Chapter -3

3. EVALUATION OF BIOLOGICAL ACTIVITY OF THE SELECTED PLANTS

This chapter deals with the evaluation of the roots of *E. echinatus* and aerial parts of *T. glaberrima*. Both the plants are sold in market under the trade name 'Brahmadandi' as aphrodisiac, generating a controversy as to which of these possess the reported activity being a correct source or both may serve as individual source of Brahmadandi. Since ample scientific data are unavailable to support the usage of Brahmadandi as aphrodisiac, it was thought initially to screen methanol extract of roots of *E. echinatus* and aerial parts of *T. glaberrima* on reproductive system of male rats.

3.1. Preparation of the total methanol extract of the selected plants

Cold maceration method was followed to prepare the total methanol extract. Coarse powder of the roots of *E. echinatus* (1 kg) and aerial parts of *T. glaberrima* (500 g) were macerated separately with sufficient methanol (95% v/v) for six hours. The contents were shaken at an interval of 30 minutes for the first six hours and then set aside for a period of 18 hours after which the extract was filtered. Two more similar extractions were carried out using the same marc with reduced volume of solvent. Filtrates collected in each extraction were then combined and concentrated on a Rotary Vacuum Evaporator (BUCHI Rotavapor R200) till a concentrated liquid is obtained which was then dried under vacuum. The vacuum dried methanol extract was subjected to chemical tests (see section 3.3.2) to determine the presence of various phytoconstituents.

3.2. Animals

Wistar albino rats of either sex (90 days old), weighing between 210–300 g were obtained from M/s Zydus-Cadilla Research Centre Ahmedabad, India. Animals were housed at a temperature of 24-28 °C with a relative humidity of 45-55% and 12/12 hours light/dark cycle. Males were placed individually and females in groups with free access to food (Amrut Laboratory animal feed, Sangli, INDIA) and water *ad libitum*. Before beginning the experiment

the local committee of ethics on animal experimentation approved all experimental procedures (No: 404/01/a/CPCSEA).

3.3. Acute toxicity studies

Healthy adult albino rats of either sex, 90 days old, weighing between 210–300 g were divided into four groups (n = 6), they were starved overnight and then fed with increasing doses (250, 500, 1000 and 2000 mg/kg body weight) of the methanol extract of the *E. echinatus* and *T. glaberrima*. The animals were observed up to 14 days for toxicity and any mortality. Finally $1/10^{th}$ of the highest tolerable dose was taken for further studies (Ghosh, 1984).

3.4. Preparation of male rats for sexual behavior study

Before experimental testing all the animals were trained for sexual experience. To provide sexual experience each male rat was allowed 30 minutes exposure (after an acclimatization period of 5 minutes) to a female rat (used as mating stimulus) in behavioral oestrous, for several times. Only the males were considered sexually active if they attempted to mount the female, when it was introduced into the cage. Sexually active males, showing ejaculation latency shorter than 15 minutes in at least the last three sessions, were selected and considered sexually experienced (Carro-Juarez et al., 2004).

3.5. Preparation of female rats for sexual behavior study

Female rats were housed in groups in cages with free access to food and water *ad libitum*. Female receptivity was induced by bringing them in oestrous phase by the sequential subcutaneous administration of ethinyl estradiol (10 µg/kg body weight) and hydroxy progesterone caproate (1.5 mg/kg body weight) 48 hours and 6 hours, respectively, before the copulatory studies (Ramachandran et al., 2004; Gauthaman et al., 2002).

3.6. Experimental protocol

Sexually active male rats were randomly divided into four groups (Group 1 – 4) of six animals each. The animals were treated with extracts and standard as per the schedule mentioned below (Table 3.1). L-dopa was used as standard in the studies (Taglimonte et al., 1974; Angrist & Gershon, 1976; Ananthakumar et al., 1994). Following parameters were studied to evaluate the activity:

- ✓ Observation of the behavior parameters (Carro-Juarez et al., 2004; Ananthakumar et al., 1994; Ageel et al., 1994).

  Coupulatory sex behavior

  Mounting frequency

  Mounting latency

  Intromission frequency

  Intromission latency

  Post ejaculatory interval

  Orientational behavior

  Licking

  Anogenital sniffing

  Genital grooming

  Non genital grooming

  Climbing
- ✓ Observation of the body weight and weight of reproductive organs
- ✓ Determination of epididymal sperm count
- ✓ Circulatory levels of testosterone
- ✓ Studies on testicular histomorphology
- ✓ Estimation of antioxidant enzymes in testicular homogenate
- ✓ In vitro study of extracts for radical scavenging properties

Table 3.1. Treatment schedule for animals

Groups (n = 6)	**Treatment schedule**
Group 1	Normal control; received 1 ml of 1% solution of sodium carboxy methyl cellulose (SCMC) in water, orally, for 28 days.
Group 2	Treated with 200 mg/kg body weight of methanol extract of the roots of *E. echinatus* in 1% solution of SCMC, orally, for 28 days.
Group 3	Treated with 200 mg/kg body weight of methanol extract of the aerial parts of *T. glaberrima* in 1% solution of SCMC, orally, for 28 days.
Group 4	Positive control; received with L-dopa (100 mg/kg body weight) in 1% solution of SCMC, orally, for 28 days.

3.6.1. Protocol for testing the sexual behavior parameters

All the sexual behavior tests were conducted 2 hours after the onset of darkness. Drugs were administered one hour before commencement of the experiment. Males were introduced into a rectangular chamber (14" x 14") and a 5 min adaptation period was allowed. Thereafter each male rat is paired with two stimulus receptive females and sexual behavior was recorded along a period of 1 hour. The experiment was conducted in a silent room under dim red light, any jerking movement of the mating area was avoided to enable the rats to chase each other and clearing of the mating area was done after each trial, as the urine trails left by one rat might alter the sexual behavior of the other rat (Carro-Juarez et al., 2004; Ananthakumar et al., 1994; Ageel et al., 1994).

The sexual behavior parameters analyzed were mounting latency (ML), time from introduction of the female until the first mount; intromission latency (IL), time from introduction of the female until the first mount with pelvic thrusting and vaginal penetration (intromission); mounting frequency (MF), number of mounts observed in 60 minutes; intromission frequency (IF) number of intromissions observed in 60 minutes and post ejaculatory interval (PEI), time from ejaculation until the next intromission. Latency data were expressed in seconds as mean ± S.E.M. and the number of mounts and intromissions as median numbers.

3.6.2. Effect on body weight and weight of reproductive organs

All the treated and control animals were weighed and the change in their body weights were recorded. At the end of treatment the animals were sacrificed by cervical dislocation method and the sexual organs (testis, vas deferens, seminal vesicles and epididymis) were dissected out carefully, freed from adhering tissues, washed in ice cold saline, blotted and weighed to the nearest 0.1 mg on a single pan electronic balance (Precisa 205 ASCS). The weights of the organs were expressed as mg/100 g of body weight.

3.6.3. Epididymal sperm count

Reagents:

Dilution fluid containing sodium bicarbonate (5%) and neutral formalin (1%), in distilled water.

Method: Measure 10 ml of dilution fluid into a clean test tube (15 ml). To this 0.5 ml of seminal fluid collected from epididymus was added. This yields a 1:20 dilution, Neubauer's chamber were filled with diluted seminal fluid, and left on a bench for two minutes so as to allow the immobilized sperms to settle down. The numbers of sperms were counted in the four corner squares covering four sq mm under the low power objective (Mukherjee, 1997).

Sperm count is calculated using the below mentioned formula and was expressed in terms of per cubic centimeter (ml).

Sperm count = (Sperm count X 20)/ (4 X 0.1)

3.6.4. Estimation of serum testosterone

Serum levels of testosterone were assayed by a competitive immunoassay using direct chemiluminescent technology on an autoanalyser (ADIVA Centaur) at Endocrine Laboratory and Invitro Allergy Testing Centre, Ahmedabad, India.

Reagents:

Reagents were obtained as ready to use pack from ADIVA Centaur (ADIVA Centaur TSTO Ready Pack, Primary reagent and ADIVA Centaur TSTO^{REL} Ready pack, ancillary reagent). The ready packs contain the following reagents.

Lite reagent (2.5 ml): containing acridinium ester labeled testosterone in buffered saline with preservatives.

Solid phase (15 ml): containing polyclonal rabbit anti testosterone antibody bound to monoclonal mouse anti rabbit antibody covalently coupled to paramagnetic particles in buffered saline with sodium azide and preservatives.

Probe wash (10 ml): containing buffered saline with sodium azide and preservatives.
Releasing agent (5 ml): containing steroid realizing agent in buffered saline with sodium azide and preservatives.

Principle: The ADIVA centaur testosterone assay is a competitive immunoassay using direct chemiluminescent technology. Testosterone in the test sample competes with the acridinium ester labeled testosterone in the lite reagent for limited amount polyclonal rabbit anti-testosterone antibody bound to monoclonal mouse anti-rabbit antibody, which is coupled to paramagnetic particles in the solid phase. The assay uses testosterone releasing agent to release bound testosterone from the endogenous binding proteins in the sample.

