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Antigonadal effect induced by hydro-methanolic extract of leaf of *Aegle marmelos* in male rat: Effect of hCG co-administration

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Antigonadal activity of hydro-methanolic (40:60) extract of leaf of *Aegle marmelos* was noted here by using male albino rat as model animal. This evaluation was performed by sperm count, sperm viability, sperm motility, plasma testosterone level; androgenic key enzyme activities in testis which were decreased significantly after the leaf extract treatment in respect to control. The levels of thiobarbituric acid reactive substance (TBARS) and catalase activities of testis and sperm pellet along with the count of different generation of germ cells at stage VII of seminiferous epithelial cycle were noted. Leaf extract treatment at the dose of 50 mg/ml /100 g body weight / day for 28 days to normal rat resulted a significant inhibition in the levels of these parameters in respect to control except the TBARS levels which were increased significantly. This extract has no toxicity in general that has been noted by plasma GOT and GPT activities. Co-treatment of hCG in the *A. marmelos* leaf extract treated rat resulted significant recovery of all these parameters towards the control level. This study focus the effect of this extract through hypothalamico-hypophysial axis for the induction of antigonadal activity that may lead to develop an herbal male contraceptive in future.

Key words: *Aegle marmelos*, hCG, androgenic key enzyme, TBARS, GOT, GPT.

INTRODUCTION

Over population is one of the serious problems in the developing countries like India and that would be increased about 9.2 billion by the year 2050 (United Nations, 2007). The census of 2005 showed that the growth rate of population in India during the previous ten years was about 1.5% (Basu and Maertens, 2007). In each year around 18 million people are adding to our total population. This increment imposes an extra burden on the community and it is also one of the leading causes of poverty and pollution in developing countries. For this purpose, the World Health Organization (WHO) has constituted a population control programme, which includes studies having traditional medical practices (WHO, 2004). From the advancement of reproductive biomedicine, several hormonal contraceptive pills have been developed but no one is free from different side effects. At present

global attempt has been taken to search out the effect of herbal product for contraceptive purposes (Chakraborti et al., 2003). Few herbal contraceptives have been developed but the potentiality of these contraceptives is very minimal and the mode of action is beyond of our knowledge, till now. Epidemiological studies indicate that combined oral contraceptives increase risks of cerebral thrombosis (Lidegaard, 2002), increase serum level of triglyceride, HDL and cholesterol (Kasture et al., 2000; Kang et al, 2000) and increase family mortality due to cardiovascular diseases (Iacobellis, 2004) as well as malignant tumors in any organs, poor glucose tolerance or diabetes, nausea, abdominal pain, headache, obesity and menstrual changes (Chakraborti et al., 2003; Sanersak et al., 2006). At present the potent spermicidal agent with a formulation of non oxynol-9 are available in the market have the tendency to create inflammation and genital ulceration which may increase the risk of HIV-I infection on repeated use (Fichorova et al., 2001; CDC, 2002). In USA some agencies =have conducted different

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USA some agencies have cosurvey works and reported that the community health affected remarkably by these contraceptive agents. The World Health Organization has set up a task force on different herbal medicinal plant research for the control of population and fertility with an aim to find out a new orally active non-steroidal contraceptive compound. For this purpose, a global attempt has been taken to search out the effect of herbal products with contraceptive efficacy from the last part of 20th century.

In India, Ayurvedic physicians usually use different indigenous plants as contraceptive for a long time. Over a decade, a large number of medicinal plants have been studied namely *Acalypha indica*, *Carica papaya*, *Praneem vici*, *Alstonia scholans* etc (Talwer et al., 1995; Hiremath et al., 1999; Raji et al., 2005; Gupta et al., 2002) for their anti-fertility properties and this has been subjected to different laboratory testing. They act either by preventing implantation or by suppressing spermatogenesis (Verma et al., 2002; Breuner, 2005). On the other hand, gossypol, a seed extract of Chinese cotton plant *Gossipium herbaceum* was studied extensively but due to its side effects namely hypokalaemia, the programme was abandoned (Qian and Wang, 1984), we have also published the anti-testicular activities of some plants parts in vivo as well as *in vitro* (Ghosh et al., 2002; Paul et al., 2006). Again, an exact human health friendly alternative of hormonal pill is not yet available, mainly due to lack of systematic research work in the field. At present worldwide attempt has been taken to develop herbal contraceptive medicine. There is a reputation of herbal folk medicine in this aspect. In West Bengal tribal people use the leaf extract of *Aegle marmelos* (Bael) for contraception of male. But the present investigation made so far for the laboratory testing about the efficacy of this leaf extract for male contraception. The primary information about the plant for contraceptive function was reported in indigenous system of medicine (Bhattacharya, 1982).

