Research Article

Studies on Spermatogenic and Aphrodisiac Potential of Standardized Ayurveda Formulation-*Gokshuradi Gugglu*

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Abstract:

Gokshuradi guggulu (GG) is an official in Ayurvedic Formulary of India. It is indicated as spermatogenic and aphrodisiac, comprised of Tribulus terrestris ,as one of the major ingredients. GG was standardized as per the method described. Acute toxicity studies were performed as per OECD guideline 423 to ensure the absence of toxic effects of GG at higher doses. The formulation was subjected to biological evaluation to assess aphrodisiac and spermatogenic potential, using rats as an experimental animal. The formulation showed significant aphrodisiac activity in male wistar rats as observed in behavioral studies. Biochemical evaluation showed significant increase in serum testosterone level at significance level p<0.001. The histological study provided evidences of enlargement of seminiferous tubule, presence of sertoli cell and Leydig cell, and different stages of spermatogenesis.

Keywords: *Gokshuradi Guggulu*, aphrodisiac,spermatogenic

INTRODUCTION

Infertility is a major concern among 25% of married couples world wide as by Zegers (2009)[1]. Poongothai *et al* (2009) stated that approximately, in such 50% of the cases, the underlying etiology lies in men alone. Hormone supplement therapy and aromatase inhibitors are prescribed to treat male factor infertility. Ikechebelu *et al* (2003) described that normal sexual intercourse in males, the sexual organs and factors relating to erection of the copulatory organ must function normally. The repeated inability of the male to perform this function, at least effectively, or a disorder that interfere with his full sexual response cycle is termed male sexual dysfunction (MSD).Yakubu *et al* (2010) reported that MSD can be caused by physical or psychological stress. The National Health and Social Life Survey performed in USA in 1992 found that 15 % of men lacked sexual interest for several months within the past one year of study. Descriptive epidemiological study by Montgomer (2008)revealed that prevalence of Male Hypo Sexual Drive Disorder (HSDD) was 2.56% among the Indian population. It was noted that, actual prevalence might be higher than this. Most of HSDD cases are treated with Sildenafil or other medicines (anti depressants).This treatment focus on the physiological mechanics to achieve and maintain erection and do little or nothing to enhance the sexual desire or libido of men suffering erectile dysfunction. Current main line therapy of western medicine offers few remedies to restore impaired spermatogenesis and to enhance the suppressed libido in male, but not without major side effects, with arguable efficacy. Shamloul (2010) described numerous products of natural origin which have been studied by modern pharmacology, on the basis of indications obtained from traditional medicine, to evaluate the possible scientific basis for their empirical use. Several such plants and their preparations play an important role in fertility regulation, a fact that has been reported in *Ayurveda*.

Gokshuradi Guggulu (GG) is official in Ayurvedic Formulary of India, indicated as aphrodisiac and spermatogenic. Fruits of *Tribulus terrestris(TT)* are incorporated as major ingredient in GG. Gauthaman

Table.1: Physicochemical standardization of GG

Quality Parameters	Mean±SD(%W/W)	Standard(%W/W)
Total ash value	3.26 ± 1.22	Not more than 5%
Acid insoluble ash	0.62 ± 0.11	Not more than 1%
Water soluble ash	0.88 ± 0.53	--
Water soluble extractives	53.33 ± 6.56	Not less than 46%
Chloroform soluble extractives	3.91 ± 0.5	--
Methanol soluble extractives	10.66 ± 2.51	Not more than 19%
*Test value mean ± SD, n=3		

et al reported that fruits of TT showed aphrodisiac and spermatogenic potential in several independent preclinical and clinical studies conducted, in normal male subjects or male subjects with compromised reproductive system functioning.

MATERIALS AND METHODS

Procurement of formulation

GG was procured from Sunder Pharmacy; GMP certified manufacturing unit associated with J and S *Ayurveda* college, Nadiad (Gujarat, India). GG was manufactured by adopting procedure given in *Ayurvedic* Formulary of India. The formulation was used to evolve physicochemical standard as well as to carry out biological studies without any further modifications.