Sample preparation: Blood samples were collected simultaneously during sacrification of animals; serum samples were separated and stored according to procedures given by National committee for Clinical Laboratory Standards (NCCLS) [Anonymous, 1999].

Method:
The system automatically performs the following steps.

1. Dispense 15 µl of sample and 50 µl of releasing agent into a cuvette.
2. Washes the reagent probe with 100 µl of probe wash, if necessary
3. Dispenses 50 µl of lite reagent and 300 µl of solid phase and incubates for 5 min at 37 °C
4. Separates aspirates and washes the cuvettes with reagent water.
5. Dispenses 300 µl each of acid reagent and base reagent to initiate the chemiluminescent reaction
6. Reports results according to the selected option, as described in the system operating instructions or in the online help system.

3.6.5. Testicular histomorphology

Testis from each group was excised quickly during the dissection of animals and fixed in 10% buffered neutral formalin. Ultra thin sections of the testicular tissue were cut and stained with Hematoxylin and Eosin (at Baroda Clinical Laboratory, Vadodara, INDIA). Histological examinations included the mean semniferous tubular diameter (STD) and germinal epithelial cell thickness (GECT). Three slides from upper, lower and mid portions of the testis were prepared and evaluated. The mean STD and GECT were determined in 20 semniferous tubules of each section using a projection microscope and the values were expressed in terms of 'μm' (micro meters). Microphotographs were made using Olympus BX 40 microscope attached with Olympus DP12 digital camera [Ulusoy et al., 2004).

3.6.6. Determination of antioxidant enzyme activity in testicular homogenate

3.6.6.1. Preparation of testicular homogenate

One testis from each animal was used for measuring the activity of antioxidant enzymes. The testis were minced and a 10% homogenate was prepared using Tris-hydrochloride buffer (10 mM; pH 7.4) which was then centrifuged at 6000 rpm for 20 minutes under cold condition (SIGMA 3K30 cooling centrifuge). The clear supernatant was used to determine the activity of the antioxidant enzymes viz., superoxide dismutase (SOD) and catalase.

3.6.6.2. Super oxide dismutase

Superoxide dismutase was estimated using the method developed by Misra and Fridovich (1972).

Reagents:

Carbonate Buffer (0.05 M, pH 10.2): Dissolve 1.68 g of $NaHCO_3$ and 2.2 g of Na_2CO_3 in 50 ml of distilled water and make up the volume to 100 ml with distilled water.

Ethylene di-amine tetra acetic acid (EDTA) solution (0.49 M): Dissolve 182 mg of EDTA in 20 ml of distilled water and make up the volume to 100 ml with distilled water.

Epinephrine solution (3 mM): Dissolve 0.99 mg of epinephrine hydrochloride in 10 ml of distilled water.

Method: Tissue homogenate (0.5 ml) was diluted with distilled water (0.5 ml) and was treated with ice cold ethanol (0.25 ml) and ice-cold chloroform (0.15 ml). The contents were mixed well and centrifuged at 2000 rpm for 5-10 minutes. To the supernatant (0.5 ml) add 1.5 ml of carbonate buffer and 0.5 ml of EDTA. The reaction was initiated by the addition of 0.4 ml of epinephrine and the change in optical density/minute was measured at 480 nm against reagent blank.

SOD activity was expressed as units/mg protein. Change in optical density per minute at 50% inhibition of epinephrine to adrenochrome transition by the enzyme is taken as the enzyme unit.

3.6.6.3. Catalase

It was estimated by the method of Aebi (1983).

Reagents:

Phosphate Buffer (0.01 M, pH 7.0):

(a) Dissolve 1.36 g of potassium dihydrogen orthophosphate (KH_2PO_4) in distilled water and make up the volume to 1000 ml with distilled water.

(b) Dissolve 0.4 g of sodium hydroxide (NaOH) in distilled water and make up the volume to 1000 ml with distilled water.

50 ml of solution (a) was placed in 200 ml volumetric flask. To this 29.1 ml of solution (b) was added and finally the volume was made up to 200 ml with distilled water.

Hydrogen Peroxide (30 mmol/l): 0.34 ml of 30% hydrogen peroxide was diluted with phosphate buffer to 100 ml.

Method: The homogenate was diluted to 20 times with phosphate buffer (0.4 ml to 8 ml). To the 2 ml of diluted homogenate 1 ml of hydrogen peroxide was added to initiate the reaction. Blank was prepared by mixing 2 ml of homogenate with 1ml of phosphate buffer. The decrease in absorbance was measured at 240 nm. Catalase activity was expressed as micro moles (µmoles) of H_2O_2 consumed/minute/mg of protein.

3.6.6.4. Determination of total proteins

The protein content of tissue homogenate was measured by the method of Lowry et al (1951).

Reagents:

Sodium hydroxide (0.1M): 4 g of sodium hydroxide was dissolved in 400 ml of distilled water and the final volume was made up to 1000 ml with distilled water.

Lowry C reagent

a) Copper sulphate in sodium potassium tartarate (1% w/v): Dissolve 1 g of copper sulphate in 1% solution of sodium potassium tartarate (prepared by dissolving 1gm of sodium potassium tartarate in 100 ml of distilled water).
b) Sodium carbonate in 0.1M sodium hydroxide (2% w/v): Dissolve 2 g of sodium carbonate in 100 ml of 0.1M sodium hydroxide.

Mix 2 ml of solution (a) with 100 ml of solution (b) just before use.

Standard protein (Bovine serum albumin): 20 mg of bovine serum albumin (Loba Chemie) was dissolved in 80 ml of distilled water and few drops of sodium hydroxide were added to aid complete dissolution of bovine serum albumin and to avoid frothing. Final volume was made up to 100 ml with distilled water and stored overnight in a refrigerator.

Folin Ciocalteu reagent: Folin Ciocalteu reagent (Qualigens fine chemicals) was diluted with distilled water in the ratio of 1:1 (1ml of Folin Ciocalteu reagent was mixed with 1 ml of distilled water).

Method: Testicular homogenate (0.1 ml) was mixed with 0.8 ml of 0.1 M sodium hydroxide and 5 ml of Lowry C reagent; the solution was allowed to stand for 15 minutes. Then 0.5 ml of Folin Ciocalteu reagent was added and the contents were mixed well on a vortex mixer. Colour developed was measured at 640 nm against reagent blank containing distilled water instead of sample.

Different concentrations (40-200 μg) of standard protein (Bovine serum albumin) were taken and processed as above for standard graph. The values were expressed as mg of protein/gm of wet tissue.

3.6.7. In vitro study of extracts for radical scavenging properties

Free radical scavenging properties of methanol extract of the roots of *E. echinatus* and aerial parts of *T. glaberrima* were examined *in vitro* using different radicals such as radical scavenging activity by DPPH reduction (DPPH assay), superoxide radical scavenging activity in riboflavin/light/NBT system and reducing power assay.

3.6.7.1. DPPH assay

Principle: DPPH (1, 1-diphenyl-1-picrylhydrazyl) radical assay was used as a quick and reliable parameter to assess the *in vitro* antioxidant activity of plant extracts. Antiradical activity was measured by a decrease in absorbance at 516 nm of a methanol solution of colored DPPH brought about by the test samples. (DPPH) a purple colored stable free radical is reduced into the yellow colored diphenyl picryl hydrazine (Vani et al., 1997; Blois, 1958).

Reagents: DPPH stock solution: 7.88 mg of DPPH was dissolved in 100 ml of methanol.

Method: The test medium was included with 200 µM of DPPH solution along with different concentrations (50, 100, 150, 200 and 250 µg) of samples in 3 ml methanol. Decrease in the absorbance was noted after 15 minutes at 516 nm against methanol as blank. Quercetin was used as positive control.

The obtained data was used to determine the concentration of the sample required to scavenge 50% of the DPPH free radicals (IC_{50}). The percent inhibition was plotted against the concentration and the IC_{50} was obtained. A lower IC_{50} denotes a more potent antioxidant. The results were expressed as the mean ± SEM of three replicates.

3.6.7.2. Assay for superoxide radical scavenging activity

Principle: The assay was based on the capacity of the drug to inhibit formazan formation by scavenging the superoxide radicals generated in

riboflavin-light- nitro blue tetrazolium (NBT) system (Beauchamp and Fridovich, 1971).

Reagents:

Phosphate buffer (0.2 M, pH 7.6)

(a) 2.74 g of potassium dihydrogen orthophosphate (KH_2PO_4) was dissolved in 60 ml of distilled water. The volume was then made up to 100 ml with distilled water.

(b) 7.23 g of di-sodium hydrogen orthophosphate (Na_2HPO_4) was dissolved in 60 ml of distilled water. The volume was then made up to 100 ml with distilled water.

15 ml of (a) was mixed with 85 ml of (b), adjust the pH to 7.6 if necessary.