The plant *A. marmelos* is a tree type and grows in almost all the districts of West Bengal, India. This is under the family of Rutaceae. The height of the plant is about 20 - 30 feet, that is, 7 - 10 m. The leaves of the plant are pinnately compound. Almost all the parts of these tree- roots, leaves, fruits and barks have a medicinal value. Drink, which prepared from Bael fruit prevent bad breaths and can be used for the treatment of bleeding gums. Eating Bael fruits is highly recommended for diarrhea, dysentery, asthma (Duke and Jo, 2002).

There is no remarkable report about the therapeutic utility of the leaves of Bael. We have reported that aqueous extract of the powder of leaf of this plant has a significant male contraceptive efficacy (Das et al., 2006).

MATERIALS AND METHODS

Preparation of hydro-methanolic extract of *A. marmelos*

Fresh leaves of *A. marmelos* were collected from local areas, these

were dried in an incubator for 2 days at 40°C, crushed in an electric grinder and then powder was formed. Out of this powder, 50 g was suspended in 250 ml of hydro-methanol (2:3) and kept at incubator at 37°C for 36 h. The slurry was stirred intermittently for 2 h and left overnight. The mixture was then filtered and filtrate was dried by low pressure and residue was collected. When required the residue was suspended in olive oil in a fixed dose and used for treatment.

Selection of animal and animal care

Twenty-four Wistar strain male albino rats (Chakraborty Traders, Midnapore, India) of 3 month of age weighing about 150 ± 10 g were taken for this experiment. Animals were acclimated for a period of 15 days in our laboratory condition prior to the experiment. Rats were housed in colony cage at an ambient temperature of 25 ± 2°C with 12 h light: 12 h dark cycle. Rats have free access to standard food and water *ad libitum*.

Initial body weight of all the animals was noted at the starting day of the experiment. For this experiment the animals were divided into three groups as per schedule given below.

Group I (Control group)

Animals of these groups were treated with 1 ml of olive oil /100 g body weight/day and this treatment was done by forceful feeding using the gavage method.

Group II (Extract treated group)

Each animal of this group were subjected to forceful feeding of hydro- methanolic extract (2:3) of the leaf of *A. marmelos* at a dose of 50 mg/ml /100 g body weight / day for 28 days.

Group III (hCG co-administered group)

Animals of these groups were subjected to treatment by the extract as group II by forceful feeding using gavage method. Leaf extract treated animals were co-administered with intramuscular injection of hCG at the dose of 25 µg/100 g body weight/day in 0.25 ml distilled water after treatment with *A. marmelos* leaf extract 50 mg/ml/100 g body weight for 28 days after 4 h of leaf extract treatment.

After completion of the duration of the treatment, animals were subjected to light ether anesthesia after taking the body weights of each animals on the 29th day. Blood was collected from the dorsal aorta using heparinized syringe and plasma was separated by centrifugation of 2,000 r.p.m. for 10 min. By decapitation animals were sacrificed one after another and both the testes, epididymides were dissected out. Sperm cells were collected from the pair of cauda epididymis. One testis of each animal was collected for histological study and for this purpose it was fixed in Bouin's fluid followed by different methodology to produce a histological section.

Sperm pellet was prepared from the washed of the epididymis and this pellet along with another testis was stored at -20°C for biochemical studies. Plasma was also stored at -20°C until it was used for hormone assay.

Sperm viability

Sperm viability was performed by the eosin nigrosin staining (WHO, 1999). One drop of epididymal fluid was mixed with two drops of 1% eosin Y. After 30 s, three drops of 10% nigrosin were added and mixed well. A smear was made by placing a drop of mixture on a clean glass slide and allowed to air dry. The prepared slide was examined using a phase contrast microscope. Pink-stained dead

sperm were differentiated from unstained live sperm, and these numbers were counted.