Physicochemical standardization

GG was used to evolve physical standards e.g. ash value, acid insoluble ash value, water soluble ash value, loss on drying. The procedure for evolving those standards was adopted from compendia.

Phytochemical Screening

Accurately weighed 50 g powdered GG, was subjected to successive solvent extraction using Soxhlet's extraction apparatus .The extracts obtained were subjected to phytochemical screening to detect the presence of various phytoconstituents e.g. alkaloids, flavonoids, saponins, carbohydrates, sterols, terpenoids, glycosides, coumarins, tannins and phenolic compounds.

TLC Fingerprint

Accurately about 40 g powdered GG was weighed and sonicated with chloroform for 45 min. The procedure was repeated twice. Chloroform extract was discarded. The marc was subjected to dry for 15 min at the room temperature and further sonicated with water for 45 min, thrice. Filtrate was collected after each sonication. The collected solvents were filtered, mixed together and evaporated to dryness using rotary vacuum evaporator. The dried extract was dissolved in 10 ml methanol and filtered using whatman filter paper. Resultant solution was used to estimate amount of PD. Standard of PD was purchased from Sigma Aldrich and used for set of experiment without any further purification.

Biological Evaluations

Animals:

The protocols to carry out acute toxicity studies, *in vivo* studies and *in vitro* studies were approved by Institutional Animal Ethics Committee (IAEC) [Reg No. 940/a/06/CPCSEA] constituted as per the norms of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The protocols were approved vide number RPCP/IAEC/2014-2015/R-38r. Healthy male wistar rats of 250 to 350 g were used for the set of experiments. Rats were received from Zydus Research Centre, Ahmedabad India. All animals were housed at 25°C ± 2°C, with relative humidity of 75% ± 5%, under 12 hr light/dark cycle. A basal diet and water was provided *ad* libitum. They were allowed to acclimatize to laboratory conditions for a week before starting the experiment.

Acute toxicity studies

Acute toxicity studies were conducted as per OECD guidelines (OECD 423, 2001). Two groups comprised three animal per group were fasted overnight and weighed. Single dose of GG (2000 mg/kg body wt) was administered with 1% acacia suspension p.o. Control group received 1% acacia solution. The animals were observed continuously for behavioral changes as well as any signs of physiological abnormalities for initial 4 hr and occasionally for next 24 hr. Any mortality during study duration of 14 days was noted. At the end of the studies, blood samples were collected from all animals through retro orbital plexus, under light ether anesthesia. Hemoglobin content, RBC count, differential WBC count and Platelet count were

Table :2 Effect GG on Sexual behavior of male animals

Behavior Parameter	Control	Testosterone	GG250	GG250	GG750	TT
Mount frequency (Number)	3.33±0.42	10.83±0.65**	3.5±0.53	5.75±0.39	6.1±0.67*	4.2±0.33
Intromission frequency (Number)	1.33±0.21	8.17±0.60**	1.2±0.12	1.5±0.24	2.2±0.33	1.5±0.24
Mount latency (seconds)	120.5±6.6	59.67±2.01**	83.25±2.09*	77±3.28**	64.75 ±4.44**	82 ±1.73*
Intromission latency (seconds)	320.8±6.02	228.17±4.08**	273.5±5.08*	248.5±2.15**	232±6.7**	271.25±3.85*
Ejaculatory latency (Seconds)	123.17±4.16	359.67±4.79**	145.6±2.79*	269.75±4.06**	326.5±2.53**	151.75±3.24*
n=6, values are mean±SEM, * P<0.01, ** P<0.001, as compared to respective control						

performed from blood. Serum GOT, serum GPT, serum creatinine, serum bilirubin and total serum protein was estimated. Vital body organs, included, heart, kidney, liver and testes were dissected out, cleansed of adhering tissues and rinsed in normal saline. The dissected organs then preserved in isotonic formalin (1% in 0.9% NaCl) solution. The specimens were used to prepare microscopic slides to study possible histological alterations. The data of hematological studies, biochemical studies as well as histological observation for the test animals were compared with control group animals.