Riboflavin: 5 mg of riboflavin was dissolved in 25 ml of phosphate buffer.

EDTA (12 mM): 402 mg of EDTA was dissolved in 5 ml of phosphate buffer. The volume was then made up to 10 ml with phosphate buffer.

Nitro blue tetrazolium (NBT) (0.1%): 5 mg of NBT was dissolved in 2 ml of buffer. The volume was then made up to 5 ml with phosphate buffer.

Method: The reaction mixture contained 2.5 ml buffer, 100 µl riboflavin solution, 200 µl of EDTA solution, 100 µl of diluted methanol extract of drug and 100 µl of NBT solution, added in the mentioned sequence. Reaction was started by illuminating the reaction mixture with different concentrations of sample extract in UV light for 5 minutes. Immediately after illumination the absorbance was measured at 590 nm. Quercetin was used as positive control. The percent inhibition by sample exposure was determined against methanol as blank.

The obtained data was used to determine the concentration of the sample required to scavenge 50% of the superoxide radicals (IC_{50}) as mentioned above.

3.6.7.3. Determination of reducing power

Principle: The measurement of reductive ability was based on Fe^{3+} to Fe^{2+} transformation in the presence of sample.

Reagents:

Phosphate buffer (0.2M, pH 6.6)

(a) 2.72 g of potassium dihydrogen orthophosphate (KH_2PO_4) was dissolved in 60 ml of distilled water. The volume was then made up to 100 ml with distilled water.

(b) 800 mg of sodium hydroxide (NaOH) was dissolved in 60 ml of distilled water. The volume was then made up to 100 ml with distilled water.

50 ml of (a) was mixed with 16.4 ml of (b). The volume was then made up to 200 ml with distilled water. Adjust the pH to 6.6 if necessary.

Potassium ferricyanide (1%): Accurately weighed 1 g of potassium ferricyanide was dissolved in distilled water and volume was made up to 100 ml.

Trichloroacetic acid (10%): Accurately weighed 10 g of trichloroacetic acid was dissolved in distilled water and volume was made up to 100 ml.

Ferric chloride (0.1%): Accurately weighed 100 mg of ferric chloride was dissolved in distilled water and volume was made up to 100 ml.

Method: The reducing power was determined according to the method of Oyaizu (1986). Samples were mixed with 5 ml phosphate buffer and 5 ml potassium ferricyanide, the mixture was then incubated at 50 °C for 20 minutes, 5 ml trichloroacetic acid was added an the mixture was centrifuged at 4000 rev./ min. The upper 5 ml solution was then mixed with 5 ml distilled water and 1 ml ferric chloride. The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

3.7. Data analysis

To determine statistical significant differences among treatment groups, the data were analyzed by using One-way ANOVA followed by Bonferroni test (GraphPad InStat version 3.05). Values of $p < 0.05$ were considered as statistically significant.

3.8. Results

Percentage yield of the total extract of *E. echinatus* and *T. glaberrima* were found to be 8.83% w/w and 14% w/w respectively. The results of acute toxicity studies showed that, methanol extract of *E. echinatus* and *T. glaberrima*, when administered orally at the maximum dose of 2000 mg/kg body weight, did not produce any signs of toxicity and mortality.

3.8.1. Effect of extracts on sexual behavior of the rats

In the first part of the present study, copulatory behavior was examined in sexually experienced male rats that were sub acutely treated with 200 mg/kg body weight of the methanol extract of the roots of *E. echinatus* and 200 mg/kg body weight of the methanol extract of aerial parts of *T. glaberrima* and the results were compared with control (vehicle treated) and standard (L-dopa).

Sexual behavior evaluation did not show any significant difference in latency values, mounting and intromission frequencies and post ejaculatory intervals of animals treated with *E. echinatus* compared to the animals in control group (Table 3.2a and Table 3.2b; Figures 3.1 – 3.5).

Methanol extract of *T. glaberrima* at the dose of 200 mg/kg body weight, showed a significant increase in mounting frequency (MF) and intromission frequency (IF) and caused a significant reduction in mounting latency (ML), intromission latency (IL) and post ejaculatory interval (PEI) compared to the animals in control group. All these effects were observed on 7th, 14th, 21st and 28th day. Mounting frequency, observed with the animals treated with *T. glaberrima*, was found to be significantly higher than that observed with the animals treated with L-dopa (used as standard) on 14th, 21st, and 28th days of observation. Results of PEI observed with the animals that are treated with *T. glaberrima* were found to be significantly lower than that

observed with the animals in the standard group (treated with L-Dopa) on all the days of observation (Table 3.2a and Table 3.2b; Figures 3.1 – 3.5).

Observation of orientational behaviors did not showed any significant difference between the animals treated with *E. echinatus* and animals in control group except a significant change in sniffing number on 7th day and a significant change in climbing frequency on zero and 14th days of observation (Table 3.3b). Overall observation however showed that there exists an augmented interest towards the females in the animals treated with *E. echinatus* extract as evidenced by their increased score of sniffing and anogenital licking (Table 3.3b; Figure 3.6 and Figure 3.7).

Treatment with the methanol extract of *T. glaberrima* showed a significant difference among the control and standard groups. A significant increase in the genital grooming was observed on 7th, 14th and 21st day's of treatment with maximum activity on 14th day in the animals treated with *T. glaberrima* compared to the animals in both control and standard groups. A significant increase in sniffing was observed on all the days of treatment with maximum activity on 14th day in the animals treated with *T. glaberrima* compared to the animals in control group and the activity was also significantly higher when compared to animals in standard group on zero and 28th days of observation. Anogenital licking was significantly increased in the animals treated with *T. glaberrima* compared to the animals in control group on all the days (except on 21st day) of observation. The results were also significantly higher than the animals in standard group on zero and 7th days of observation (Table 3.3b; Figure 3.6 and Figure 3.7).

3.8.2. Observation of the body weight and weight of reproductive organs

A significant increase in the body weight was observed in all the groups but the increase was found to be much higher in *T. glaberrima* treated groups compared to that of the animals in control (Table 3.4).

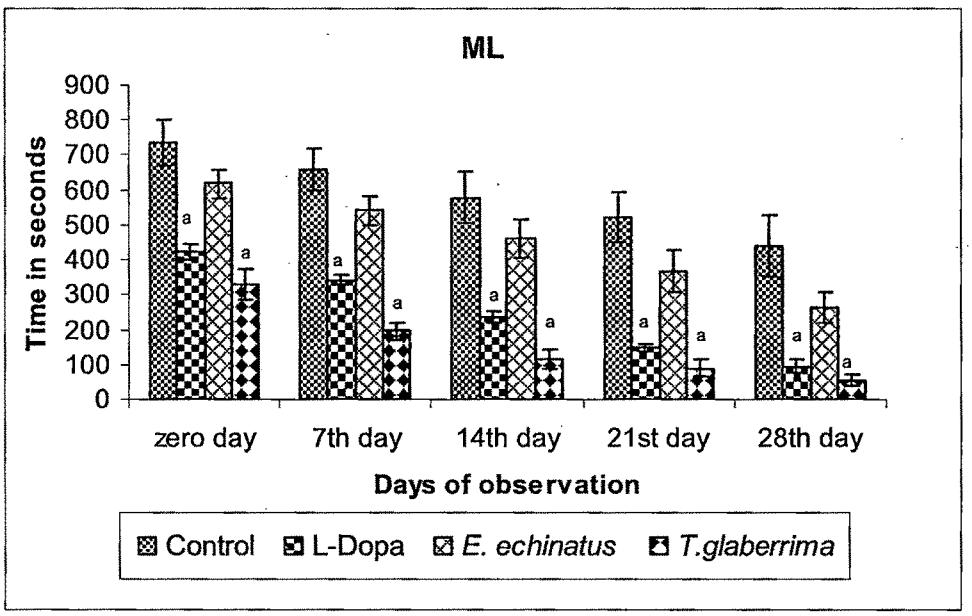

Figure 3.1. Effect of methanol extract of the roots of *E. echinatus* and aerial parts of *T. glaberrima* on mounting latency (ML)

Results are expressed as mean ± S.E.M (n = 6).

a $P < 0.001$ versus control; b $P < 0.01$ versus control; c $P < 0.05$ versus control; d $P < 0.001$ versus standard; e $P < 0.01$ versus standard; f $P < 0.05$ versus standard.