Estimation of testicular $\Delta^5,3\beta$ -hydroxysteroid dehydrogenase ($\Delta^5,3\beta$ HSD) and 17β -hydroxysteroid dehydrogenase (17β -HSD) activities

Testicular $\Delta^5,3\beta$ HSD activity was measured spectrophotometrically (Talalay, 1962). One testis from each animal was homogenized carefully at 4°C in 20% spectroscopic grade glycerol containing 5 mM potassium phosphate and 1 mM EDTA at a tissue concentration 100 mg/ml homogenizing mixture. This mixture was centrifuged at 10,000 x g for 30 min at 4°C. The supernatant (1 ml) was mixed with 100 μ M sodium pyrophosphate buffer (pH 8.9), 40% ethanol containing 30 μ g dehydro epiandrosterone and 960 μ l of 25 mg% BSA, bringing the incubation mixture to a total of 3 ml. Enzyme activity was measured after addition of 0.5 μ M NAD to the tissue supernatant mixture in a spectrophotometer cuvette at 340 nm against a blank (without NAD). One unit of enzyme activity was the amount causing a change in absorbance of 0.001/min at 340 nm.

For the measurement of testicular 17β -HSD activity, another 1 ml supernatant from the same homogenizing mixture was added to 440 μ M sodium pyrophosphate buffer (pH 10.2), 40 μ l ethanol containing 0.3 μ M testosterone and 960 μ l of 25 mg% BSA, bringing the incubation mixture to a total of 3 ml. Enzyme activity was measured according to the method of Jarabak et al. (1962) after addition of 1.1 μ M NAD to the tissue supernatant mixture in a spectrophotometer at 340 nm against a blank (without NAD). One unit of enzyme activity was equivalent to a change in absorbency of 0.001/min at 340 nm.

Plasma testosterone

Plasma testosterone level was measured using a solid phase-conjugated assay testosterone kit supplied by Lilac Medicare (P) Ltd, Mumbai, India (Srivastava, 2001). The optical densities of standard and unknown samples were measured using 480 nm selective filter and 650 nm as differentiating filter. The intra assay variation was 5.2%. No inter assay variation occurred as all samples were assayed at the same time.

Testicular cholesterol level

Testicular cholesterol was estimated according to method of Plummer (1995). In a centrifuge tube, 10 ml of alcohol – acetone mixture and 0.2 ml tissue homogenate was taken. Tubes were immersed in a boiling water bath till the solvent begins to boil. They were then cooled to room temperature and centrifuged. The supernatant was collected and allowed to evaporate to complete dryness. The residue was dissolved in 2 ml of chloroform. Series of cholesterol standards were prepared. In the test tube marked as blank, 2 ml of chloroform was taken. In each tube of sample, standard and blank, 2 ml of acetic anhydride sulfuric acid mixture was added and mixed thoroughly. All the tubes were placed in a dark place at room temperature for 15 min. Reading was noted at 680 nm. From standard curve, concentration of cholesterol in unknown sample was calculated.

Biochemical assay of catalase in testis and sperm pellet

The activity of catalase of the above mentioned tissues were measured biochemically (Beers and Sizer, 1952). For the evaluation of catalase activities, testis and sperm pellet of each animal were homogenized separately in 0.05 M Tris-HCl buffer solution (pH 7.0)

at the tissue concentration of 50 mg/ml. These homogenate were centrifuged at 10,000 g at 4°C for 10 min. In spectrophotometric cuvette, 0.5 ml of 0.00035 M H₂O₂ and 2.5 ml of distilled water were mixed and reading of absorbance was noted at 240 nm. Supernatant of testicular sample were added at a volume of 40 μ l and the subsequent six readings were noted at 30 s interval.