In vivo Studies

In vivo studies were performed by adopting the reported methodology (Vyas et al, 2016). Briefly, animals were divided in six groups, each contained six rats. Group-I served as vehicle control, administered with 1% gum acacia solution, p.o., for 28 days. Group-II served as positive control, administered with 0.5 mg/kg dose of testosterone propionate in arachis oil intramuscularly twice a week for 28 days. Group-III received 250 mg/kg, Group-IV received 500 mg/kg and Group-V received 750 mg/kg GG suspended in 0.3% gum acacia solution p.o for 28 days. Group- VI received 50 mg/kg of body weight of TT fruit powder suspended in acacia suspension p.o.. Female rats were made sexually receptive by administering single subcutaneous dose of 500 µg/ rat of progesterone prior to 48 h as well as single s. c. dose of 5 µg/rat of estradiol benzoate prior to 8 h, of mating experiments for sexual behavior studies performed on 29th day. Male to female animal ratio was maintained 1:1 in the experiment.

a) Biochemical studies

Amount of cholesterol and testosterone after treatment period was determined. Blood was collected from retro orbital plexus from each animal under light ether anesthesia. Serum was separated by centrifugation at 10000 rpm at 4°C and stored at -80°C till further analysis. Serum cholesterol (Span diagnostics, CH1022, India), and testosterone (Cal Biotech, CE1875, USA) was estimated using kits available commercially.

b) Histological studies

Testes of all animals from all the groups were dissected out and preserved in 10% formaldehyde buffer (10 % formaldehyde in PBS) individually, for 24 hours. These preserved specimens were washed off and then stored in 70% alcohol. They were further dehydrated using alcohol series and embedded in paraffin wax. Digital microtome (Leica RM2265) was used to get ribbon of sections (thickness 5µm). Sections were stained with hematoxylin–eosin solution. Stained sections were fixed on glass slides and observed under light microscope Zeiss Axio Lab attached with Zeiss Axiocam 105 camera. Various structural observations like sperm density in seminiferous tubules, shape of seminiferous tubules, density of matured spermatozoa, Epithelial lining of seminiferous tubules, density of Sertoli cells and Leydig cells were analyzed for variations of these vital organs as compared to those of control group was observed.

Total sperm count

Epididymis was dissected out from all the animals on 29th day. Caudal part was cut from dissected epididymis and tubules were dispersed using medium

199 (contained Hank's salts supplemented with 0.5% w/v BSA, pH 7.4) in a 35 mm plastic petri dish labeled with corresponding animal number. Fluid obtained was incubated at 37 °C for 10 minutes to disperse the sperms. The supernatant containing sperm was diluted 20 times with PBS. Total sperm count was measured manually using a haemocytometer

c) Sexual behavior studies

Vyas et al (2016) described the method to conduct sexual behavior studies in rats. As per the method referred ,on 29th day of the experiment, each male rat was placed in a glass chamber (20 cm X 40 cm X 60 cm) having a top lid, individually. Three sides of chamber were covered with black sheet. The rat was kept inside for 10 min to acclimatize with the cage environment. A receptive female was introduced from one side of the glass chamber. The sexual behavior of male rat toward female was observed. Observations for various parameters were made as follows:

Mount frequency (MF): The number of mounts without intromission from the time of introduction of the female until ejaculation,

Intromission frequency (IF): The number of intromissions from the time of introduction of the female until ejaculation

Mount latency (ML): The time interval between the introduction of the female and the first mount by the male,

Intromission latency (IL): The time interval from the time of introduction of the female to the first intromission by the male

Ejaculatory latency (EL): The time interval between the first intromission and ejaculation.