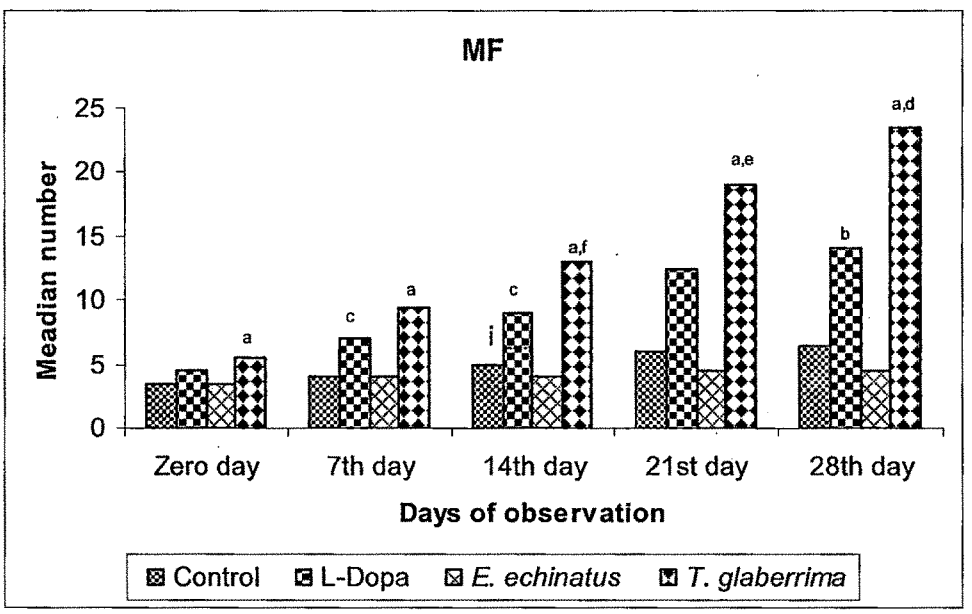

Figure 3.2. Effect of methanol extract of the roots of *E. echinatus* and aerial parts of *T. glaberrima* on mounting frequency (MF)

Results are expressed as median numbers (n = 6).

a $P < 0.001$ versus control; b $P < 0.01$ versus control; c $P < 0.05$ versus control; d $P < 0.001$ versus standard; e $P < 0.01$ versus standard; f $P < 0.05$ versus standard.

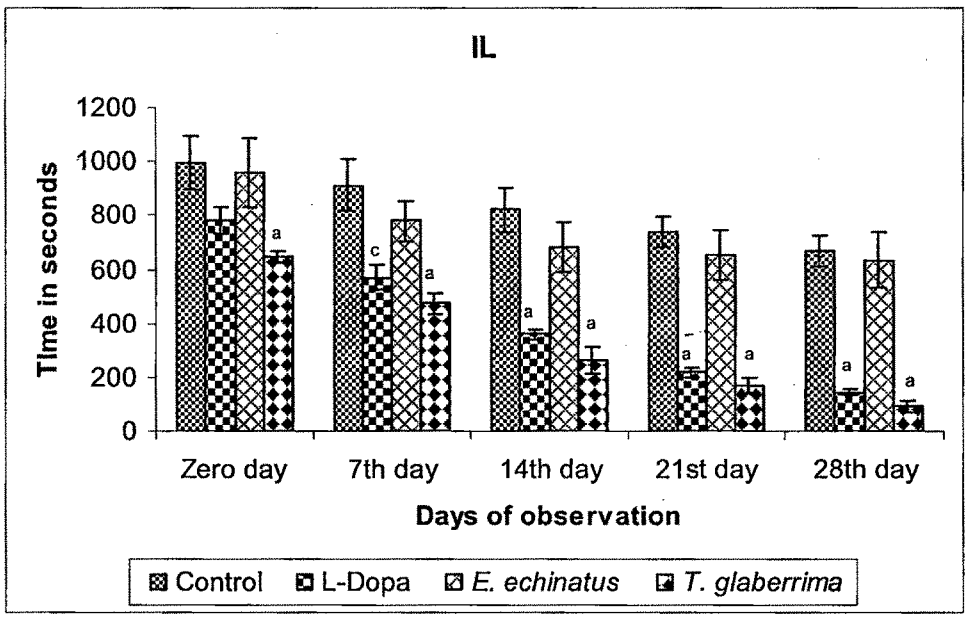

Figure 3.3. Effect of methanol extract of the roots of *E. echinatus* and aerial parts of *T. glaberrima* on intromission latency (IL)

Results are expressed as mean ± S.E.M (n = 6).

a $P < 0.001$ versus control; b $P < 0.01$ versus control; c $P < 0.05$ versus control; d $P < 0.001$ versus standard; e $P < 0.01$ versus standard; f $P < 0.05$ versus standard.

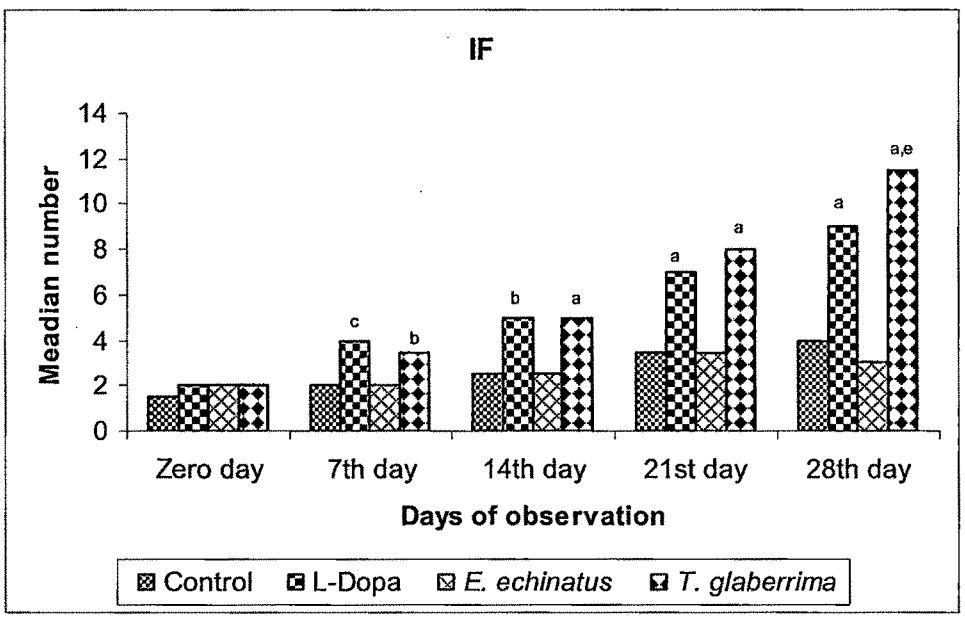

Figure 3.4. Effect of methanol extract of the roots of *E. echinatus* and aerial parts of *T. glaberrima* on intromission frequency (IF)

Results are expressed as median numbers (n = 6).

a $P < 0.001$ versus control; b $P < 0.01$ versus control; c $P < 0.05$ versus control; d $P < 0.001$ versus standard; e $P < 0.01$ versus standard; f $P < 0.05$ versus standard.

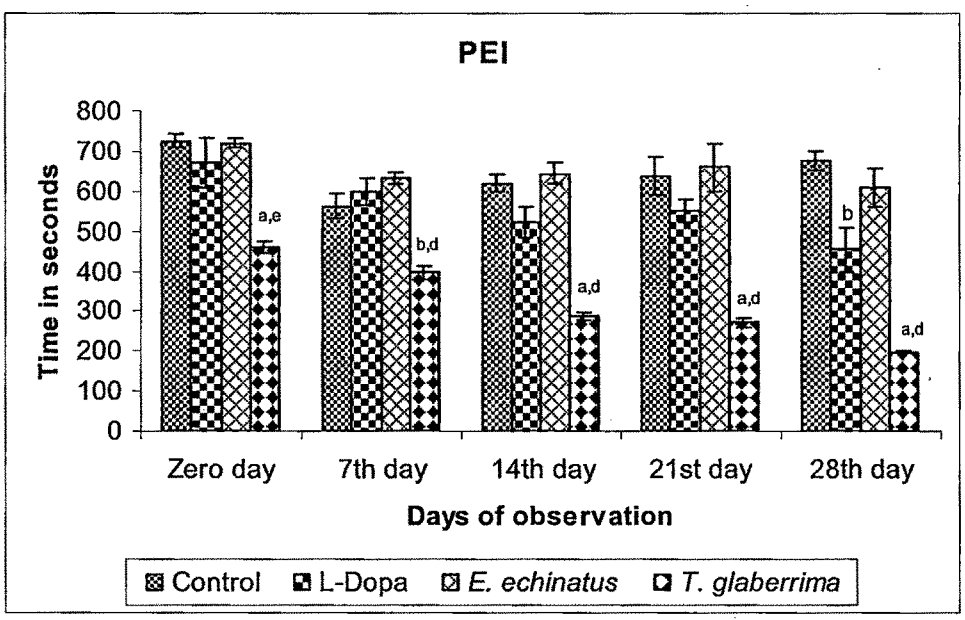

Figure 3.5. Effect of methanol extracts of the roots of *E. echinatus* and aerial parts of *T. glaberrima* on post ejaculatory interval (PEI)

Results are expressed as mean ± S.E.M (n = 6).

a $P < 0.001$ versus control; b $P < 0.01$ versus control; c $P < 0.05$ versus control; d $P < 0.001$ versus standard; e $P < 0.01$ versus standard; f $P < 0.05$ versus standard.