Estimation of thiobarbituric acid reactive substance in testis and sperm pellet

Testis and sperm pellet were homogenized separately at the tissue concentration of 50 mg/ml in 0.1 M of ice-cold phosphate buffer (pH 7.4) and the homogenates were centrifuged at 10,000 g at 4°C for 5 min individually. Each supernatant was used for the estimation of TBARS and CD. For the measurement of TBARS, the homogenate mixture of 0.5 ml was mixed with 0.5 ml of normal saline (0.9 g% NaCl) and 2 ml of TBA-TCA mixture (0.392 g thiobarbituric acid in 75 ml of 0.25 N HCl with 15 g trichloroacetic acid). The volume of the mixture was made up to 100 ml by 95% ethanol) and boiled at 100°C for 10 min. This mixture was then cooled at room temperature and centrifuged at 4000 g for 10 min. The whole supernatant was taken in spectrophotometer cuvette and read at 535 nm (Okhawa et al., 1979).

Quantification of sperm cells at stage VII in seminiferous epithelial cycle

The hematoxylin–eosin stained slides were scanned under light microscope. Quantification of different generation of germ cell at stage VII was performed according to the method of Leblond and Clermont (Leblond and Clermont, 1952). The cells present in this stage are spermatogonia-A (ASg), preleptotene spermatocyte (pLSc), midpachytene spermatocyte (mPSc), step 7 spermatid (7Sd) and 19 spermatid (19Sd). The different nuclei of the germ cells (except step 19 spermatids, which can not be precisely counted) were counted at 20 round tubular cross-sections in each rat. All the nuclear count of germ cells were corrected for differences in nuclear diameter by the formulae of Abercrombie (Abercrombie, 1947), True count = (Crude count x section thickness) / (section thickness + diameter of germ cell), and tubular shrinkage by the Sertoli cell correction factor (Clermont and Morgentaler, 1955).

Estimation of plasma glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) activities

Plasma GOT and GPT activities were measured according to the kit method supplied by Reekon Diagnostics PVT. LTD, Baroda India and the activities of these enzymes were measured according to standard protocol (Henry et al., 1960).

RESULTS

Body weight

Body growths of all the animals were assessed from the recording of initial and final body weight of each animal of all the three groups. It has been recorded that there was no change of body weight in initial and final among all the groups (Table 1).

Epididymal sperm count, sperm viability and motility

Epididymal sperm count, viability and motility were de-

Table 1. Effect of hydro-methanolic extract of leaf of *A. marmelos* followed by hCG co-administration on body weight, number different generation of germ cells in albino rat.

Group	Body weight (gm)		Germ cell count at stage VII			
	Initial	Final	ASg	pLSc	mPSc	7Sd
Control	150±5.0 ^a	172.0±4.0 ^a	1.6±0.02 ^a	20.52±0.31 ^a	25.62±1.1 ^a	65.91±1.8 ^a
<i>A. marmelos</i> reated	155±4.0 ^a	169.0±5.0 ^a	0.8±0.03 ^b	14.32±0.42 ^b	17.32±1.2 ^b	41.32±1.2 ^b
<i>A. marmelos</i> + hCG	148±5.0 ^a	171.0±5.0 ^a	1.20±0.02 ^c	18.51±0.30 ^c	21.52±1.0 ^c	52.32±2.1 ^c

Data are expressed as Mean ± SEM, n = 8. Different superscripts (a, b, c) in each vertical column significantly differ from each other (p > 0.05).

