Table 2). However, a significant decrease in the weights of reproductive organs i.e. testes, epididymis, seminal vesicle, ventral prostrate were noted in all the treated groups when compared to the control group (Table 2).

Table 2: Effects of Methanolic extract of *C. siamea* (stem bark) on the body and organ weights

	Body Weight (g)		Organs Weight (mg/100g body weight)			
Groups	**Initial**	**Final**	**Testes**	**Epididymides**	**Seminal Vesicle**	**Ventral Prostate**
Group I	169.64±4.41	200.42±3.96	1011.12±30.02	334.99±14.84	704.69±11.26	400.92±8.83
Group II	166.33±4.49	213.95±3.11	894.85±22.31[b]	298.41±6.24[a]	640.50±19.47[a]	366.57±8.26[a]
Group III	176.40±6.73	223.91±8.21	806.35±13.13[c]	277.11±6.72[b]	505.56±14.25[c]	350.22±7.74[b]

Values are expressed as mean ± SEM (n= 6); ns= non-significant.

Levels of significance: [a] $p<0.05$; [b] $p<0.01$; [c] $p<0.001$ compared with group I.

Group I: Control group; Group II: CSME 50mg/kg body weight; Group III: CSME 100mg/kg body weight

Sperm motility in epididymis reduced by 10.46 and 15.70 % in groups II and III respectively when compared the control group (Table 3). A significant reduction of sperm density in testes and cauda epididymis was also noted in group II ($p \leq 0.05$ and $p \leq 0.01$) and group III ($p \leq 0.01$ and $p \leq 0.001$) in comparison to the control group (Table 3).

Concentration of androgenic hormone testosterone in serum decreased significantly in group II ($p \leq 0.05$) and group III ($p \leq 0.01$) when compared to the control group (Table 3). There was also significant reduction in pituitary gonadotropin levels in serum i.e., LH, FSH in comparison to the control group (Table 3).

Table 3: Effects of Methanolic extract of *C. siamea* (stem bark) on sperm dynamics and serum hormonal assay

Groups	Sperm Motility (%)	Sperm Density (millions ml^{-1})		Testosterone	LH	FSH
	Cauda epididymides	Testes	Cauda epididymides	(mg ml^{-1})	(mlU ml^{-1})	(mlU ml^{-1})
Group I	81.90±1.23	6.33±0.28	40.49±1.15	8.08±0.20	4.38±0.24	3.59±0.16
Group II	73.33±1.43^{a}	5.08±0.28^{b}	35.60±1.13 ^{a}	6.77±0.14 ^{a}	3.56±0.16 ^{b}	2.91±0.20 ^{a}
Group III	69.04±3.29 ^{b}	2.54±0.08^{c}	32.76±0.77^{b}	6.43±0.32 ^{b}	2.99±0.09 ^{c}	2.69±0.13 ^{b}

Values are expressed as mean ± SEM (n= 6); ns= non-significant.
Levels of significance: ^{a} $p<0.05$; ^{b} $p<0.01$; ^{c} $p<0.001$ compared with group I.
Group I: Control group; Group II: CSME 50mg/kg body weight; Group III: CSME 100mg/kg body weight

In the treated group II ($p \leq 0.01$) and III ($p \leq 0.001$) cholesterol levels in testes showed highly significant when compared to the control group (Table 4), the glycogen content of testes in group II ($p \leq 0.05$) and group III ($p \leq 0.001$)decreased significantly in comparison to the control group (Table 4). The levels of sialic acid and protein also significantly reduced in group II ($p \leq 0.05$) and group III ($p \leq 0.01$) when compared to the control group (Table 4).

Table 4: Effects of Methanolic extract of *C. siamea* (stem bark) on tissue biochemistry

Groups	Cholesterol (mg/g)	Glycogen (mg/g)	Sialic acid (mg/g)	Protein (mg/g)
	Testes	Testes	Epididymides	Testes
Group I	7.50±0.15	5.46±0.19	4.90±1.14	236.79±3.27
Group II	8.63±0.37^{b}	4.86±0.21^{a}	4.39±0.16^{a}	217.83±4.61^{a}
Group III	11.01±0.13^{c}	4.55±0.13^{b}	4.23±0.08^{b}	189.31±1.97^{b}

Values are expressed as mean ± SEM (n= 6); ns= non-significant.
Levels of significance: ^{a} $p<0.05$; ^{b} $p<0.01$; ^{c} $p<0.001$ compared with group I.
Group I: Control group; Group II: CSME 50mg/kg body weight; Group III: CSME 100mg/kg body weight

The histoarchitecture of testes of control rats showed active spermatogenesis with normal round or oval seminiferous tubules (Figure 1). The histology of group II rats showed more degeneration of spermatogonial cells with incomplete spermatogenic activity when compared to control group (group) whereas the histology of group III showed most degenerated spermatogonial cell lining. The spermatogonial cells and sloughing of degenerated germ cells were observed (Figure 2 & 3).