In vitro Studies

Preparation of rat Leydig cells:

Male animals were sacrificed and testes were dissected aseptically. Testicular Leydig cells were isolated by method previously described by Sharp (1982) and Sharma (2006).As per the method described, testes were decapsulated and placed in digestion medium (0.005% trypsin inhibitor, 0.001% DNAase, 0.025% collagenase and 0.1% BSA in medium 199) at 34°C for 20 mins in shaking water bath, set at 90 cycles/min shaking movement. Digestion was terminated by addition of separation buffer (0.071% sodium bicarbonate, 0.21% HEPES, 0.025% trypsin inhibitor and 1% BSA in medium 199). Tubes were capped tightly, inverted few times and kept at 4°C for 10 mins in refrigerator. Seminiferous tubules settled at bottom and supernatant was separated. Clear supernatant was carefully siphoned off and collected from the top with Pasteur pipette. Supernatant was then centrifuged at 1500 g for 10 min at 4°C in cooling centrifuge. The cell pellet obtained at bottom, was collected in centrifuge tubes and kept in incubation medium (0.01% trypsin inhibitor and 1% BSA in medium 199). Purity of isolated Leydig cells was checked using positive staining of 3 β - hydroxysteroid dehydrogenase (HSD). Leydig cell suspension was diluted using incubation medium to set Leydig cell concentration of 2 X 10^6 cells/ml. Viability of cells was checked using trypan blue cell exclusion method.

Effect of GG on testosterone production using rat Leydig cells:

The protocol was adopted as described by Raval et al (2016). Briefly, the tubes with equal volume of suspended isolated rat leydig cells were divided in groups of 1) Blank (incubation medium without isolated Leydig cells), 2) Control (1 ml isolated Leydig cells, diluted up to 2 ml using incubation medium), (concentration of 2 X 10^6cells/ml) were mixed with incubation medium to make final volume up to 2 ml) 3) Positive standard (1 ml isolated Leydig cells were mixed with 0.1 ml of 10, 100, and 1000 µg/ml concentrations of Dehydroepiandrosterone (DHEA) diluted up to 2 ml using incubation medium) 4) Test (1 ml isolated Leydig cells were mixed with 0.1 ml of 10, 100, and 1000 mg/ml concentration of aqueous extract of GG(GGJA) diluted up to 2 ml using incubation medium). The cells were incubated for 3 h under atmosphere of 95% carbon dioxide condition using carbon dioxide incubator at 37°C. The content was centrifuged 1500g for 10 min at 4ºC and supernatant was separated. The supernatant was portioned with 1 ml chloroform, individually. 0.5 ml chloroform layer was separated and subjected to HPTLC analysis to estimate the amount of testosterone. Amount of testosterone was determined quantitatively by adopting methodology reported by Stahl (1969) with incorporation of minor modifications. Briefly 50 µl of chloroform portion containing testosterone, was spotted on silica gel G F254 coated TLC plate. Plates were developed using benzene: ethyl acetate (5:5 v/v) as mobile phase at 27° C ± 5° C. Sample solution was spotted on the plate in form of a narrow band (6 mm X 0.45 mm) using Linomat-V semiautomatic spotter. Quantitative analysis was performed by Scanner 4 using reflectance absorbance mode at 240 nm using deuterium lamp. The data was integrated using win-CATS software. The amount of testosterone present in each sample solution was estimated after determining

the regression equation obtained for the area for each spot represented one concentration amongst set of two fold concentrations of external standards spotted on same TLC plate.

Statistical Analysis

Results are expressed as Mean ± SEM. The statistical significance for difference between the mean of test group and respective control group was determined by one-way Analysis of Variance (ANOVA) followed by Dunnett's test. In all statistical tests, a value of $p \leq 0.05$ was considered significant. All analysis was performed using Microsoft Excel 2010.

RESULTS AND DISCUSSION

The results of studies performed to evolve physical parameters are shown in Table 1. The studies confirmed that the values obtained for the sample of GG received, complied with the set limits (Anonymous 2008). Phytochemical screening showed presence of alkaloid, flavonoids, phenolic, carbohydrate, sterols in different extracts of the formulation.