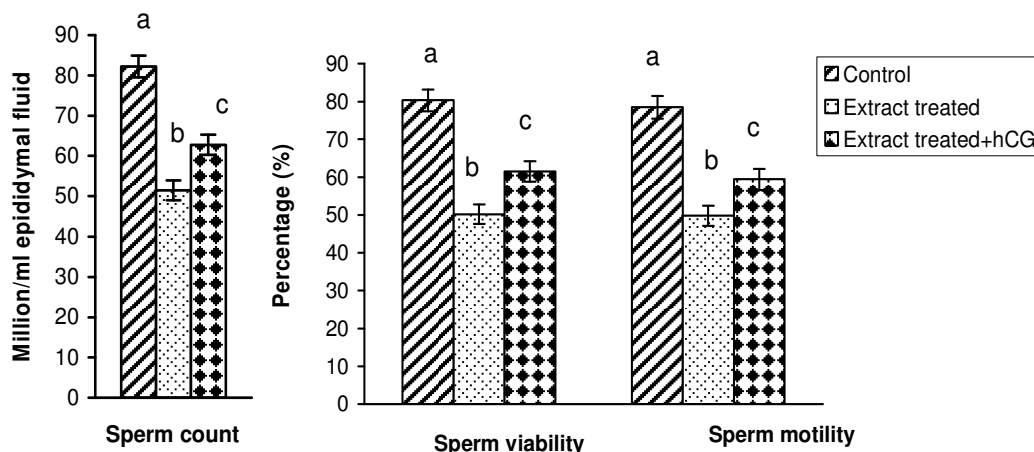


Figure 1. Treatment of hydro-methanolic extract of leaf of *A. marmelos* followed by hCG co-administration on sperm count, viability and motility in male albino rat. Data expressed as Mean ± SEM, n = 8, Bars with different superscripts (a, b, c) significantly differ from each other (p < 0.05).

creased significantly in the extract treated group in comparison to the corresponding control group, that is, group I (Figure 1). Sperm count was increased significantly in respect to the extract treated group after co-administration of hCG in group III (Figure 1).

Testicular Δ^5 , 3β - HSD and 17β - HSD activities

Activities of testicular Δ^5 , 3β - HSD and 17β - HSD were decreased significantly in the extract treated group, that is, group II in respect to the control group, that is, group I (Figure 2). After hCG co-administration, that is, group III, the activities of the two enzymes were significantly recovered towards the control level (Figure 2).

Plasma testosterone

Plasma levels of testosterone were significantly decreased in the extract treated group, that is, group II in comparison to the control group, that is, group I (Figure 3). It has been indicated that the plasma levels of testosterone was increased significantly after hCG co-administered group, that is, group III when comparison made with group II (Figure 3).

Testicular cholesterol level

Quantity of testicular cholesterol was increased in extract treated groups, that is, group II in respect to the control groups, that is, group I (Figure 3). After the hCG co-administration testicular cholesterol level was recovered towards the control level significantly (Figure 3).

Catalase activities in testis and sperm pellet

Catalase activities in testis and sperm pellet were decreased significantly in extract treated group in compare to control group. Significant recovery was noted in both these tissues after co-administration of extract with hCG injection (Figure 4).

Thiobarbituric acid reactive substance level in testis and sperm pellet

Thiobarbituric acid reactive substance levels in above mentioned tissue sample were noted in all the above groups of this experiment. Levels of these parameter were increased significantly in all the above mentioned tissue samples in the extract treated group, that is, group II in respect to control, that is, group I (Figure 5). The

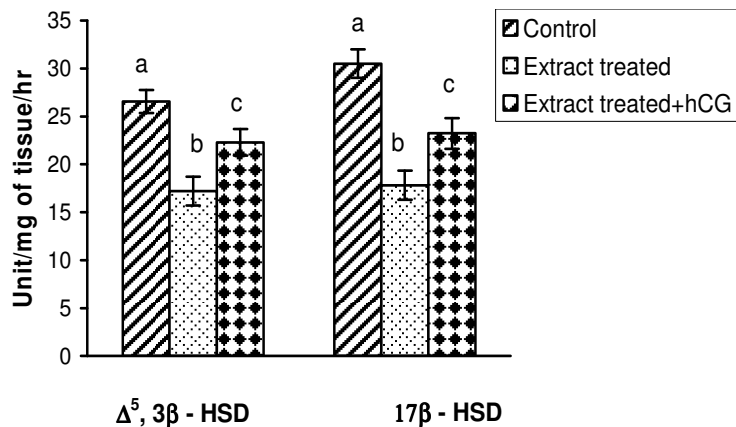


Figure 2. Changes in testicular $\Delta^5, 3\beta$ - HSD and 17 β - HSD activities in hydro-methanolic extract of *A. marmelos* treatment followed by hCG co-administration in albino rat. Data were expressed as Mean \pm SEM, n = 8, Bars with different superscripts (a, b, c) significantly differ from each other (p < 0.05).