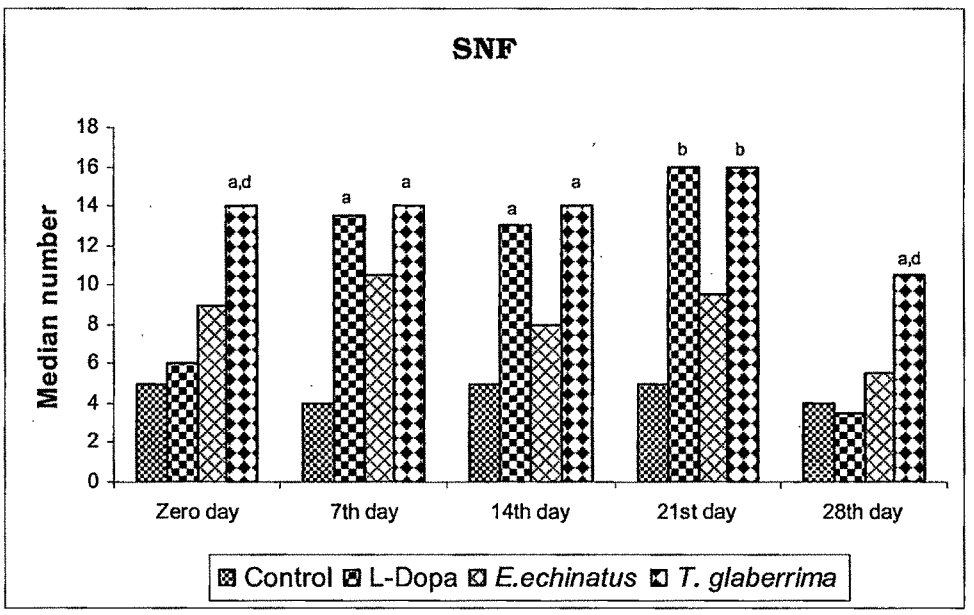

Figure 3.6. Effect of methanol extract of the roots of *E. echinatus* and aerial parts of *T. glaberrima* on anogenital sniffing (SNF)

Values are expressed as median numbers (n = 6).

a $P < 0.001$ versus control; b $P < 0.01$ versus control; c $P < 0.05$ versus control; d $P < 0.001$ versus standard; e $P < 0.01$ versus standard; f $P < 0.05$ versus standard (See Table 3.3a).

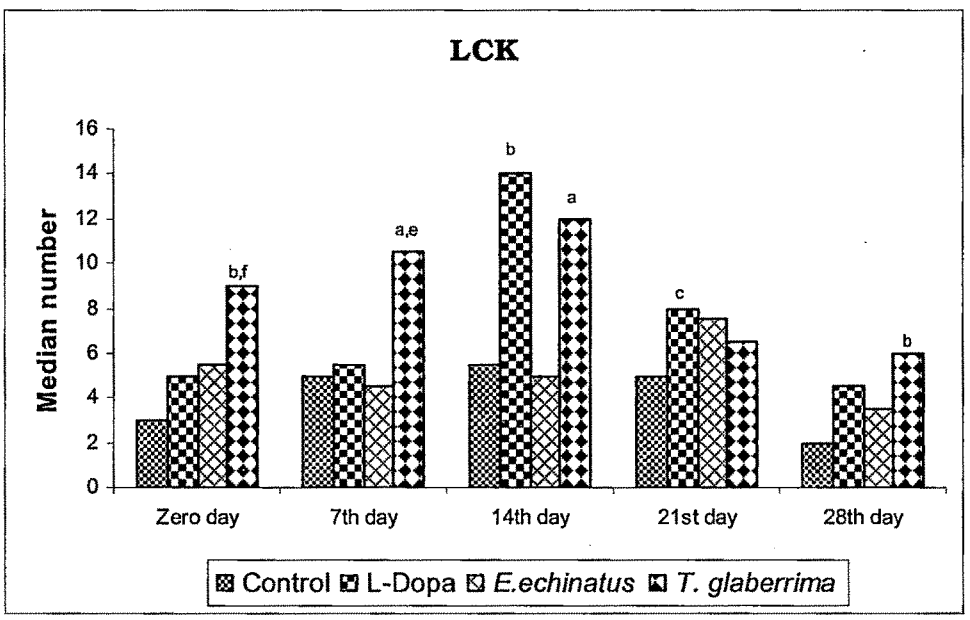

Figure 3.7. Effect of methanol extract of the roots of *E. echinatus* and aerial parts of *T. glaberrima* on licking (LCK)

Values are expressed as median numbers (n = 6).

a $P < 0.001$ versus control; b $P < 0.01$ versus control; c $P < 0.05$ versus control; d $P < 0.001$ versus standard; e $P < 0.01$ versus standard; f $P < 0.05$ versus standard.

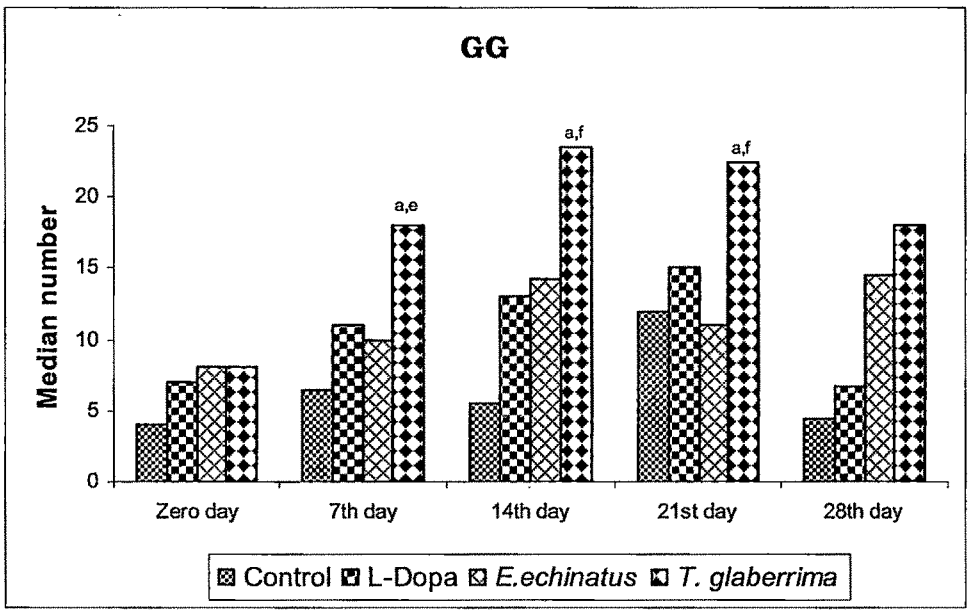

Figure 3.8. Effect of methanol extract of the roots of *E. echinatus* and aerial parts of *T. glaberrima* on genital grooming (GG)

Values are expressed as median numbers (n = 6).

a $P < 0.001$ versus control; b $P < 0.01$ versus control; c $P < 0.05$ versus control; d $P < 0.001$ versus standard; e $P < 0.01$ versus standard; f $P < 0.05$ versus standard.

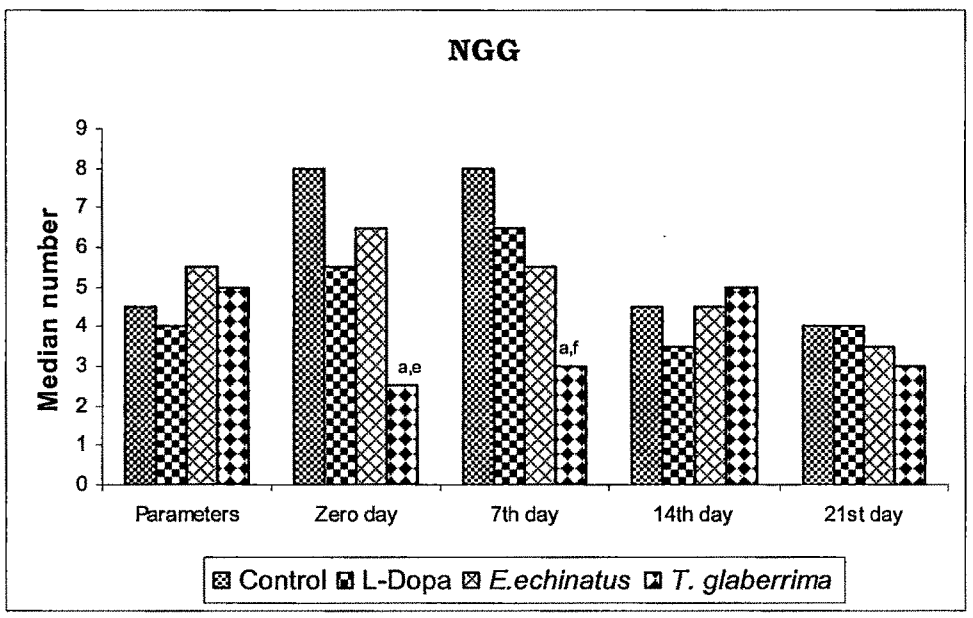

Figure 3.9. Effect of methanol extract of the roots of *E. echinatus* and aerial parts of *T. glaberrima* on non genital grooming (NGG)

Values are expressed as median numbers (n = 6).

a $P < 0.001$ versus control; b $P < 0.01$ versus control; c $P < 0.05$ versus control; d $P < 0.001$ versus standard; e $P < 0.01$ versus standard; f $P < 0.05$ versus standard.