The samples were spotted in the form of bands of width 8mm with a Camag microlitre syringe on pre-coated silica gel plate $60^{F}254$ (10cm × 10cm) (E.Merck, India) using a Camag Linomat V sample applicator (Switzerland). The Slit dimension was 4mm x 0.30 mm, and scanning speed was 20mm/s. The composition of mobile phase was n-butanol: GAA: methanol: water (5:3:0.1:1.5 v/v/v/v). Linear ascending technique was used for development for TLC plates in a twin trough glass chamber saturated with mobile phase. The chamber was previously saturated with the solvent system for 20 min at room temperature. The length of chromatogram run was 80mm. Subsequent to the development, TLC plates were air dried. The plates were subjected to post chromatographic derivatization using Anisaldehyde – Sulphuric Acid reagent involving post heating in laboratory oven at 110 °C for 10 min.(Fig .1)

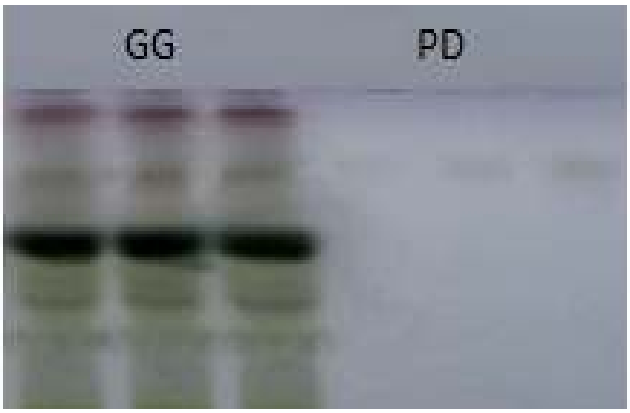

Fig.1:TLC fingerprinting of PD

No toxic symptoms or mortality observed in any of the test animals subjected to toxicity studies. All the animals lived up to 14 days after the administration of GG at single dose level of 2000 mg/kg body weight. The animals did not show any changes in the general appearance during the observation period. Morphological characteristics (fur, skin, eyes, and nose) were also unchanged. The treated animals did not show any tremors, convulsion, salivation, diarrhea, lethargy, or unusual behaviors such as self-mutilation, walking backward. There was no significant difference in body weights before and after the study period. The studies showed that there was no statistically significant alteration in mean value of selected hematological parameters as well as other biochemical parameters, confirmed no toxic effects of GG on haemopoietic system as well as on liver and kidney functions. Histological studies of heart, kidney, and liver showed no notable alteration in histo architect. The studies confirmed that GG at dose of 2000 mg/kg body weight might be safe dose that animals could tolerate. The studies also ruled out the detrimental effects of GG on vital organ histoarchitect as well as functioning of those organs.

Effect of GG on spermatogenesis was evaluated by performing behavioral, biochemical studies, comparative histological studies and determining the sperm concentration in fluid collected from cauda region of vas duct.

The result showed dose dependent increase in serum testosterone and serum cholesterol concentration in GG treated animals. The similar rise in serum concentration of testosterone was also observed in TT treated animals, served as positive control while cholesterol remained unaltered in testosterone treated animals (Fig 2 and Fig 3). Concentration of spermatozoa in fluid collected from caudal region of epididymis was increased in GG treated animals as well as animals of positive control group (Fig 4).