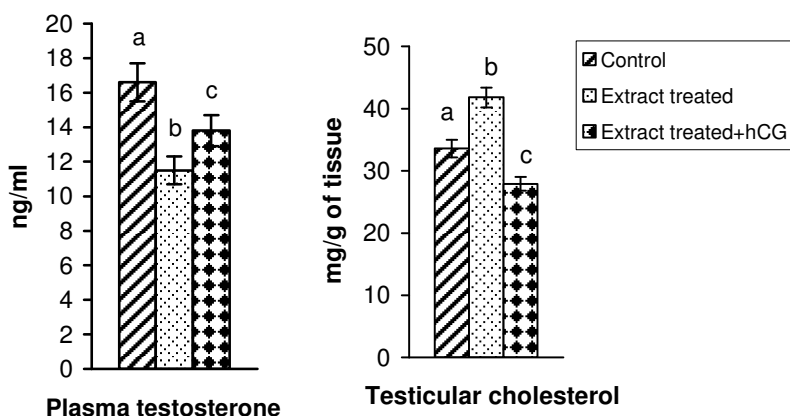


Figure 3. Effect of hydro-methanolic extract of leaf of *A. marmelos* treatment followed by hCG co-administration on plasma testosterone and testicular cholesterol levels in albino rat. Data are expressed as Mean \pm SEM, n = 8, Bars with different superscripts (a, b, c) significantly differ from each other (p < 0.05).

above mentioned parameters were resettled towards the control group in hCG co-administered group, that is, group III.

Quantification of spermatogenesis

Quantitative analysis of hormone sensitive germ cells at stage VII of seminiferous epithelium cycle revealed that the number of ASg, pLSc, mPSc and 7Sd were decreased significantly in extract treated groups in comparison to control group, that is, group I (Plate 1). But after hCG co-administration the number of the above mentioned germ cells was restored towards the control level.

Plasma glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) activities

Plasma GOT and GPT activities in extract treated groups as well as hCG co-administered group did not show any significant change in comparison to control.(Figure 6)

DISCUSSION

From the study it has been stated that the hydro-methanolic extract of the leaf of *A. marmelos* resulted a significant inhibition on testicular activity with an effective dose of 50 mg /100 g body wt/day with a minimum duration of 28 days. This dose has been standardized and

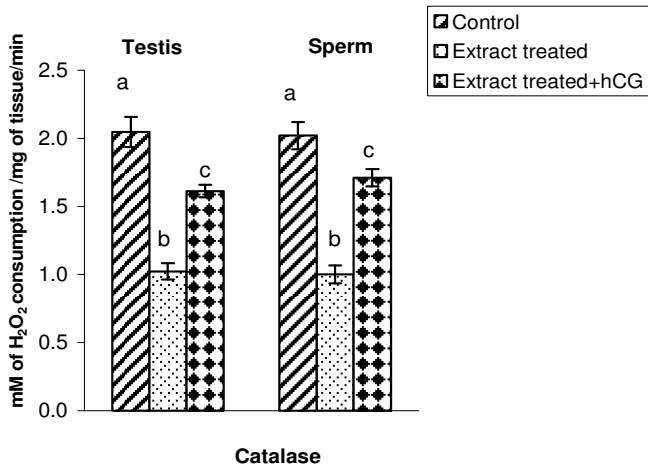


Figure 4. Changes in the activities of catalase in testis and sperm pellet in hydro-methanolic extract of *A. marmelos* treatment followed by hCG co-administered albino rat. Data are expressed as Mean \pm SEM, n = 8, Bars with different superscripts (a, b, c) significantly differ from each other (p < 0.05).

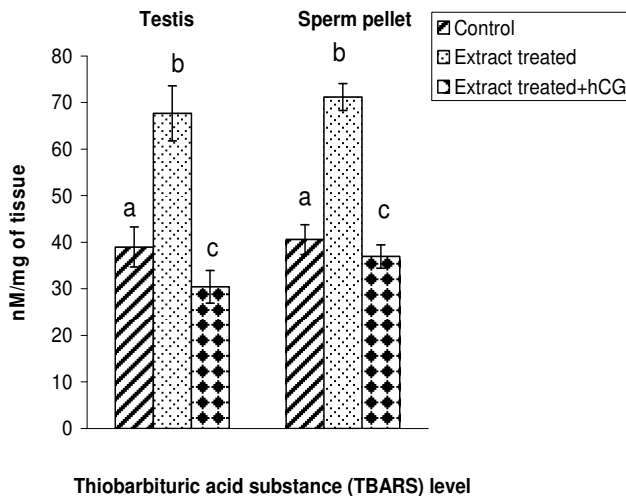


Figure 5. Effect of hydro-methanolic extract of *A. marmelos* treated followed by hCG co-administration on thiobarbituric acid reactive substance levels in testis and sperm pellet in albino rat. Data are expressed as Mean \pm SEM, n = 8, Bars with different superscripts (a, b, c) significantly differ from each other (p < 0.05).