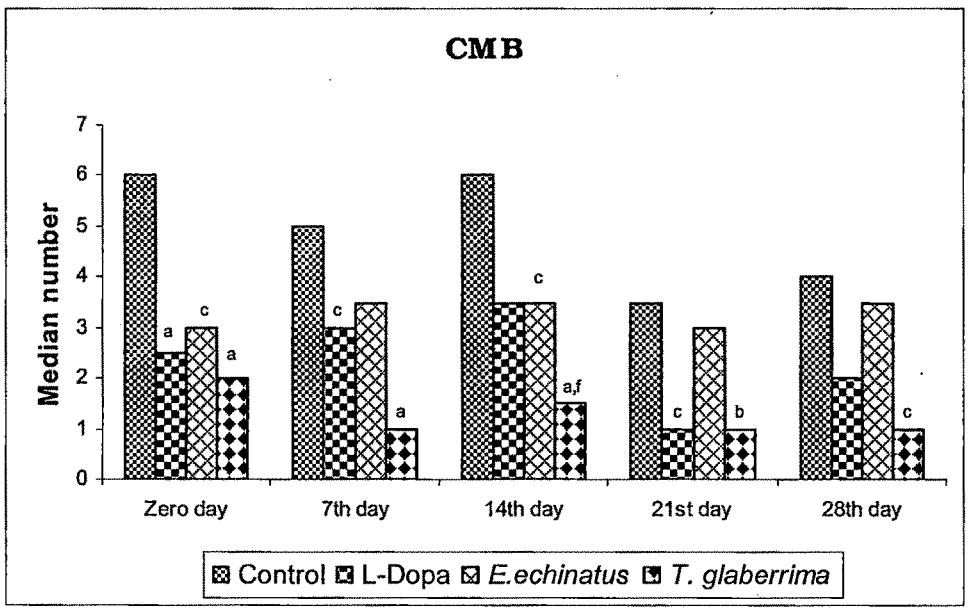

Figure 3.10. Effect of methanol extract of the roots of *E. echinatus* and aerial parts of *T. glaberrima* on climbing (CMB)

Values are expressed as median numbers (n = 6).

a $P < 0.001$ versus control; b $P < 0.01$ versus control; c $P < 0.05$ versus control; d $P < 0.001$ versus standard; e $P < 0.01$ versus standard; f $P < 0.05$ versus standard.

There was no significant difference in body weight of the animals treated with the methanol extract of *E. echinatus* compared to the animals in control group. A marked decrease in the relative weights of testis, epididymus and vas deferens was observed in the animals treated with *E. echinatus* compared to control group. On the other hand treatment with *T. glaberrima* showed a significant increase in the weight of seminal vesicles and a marked increase in weight of the other reproductive organs (testis, epididymus and vas deferens) compared to that in control group (Table 3.4).

3.8.3. Determination of epididymal sperm count

A significant decrease in the epididymal sperm count was observed in the animals treated with *E. echinatus* compared to the animals in both control group ($P < 0.05$) and standard group ($P < 0.01$) (Table 3.5). On the other hand treatment with *T. glaberrima* was found to enhance sperm count compared to the animals in control group (Table 3.5).

3.8.4. Estimation of serum testosterone levels

A significant decrease in serum testosterone levels ($P < 0.001$) was observed in the animals treated with *E. echinatus* compared to that of the animals in control and standard groups. Treatment with *T. glaberrima* showed an increase in the serum testosterone levels compared to the animals in control group (Table 3.5).

3.8.5. Histopathology

Histology of the testis in control group showed normal features with successive stages of transformation of the seminiferous epithelium into spermatozoa (Figure 3.12). Whereas histological examination of the testis after 28 days of treatment with the methanol extract of the roots of *E. echinatus* showed the presence of severe lesions in seminiferous epithelium (Figure 3.12) with a marked reduction in the seminiferous tubular diameter (STD) and germinal epithelial cell thickness (GECT)(Figure 3.11; Table 3.5).

Table 3.2a. Effect of oral administration of the methanol extracts of the roots of *E. echinatus* and aerial parts of *T. glaberrima* on copulatory behavior in male rats.

Groups	Parameters	Mean ± S.E.M				
		0 day	**7th day**	**14th day**	**21st day**	**28th day**
Control (Vehicle treated)	ML	734 ± 67.2	656.8 ± 60.0	578.6 ± 72.6	523.6 ± 71.5	441.0 ± 87.4
	IL	993.1 ± 101.6	910.6 ± 94.8	821.0 ± 83.1	737.6 ± 56.2	667.5 ± 54.0
	MF	3.5	4.0	5.0	6.0	6.5
	IF	1.5	2.0	2.5	3.5	4.0
	PEI	724.6 ± 17.5	561.1 ± 31.3	619.8 ± 21.2	637.1 ± 46.5	676.6 ± 23.9
L-dopa (100 mg/kg body weight)	ML	422.5 ± 24.6[a]	341.3 ± 14.6[a]	238.1 ± 16.8[a]	147.6 ± 12.0[a]	95.5 ± 18.2[a]
	IL	779.6 ± 47.9	569.5 ± 44.8[c]	358.6 ± 17.7[a]	219.0 ± 17.6[a]	145.3 ± 13.9[a]
	MF	4.5	7.0[c]	9.0[c]	12.5	14.0[b]
	IF	2.0	4.0[c]	5.0[b]	7.0[a]	9.0[a]
	PEI	672.6 ± 62.1	598.5 ± 33.2	523.1 ± 39.4	551.1 ± 26.6	453.3 ± 53.6[b]

Frequency results are expressed as median numbers and other data were expressed as mean ± S.E.M (n = 6).

a $P < 0.001$ versus control; b $P < 0.01$ versus control; c $P < 0.05$ versus control.

ML: mounting latency; IL: intromission latency; MF: mounting frequency; IF: intromission frequency; PEI: post ejaculatory interval.

Table 3. 2b. Effect of oral administration of the methanol extracts of the roots of *E. echinatus* and aerial parts of *T. glaberrima* on copulatory behavior in male rats.

Groups	Parameters	Mean ± S.E.M				
		0 day	7th day	14th day	21st day	28th day
	ML	619.1 ± 40.8	541.1 ± 40.8	461.1 ± 54.2	367.6 ± 60.8	263.5 ± 45.7
E. echinatus	IL	960.3 ± 129.1	778.6 ± 73.6	680.0 ± 93.5	651.3 ± 92.3	634.3 ± 101.9
(200 mg/kg	MF	3.5	4.0	4.0	4.5	4.5
body weight)	IF	2.0	2.0	2.5	3.5	3.0
	PEI	720.1 ± 13.0	632.6 ± 16.2	643.5 ± 26.5	659.1 ± 61.5	607.5 ± 47.5
	ML	328 ± 43.9[a]	197.1 ± 24.7[a]	116.6 ± 28.5[a]	90.5 ± 24.7[a]	55.1 ± 17.2[a]
T. glaberrima	IL	646.6 ± 24.2[a]	472.8 ± 37.1[a]	262.8 ± 49.2[a]	172.5 ± 28.1[a]	92.5 ± 18.0[a]
(200 mg/kg	MF	5.5[a]	9.5[a]	13.0[a,f]	19.0[a,e]	23.5[a,d]
body weight)	IF	2.0	3.5[b]	5.0[a]	8.0[a]	11.5[a,e]
	PEI	461.1 ± 13.7[a,e]	397.1 ± 16.8[b,d]	287.1 ± 8.5[a,d]	270.8 ± 12.0[a,d]	198.8 ± 4.6[a,d]

Frequency results are expressed as median numbers and other data were expressed as mean ± S.E.M (n = 6).

a $P < 0.001$ versus control; b $P < 0.01$ versus control; c $P < 0.05$ versus control (See Table 3.2a).

d $P < 0.001$ versus standard; e $P < 0.01$ versus standard; f $P < 0.05$ versus standard (See Table 3.2a).

ML: mounting latency; IL: intromission latency; MF: mounting frequency; IF: intromission frequency; PEI: post ejaculatory interval.

Table 3.3a. Effect of oral administration of the methanol extracts of the roots of *E. echinatus* and aerial parts of *T. glaberrima* on orientational behavior of animals.