Histological studies of testes, as shown in Fig 5 showed presence of seminiferous tubules, which were circular in shape and contained with optimal number of spermatozoa present in lumen for control animals (Fig.5a). The sertoli cells present in epithelium of seminiferous tubule were not stained excessively as well as appeared as less dense mass. Photomicrograph of histo architect of testis from animals treated with Testosterone, GG 500, GG 750 and GG750, figure 5b, 5d and 5e respectively, showed increased diameter of seminiferous tubules. The lumen of seminiferous tubule was seen often, oval in shape and contained

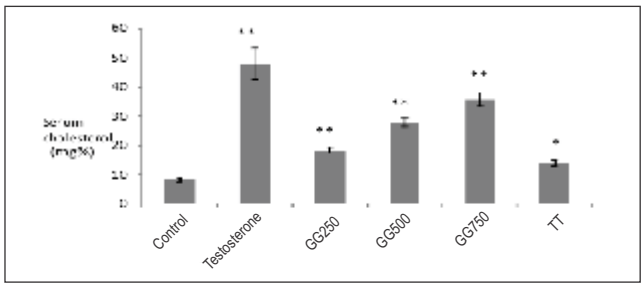

Fig.2 :Serum cholesterol, Results are expressed as mean ±SEM, n=3, *p<0.05,p<0.01**

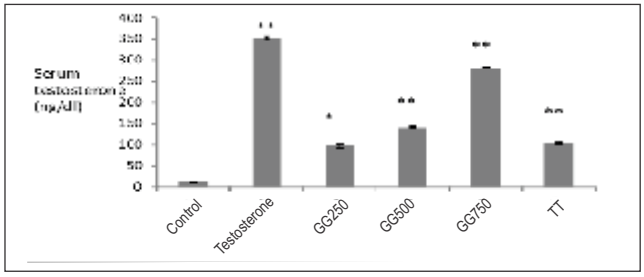

Fig.3:Serum testosterone, Results are expressed as mean ±SEM,n=3,*P<0.01,P<0.001**

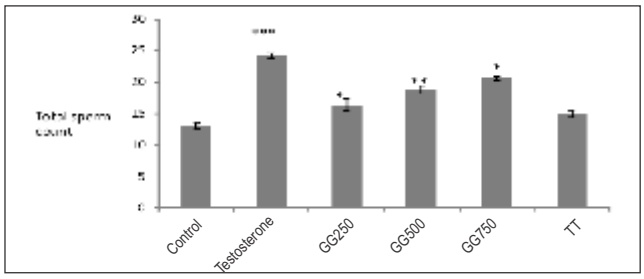

Fig. 4: Total sperm count ,Results are expressed as mean ±SEM, n=5, significance *P<0.05, p<0.01, ***p<0.001**

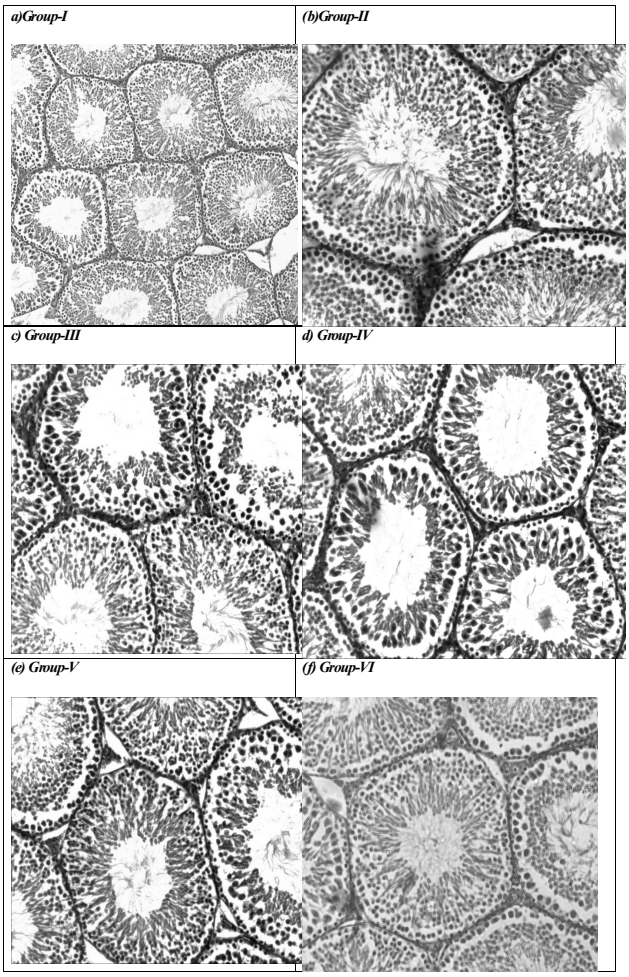

Fig.5: Histopathology of testes of male animals (Rats).

excessive spermatozoa present in lumen. The flagella of mature sperms were seen in the lumen of the tubules. The Sertoli cells and other germinal cells were easily visible in lining of epithelium of seminiferous tubules. Sertoli cells appeared as pyramidal in shape and resting on the basement membrane.