and published in our previous report (Das et al., 2006). To find out the mode of action of this extract for induction of anti-testicular activities, the hCG co-administration in extract treated rat was performed with special emphasis whether this drug has any inhibitory effect on hypothalamico-hypophyseal axis for gonadotrophin secretion in connection to regulation of testicular activities.

Testicular androgenic key enzymes activities, that is, Δ^5 , 3β -HSD and 17β -HSD activities (Mallick et al., 2007) were decreased significantly after extract treatment,

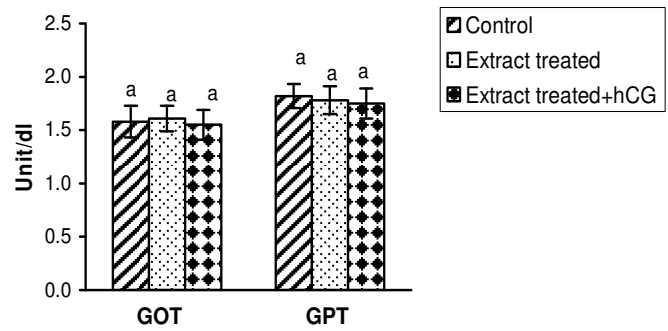


Figure 6. Effect of hydro-methanolic extract of *A. marmelos* treated followed by hCG co-administration on plasma glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) activities in albino rat. Data are expressed as Mean \pm SEM, n = 8, Bars with same superscript (a) did not differ from each other significantly (p < 0.05).

which is consistent with the results of our previous study (Das et al., 2006). After hCG co-administration, the activities of the androgenic key enzymes were recovered significantly but not to the level of control. This has been indicated here that this significant recovery may be due to the effect of hCG as hCG acts as LH which is the regulator of androgenic key enzymes activities (Hedger and Kretser, 2000; McLachlan et al., 2002a). So, this significant protection is due to the effect of hCG. But after hCG co-administration at standard dose, the activities of the androgenic key enzymes were not resettled to the control level. This indicates the possibility of direct effect of this extract on testicular tissue besides its effects on hypophysis. This direct effect further supported here from plasma testosterone level, where it was noted that after hCG co-administration in the extract treated rat, the plasma testosterone level was recovered significantly but not resettled to the control level.

From the biochemical assay of the testicular cholesterol it has been noted that after treatment with the extract of leaf of *A. marmelos* there was a maximum elevation in testicular cholesterol level that may be due to the diminution of androgenesis (Das, 2003). After hCG co-administration there was a significant diminution in the testicular cholesterol level than the extract treated group as hCG utilized cholesterol for androgenesis (Das, 2003). This result further supports the effects of this extract on pituitary for such antagonadal activity.

From the analysis of spermatokinetics, the same type of results was observed in the field of quantitative study of spermatogenesis after the treatment of hydro-methanolic extract of leaf of *A. marmelos*. A significant diminution was noted in the number of A₅g, mPSc, pLSc, and 7Sd, the different generation of germ cell at stage VII of seminiferous cycle. As these germ cells are hormone sensitive (de Franca et al., 1994; McLachlan et al., 2002b) as well as this is the mid point of spermatogenesis process (Segatelli et al., 2004) so, this count focused