Groups	Parameters		Zero day	7th day	14th day	21st day	28th day
Control (Vehicle treated)	Towards female	LCK	3	5	5.5	5	2
		SNF	5	4	5	5	4
	Towards self	GG	4	6.5	5.5	12	4.5
		NGG	4.5	8	8	4.5	4
	Towards environment	CMB	6	5	6	3.5	4
L-Dopa (100 mg/kg body weight)	Towards female	LCK	5	5.5	14[b]	8[c]	4.5
		SNF	6	13.5[a]	13[a]	16[b]	3.5
	Towards self	GG	7	11	13	15	6.7
		NGG	4	5.5	6.5	3.5	4
	Towards environment	CMB	2.5[a]	3[c]	3.5	1[c]	2

Values are expressed as median numbers (n = 6).

a $P < 0.001$ versus control; b $P < 0.01$ versus control; c $P < 0.05$ versus control.

GG: Genital grooming; NGG: non genital grooming; SNF: sniffing; LCK: anogenital licking; CMB: climbing.

Table 3.3b. Effect of oral administration of the methanol extracts of the roots of *E. echinatus* and aerial parts of *T. glaberrima* on orientational behavior of animals.

Groups	Parameters		Zero day	7th day	14th day	21st day	28th day
E. echinatus	Towards female	LCK	5.5	4.5	5	7.5	3.5
(200 mg/kg		SNF	9	10.5[c]	8	9.5	5.5
body weight)	Towards self	GG	8	10	14.2	11	14.5
		NGG	5.5	6.5	5.5	4.5	3.5
	Towards environment	CMB	3[c]	3.5	3.5[c]	3	3.5
T. glaberrima	Towards female	LCK	9[b,f]	10.5[a,e]	12[a]	6.5	6[b]
(200 mg/kg		SNF	14[a,d]	14[a]	14[a]	16[b]	10.5[a,d]
body weight)	Towards self	GG	8	18.5[a,e]	23.5[a,f]	22.5[a,f]	18
		NGG	5	2.5[a,e]	3[a,f]	5	3
	Towards environment	CMB	2[a]	1[a]	1.5[a,f]	1[b]	1[c]

Values are expressed as median numbers (n = 6).

a $P < 0.001$ versus control; b $P < 0.01$ versus control; c $P < 0.05$ versus control; d $P < 0.001$ versus standard; e $P < 0.01$ versus standard; f $P < 0.05$ versus standard (See Table 3.3a).

GG: Genital grooming; NGG: non genital grooming; SNF: sniffing; LCK: anogenital licking; CMB: climbing.

Table 3.4. Changes in the body weight and weight of reproductive organs of rats after treatment with the extracts of *E. echinatus* and *T. glaberrima*.

Groups	Body weight (g)			Weight of reproductive organ mg/100 g body weight (Mean ± SEM)			
	Initial	Final	% increase	Testis	Epididymus	Vas deferens	Seminal vesicles
Control (Vehicle)	251.6 ± 7.0	263.3 ± 8.4[a]	4.6	569.8 ± 34.6	251.7 ± 6.5	64.5 ± 3.5	123.9 ± 15.5
L-Dopa (100 mg/kg)	265.0 ± 9.4	279.1 ± 8.5[b]	5.3	600.9 ± 26.2	271.3 ± 15.7	73.2 ± 4.4	125.8 ± 17.5
E. echinatus (200 mg/kg)	278.3 ± 11.3	295.8 ± 11.2[b]	6.2	502.6 ± 11.9	246.8 ± 6.6	55.0 ± 2.4	154.4 ± 9.9
T. glaberrima (200 mg/kg)	236.1 ± 7.1	287.5 ± 12.4[b]	21.7	641.2 ± 21.7	259.3 ± 7.7	68.5 ± 5.1	280.2 ± 26.0[c,d]

Results of Body weight were analyzed by paired t-test; Values are mean ± SEM (n = 6).

a $P < 0.001$ versus initial body weight; b $P < 0.01$ versus initial body weight.

c $P < 0.001$ versus control; d $P < 0.001$ versus Standard.

Table 3.5. Effect of oral administration of the methanol extract of roots of *E. echinatus* and aerial parts of *T. glaberrima* on testicular histomorphology, serum levels of testosterone and epididymal sperm concentration.

Groups	Semniferous tubular diameter (µm)	Germinal epithelial cell thickness (µm)	Serum testosterone (ng/ml)	Sperm count 10^6/ ml
Control (Vehicle)	231.8 ± 3.0	58.1 ± 1.3	3.1 ± 0.27	3.4 ± 0.17
L-Dopa (100 mg/kg body weight)	325.1 ± 6.1[a]	77.3 ± 2.0[a]	4.1 ± 0.29	3.7 ± 0.12
E. echinatus (200 mg/kg body weight)	237.8 ± 5.7	50.6 ± 1.2	1.3 ± 0.22[a,d]	2.19 ± 0.30[c,e]
T. glaberrima (200 mg/kg body weight)	344.4 ± 7.0[a]	96.3 ± 3.2[a,d]	3.4 ± 0.18	4.3 ± 0.32

Values are mean ± SEM (n = 6).

a $P<0.001$ versus control; b $P < 0.01$ versus control; c $P < 0.05$ versus control;

d $P < 0.001$ versus standard; e $P < 0.01$ versus standard; f $P < 0.05$ versus standard.

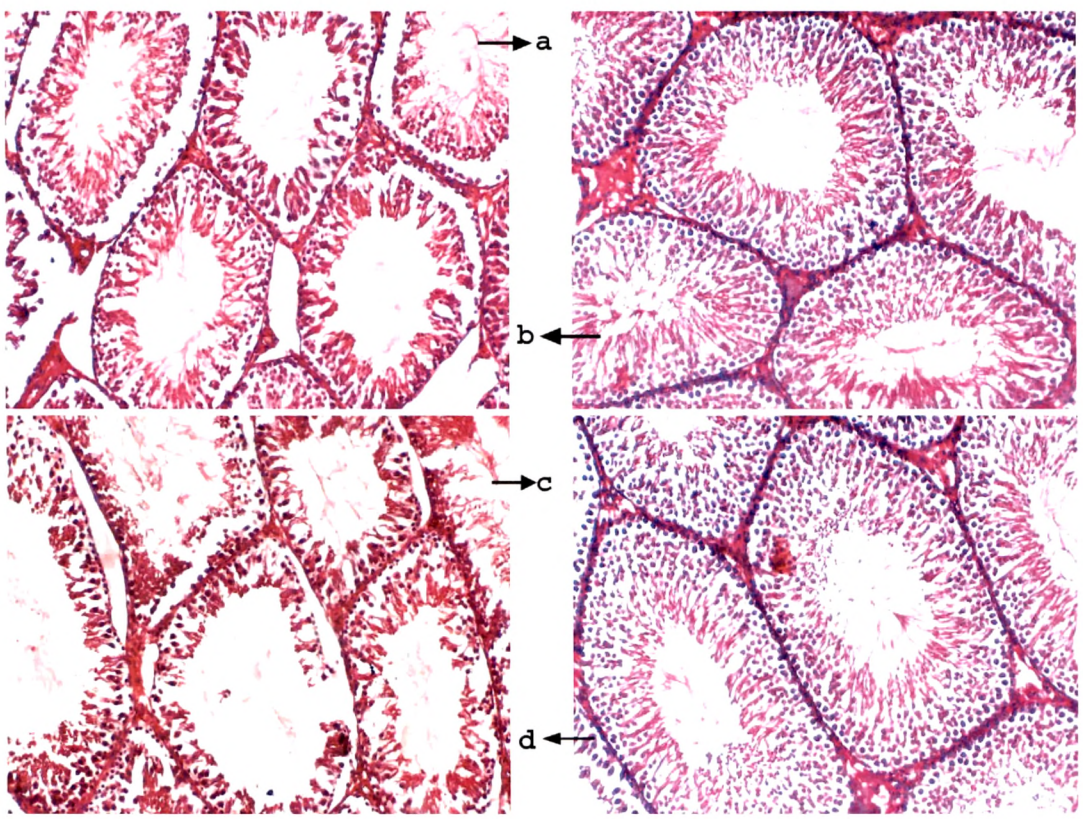

Figure 3.11. Effect on seminiferous tubular diameter and germinal cell epithelial cell thickness after treatment in different groups (a) control (b) L-dopa (c) *E. echinatus* (d) *T. glaberrima* (X10).