Testosterone is mostly produced by Leydig cell in testes through biosynthesis termed as steroidogenesis. Cholesterol is precursor for sterodogenesis. Increased concentration of serum testosterone and cholesterol in treated animal suggested the possibility of stimulation to steroidogenesis in Leydig cells.

Testosterone should be present for optimal growth of the newly formed spermatogonia and subsequent conversion to spermatids. Mclachlan et al (2002) proved that, testosterone affected formation of ectoplasmic specialization (ES) in sertoli cells. ES was found responsible for attachment of spermatids as well as elongated spermatids, which was essential for their attachment to serotoli cells. It was proved that, at low concentration of testosterone, process of spermatogenesis was proceeding at a reduced rate, primarily due to compromised attachment of spermatids with sertoli cell processes. The increased concentration of testicular testosterone in this set of experiments thus, influenced the process of spermatogenesis, which in turn, might be attributed to spermatogenic potential of GG.

The studies performed on isolated rat Leydig cells, showed that, the culture media obtained from cells incubated with GGJA contained inflated concentration of testosterone as compared to that of control group. Testosterone is synthesized from DHEA under the influence of enzyme, 3-beta- hydroxysteroid dehydrogenase (3-b-HSD). Leydig cells treated with DHEA showed higher concentration of testosterone in culture media, confirmed that, the cells were treated appropriately and could be able to convert precursors in testosterone throughout the duration of experiment. Aqueous extract of TT also showed increased concentration of testosterone in culture media, suggested that, apart from other ingredients plants present in the formulation, TT might possessed action on leydig cells too(Fig.6). *In vitro* studies, in association

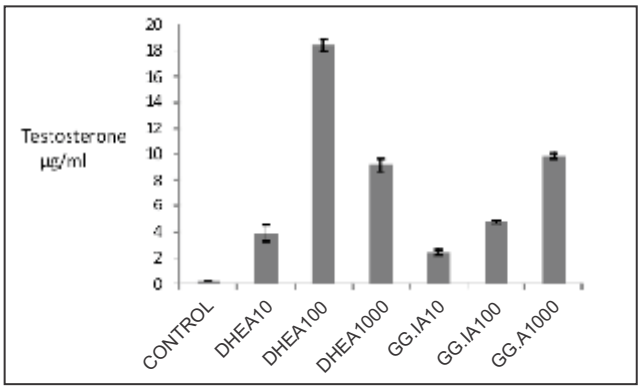

Fig.6: Testosterone secretion by Leydig cell, n=3,*p<0.05, ** p<0.01,*p<0.001 as compared to control**

with *in vivo* studies thus, confirmed that, GG might stimulate biosynthesis of testosterone, through direct action on Leydig cells as per the McLachlan et al (1996).

Aphrodisiac effect of GG was evaluated by performing sexual behavioral studies using rats. The effect of GG on selected parameters of sexual behavior is shown in Fig. 7 and 8. The studies revealed that animals treated with GG exhibited improved mounting frequency and intromission frequency in dose dependent manner, as compared to control group animals. The mount latency period and intromission latency period are decreased while ejaculatory latency period was increased in all treated animals as compared to control group animals. Testosterone and