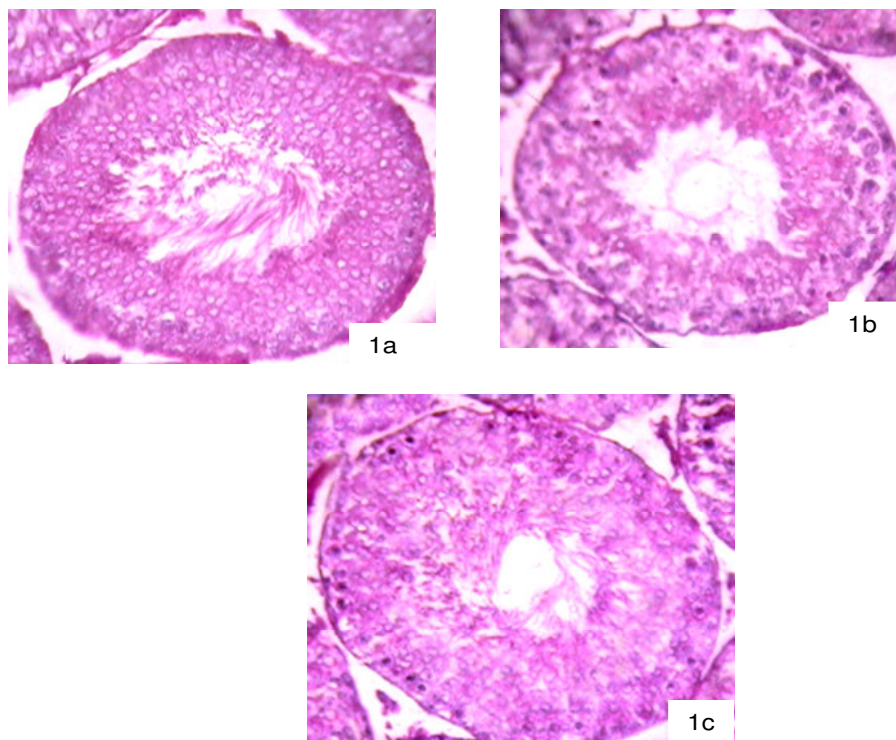


Plate 1. Representative microphotographs of seminiferous epithelial cycle at stage VII of spermatogenesis a. Focus the different generation of germ cells at stage VII of spermatogenic cycle in control group. b. Diminution in the number of different generation of germ cells at stage VII after the treatment of leaf extract of *A. marmelos* in rat. c. Significant recovery in the count of different generation of germ cells at stage VII after hCG treatment in leaf extract of *A. marmelos* treatment in rat: Heamatoxylin –eosin stain, 400 X.

the inhibitory effect of this extract on spermatogenesis. After hCG co-administration, the number of these germ cells was increased significantly but not to the control level which focused the possibility of some direct effect of this extract on germ cell for this purpose.

By measuring the activities of catalase, the important antioxidant enzyme as well as the TBARS level, product of free radical (DeJong et al., 2007; Das et al., 2006), the oxidative stress in testis and sperm pellet were assessed which focus the direct effect of extract of the leaf of *A. marmelos*. It has been noted that after the extract treatment the activity of catalase, was decreased along with the elevation in testicular TBARS level in comparison to the control. There was a significant elevation in the activity of catalase after hCG co-administration in extract treated rat, which may be due to significant recovery of plasma testosterone as testosterone is a remarkable antioxidant (Ahlbom et al., 2001). The levels of testicular catalase and TBARS were not restored to control level, which focus the direct effect of this extract on testicular tissue for such purpose. This hydro-methanolic extract of leaf of *A. marmelos* has no general toxicity at the applied dose that has been proved by the monitoring of GOT and GPT activities of plasma as these are the indicators of general toxicity (Ghosh, 2001).

From the above results two hypothesis may be proposed about the mode of action of extract for induction of antigonadal activities. One way is through hypothalamico-hypophyseal tract as hCG co-administration can able to protect the antitesticular activities at a significant level but not totally. Another way is its direct effect for oxidative stress generation on testicular tissue as well as on mature sperm. Due to its direct action, hCG co-administration becomes unable to protect the concern parameters totally.

Results of this experiment conclude that the hydro-methanolic extract of leaf of *A. marmelos* has the potentiality for the induction of antitesticular activities by inhibiting pituitary gonadotropin secretion. Besides its site of action of hypothesis, the extract has some direct effect on testicular tissue and sperm cell for induction of oxidative injury that may suppress the testicular steroidogenic and gametogenic activities. The actual ingredient present in this extract responsible for such action is not known and it would be cleaned from future work in this line.

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