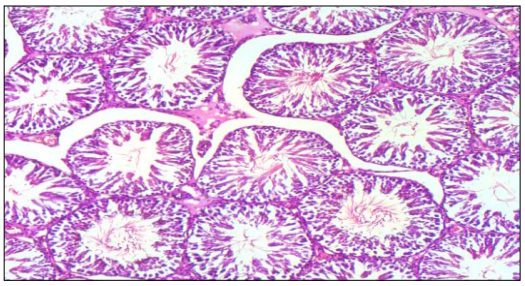

Fig 1: Photomicrograph of rat testes of control group showing normal round seminiferous tubules with complete spermatogenesis

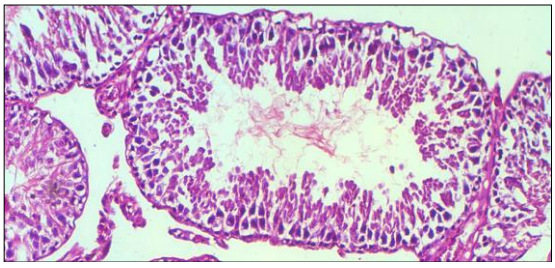

Fig 2: Photomicrograph of rat testes of treated group (group 2) showing degenerated spermatogonial cells and incomplete spermatogenesis

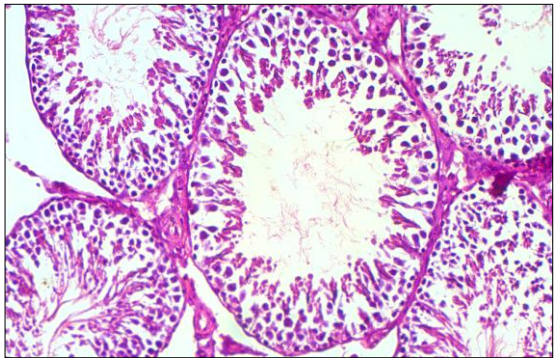

Fig 3: Photomicrograph of rat testes of treated group (group 3) showing sloughing of degenerated germ cells with disturbed spermatogenesis.

Discussion

The present investigation was assessed to evaluate the antifertility activity of methanolic extract of *Cassia siamea* (stem bark) in male albino rats. There was no significant change in the body weight of treated groups whereas the weight of the reproductive organs reduced significantly. This observation attributes to antifertility activity of CSME with significant alteration in androgen levels and sperm dynamics [12]. Sperm motility and sperm density in epididymidis decreased significantly that affects the process of spermatogenesis. This inturn suggests the reduced of testosterone supply to the epididymis affecting spermatogenesis [13]. The process of spermatogenesis and androgen production specifically testosterone are mainly under the control of anterior gonadotropins LH and FSH that are released from anterior pituitary [14]. In the present study the administration of the drug decreased the testosterone levels significantly suggesting

the lowering of LH and FSH levels releasing from anterior pituitary and affecting the spermatogenic activity.

Following the CSME treatment to male rats the testicular cholesterol content increased significantly high. This increase of cholesterol levels attributes the low levels of androgens [15] as cholesterol is a precursor of androgens. The levels of glycogen and sialic acid also reduced significantly when administrated with the drug. Reduced concentration of sialic acid in epididymis reflects low androgen levels [16] affecting the fertility in males by decreasing the metabolism and fertilizing capacity of spermatozoa. The other important parameter that affects the androgen levels is the protein concentration in reproductive organs. The protein content reduced significantly after administration of antiandrogen which was noted by Brooks and Higgins [17] and similar results were observed in the present study. The protein levels decreased significantly after administering the drug in the male rats.

Conclusion

It may be concluded that *Cassia siamea* methanolic extract suppressed the spermatogenesis and interfered with the androgen levels. This interference suggests the antifertility activity of CSME without affecting the normal metabolism. Further studies on active phytoconstituents of the plant posing antifertility activity are in progress.

Declaration of interest statement

We declare that we have no conflict of interest.

Acknowledgement

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