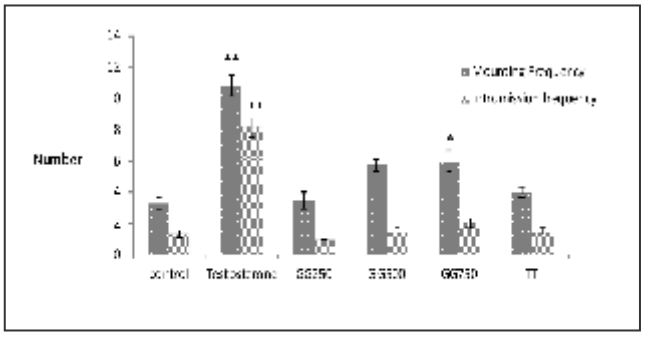

Fig 7: Effect GG on Sexual behavior of male animals n=6, values are mean±SEM, * P<0.01, ** P<0.001, as compared to respective control

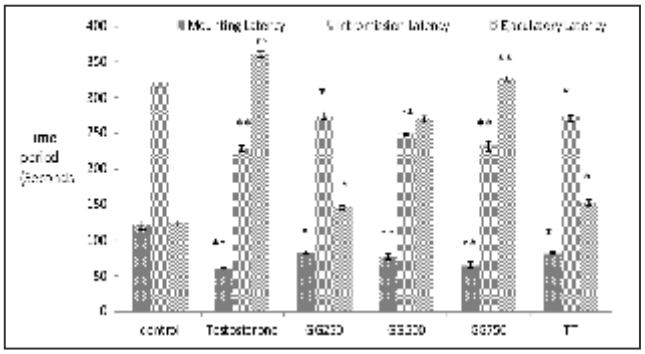

Fig 8: Effect GG on Sexual behavior of male animals n=6, values are mean±SEM, * P<0.01, ** P<0.001, as compared to respective control

TT treated animals showed comparable results for all the parameters as of GG treated animals.

Chauhan et al (2014) described that the drugs affecting sexuality is believed to act, either on the central nervous system (brain) and/or on the peripheral nervous system (indirect). Though, sexual activity is broadly divided in two distinct components, sexual arousal and sexual function, the drugs act on peripheral nerves may not affect arousal directly, but may affect sexual function only. Drugs possess direct action, involves chemical alteration of neurons, which governs sexual arousal or functions. Testosterone increases nitric oxide synthase in the medial preoptic area (MPOA). Du and Hull (1999) observed that, administration of testosterone to castrated male rats increased the number of NO synthase- labeled neurons in MPOA, indicating an increase in NO synthesis. NO is capable of stimulating dopamine release in the MPOA, which in turn stimulates penile erection. They proposed that, the mechanism described above, might constitute a basis for explaining the role of testosterone in sexual arousal.

The set of studies thus showed that GG could stimulate serum testosterone concentration in dose dependant manner. This might be directly linked with alteration in sexual behavior of animals during the studies.The results of these studies were in accordance of other studies confirming testosterone supplementation enhanced male libido. It was thus prooposed that, aphrodisiac potential of GG, as evaluated in rats, might be attributed to ability the formulation to stimulate testosterone from Leydig cells, which in turn, acted in CNS and altered animal behavior and promoted sexual arousal, which could be represented especially through, mount latency and mount frequency as TT showed, similar activity performed by Gauthman et al (2003) in animals in past as well as in present set of experiments, it might be considered as one of the ingredients responsible for spermatogenic and aphrodisiac potential of GG.

CONCLUSION

The studies confirmed spermatogenic and aphrodisiac potential of standardized *Ayurveda* formulation GG. As GG and TT could act on isolated rat leydig cells and stimulated testosterone concentration in culture media, it was concluded that GG might act on leydig cell in testis. The studies also suggested that, GG could stimulate serum testosterone concentration with concurrent rise in serum cholesterol content in rats. This might be attributed to action of GG on Leydig

cells. It was thus, proposed that GG stimulated synthesis of testosterone from precursors in Leydig cells *in - vitro* and *in- vivo*. Resulting increase in serum testosterone concentration might be responsible for spermatogenic and aphrodisiac action of GG.

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