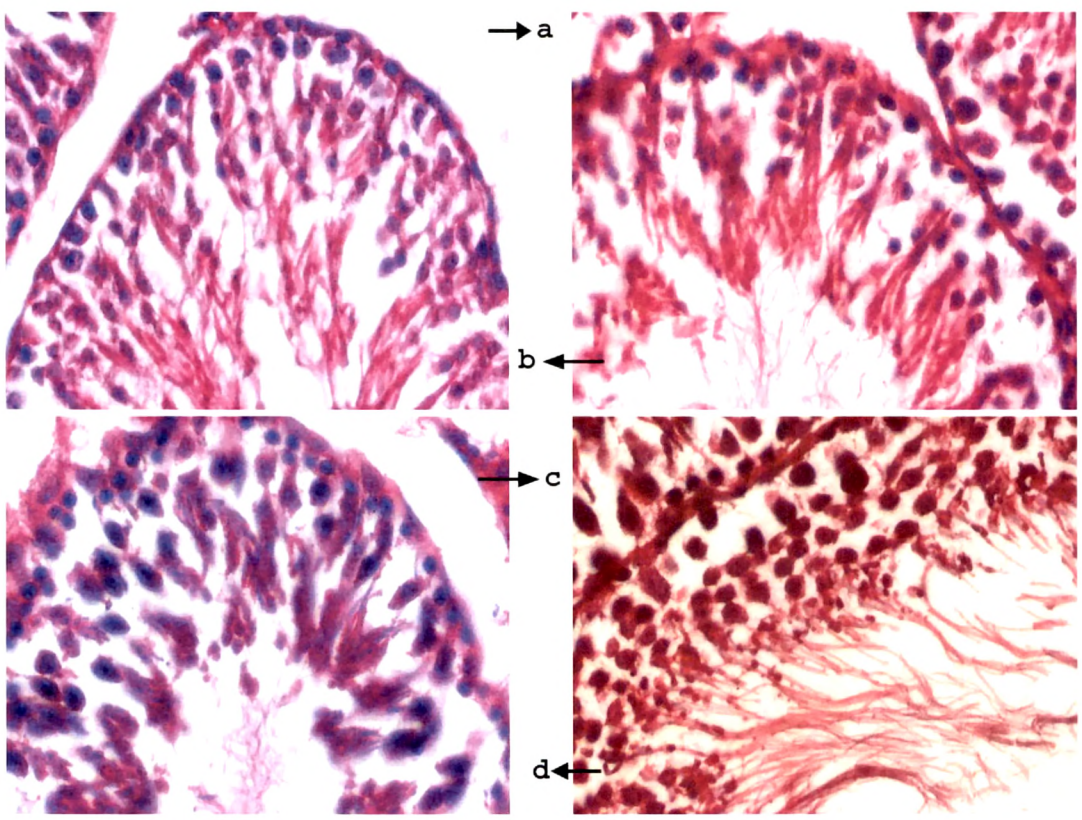

Figure 3.12. Histology of testis in different groups after treatment with (a) control (b) L-dopa (c) *E. echinatus* (d) *T. glaberrima*, showing successive stages of transformation of seminiferous epithelium into spermatozoa (X40).

Histological examination of the testis in the animals treated with the methanol extract of aerial parts of *T. glaberrima* showed a better proliferation of the testicular tissues (Figure 3.12) and thereby a significant increase in STD and GECT values ($P < 0.001$) was observed compared to the animals in control group. The GECT was also significantly higher than that observed with the animals in standard group ($P < 0.001$).

3.8.6. Determination of antioxidant enzyme activity in testicular homogenate

In view of the beneficial effects that have been observed with SOD and catalase in preserving human sperm function (Aitken et al., 1993; Griveau and Le Lannou, 1994), experiments were conducted to assess the ability of these antioxidant enzymes to suppress the peroxidative damage by endogenously generated ROS (Reactive oxygen species). From the results it was observed that, treatment with *E. echinatus* and *T. glaberrima* can influence on the activity of antioxidant enzymes. Treatment with *E. echinatus* showed a significant increase in the activity of CAT ($P < 0.01$) but only a marked increase in the SOD activity was observed compared to the animals in the control group. However, treatment with *T. glaberrima* showed a significant increase in the activity of SOD ($P < 0.01$) and CAT ($P < 0.05$) compared to that of the animals in control group (Table 3.6).

3.8.7. In vitro study of extracts for radical scavenging properties

Both the extracts have exhibited different levels of antiradical activity in all the three models studied.

Because reducing power is directly associated with the antioxidant activity of a substance, and both the extracts under investigation showed a concentration dependent increase in reducing power by reducing $Fe^{+3} \Rightarrow Fe^{+2}$ (Figure 3.13).

DPPH is a stable free radical and the measurement of its scavenging property has been largely used as a quick and reliable parameter to assess the in vitro antioxidant activity of plant extracts (Soares et al., 1997; Munasinghe et al., 2001). Methanol extract of *E. echinatus* and *T. glaberrima* exhibited a concentration dependent free radical scavenging activity by decreasing the absorbance of colored DPPH solution (Figure 3.14). *E. echinatus* was found to be more potent scavenger than *T. glaberrima* (IC_{50} values of 53.51 and 72.32 µg /ml respectively), and their activity was comparable to that of quercetin (IC_{50} 11.81 µg/ml).

Super oxide is a significant agent of oxygen toxicity in the body. It has been shown to induce lipid peroxidation, cause damage to membranes and kill cells (Halliwell and Gutteridge, 1984). Methanol extract of *E. echinatus* and *T. glaberrima* showed a concentration dependent inhibition of NBT reduction by superoxide radicals (Figure 3.15). *E. echinatus* was found to be more potent scavenger (with an IC_{50} of 45.26) than the *T. glaberrima* (with an IC_{50} of 56.01 µg/ml), the activity was comparable to that of quercetin (IC_{50}12.62 µg/ml).

Table 3.6. Effect of oral administration of the methanol extracts of *E. echinatus* and *T. glaberrima* on activity of antioxidant enzyme in testicular tissues.

Groups	Antioxidant enzymes	
	SOD (ng/mg)	CAT (ng/mg)
Control (Vehicle)	8.3 ± 1.1	252.8 ± 16.1
L-Dopa (100 mg/kg body weight)	14.9 ± 1.6	476.1 ± 46.9[b]
E. echinatus (200 mg/kg body weight)	16.2 ± 1.8	484.1 ± 45.9[b]
T. glaberrima (200 mg/kg body weight)	22.4 ± 3.7[b]	433.9 ± 49.2[c]

Values are mean ± SEM (n = 6).

b $P < 0.01$ versus control; c $P < 0.05$ versus control.

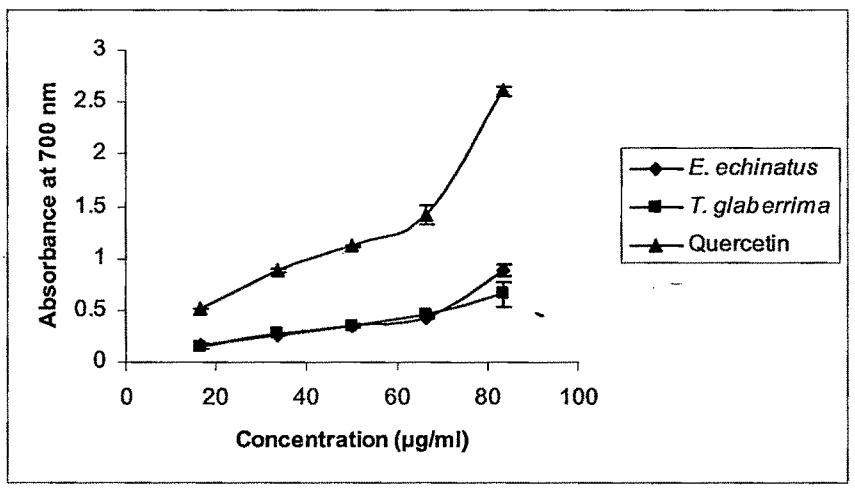

Figure 3.13. Reducing power activity of the methanol extract of *E. echinatus* and *T. glaberrima*

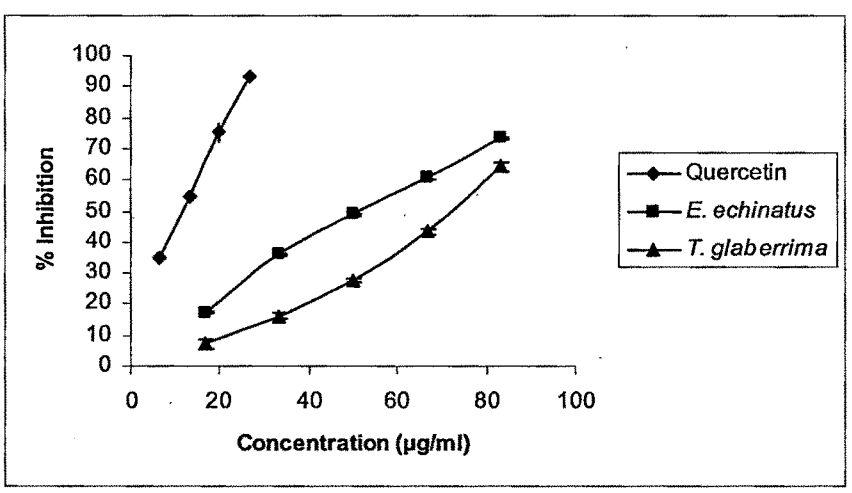

Figure 3.14. DPPH scavenging activity of the methanol extract of *E. echinatus* and *T. glaberrima*

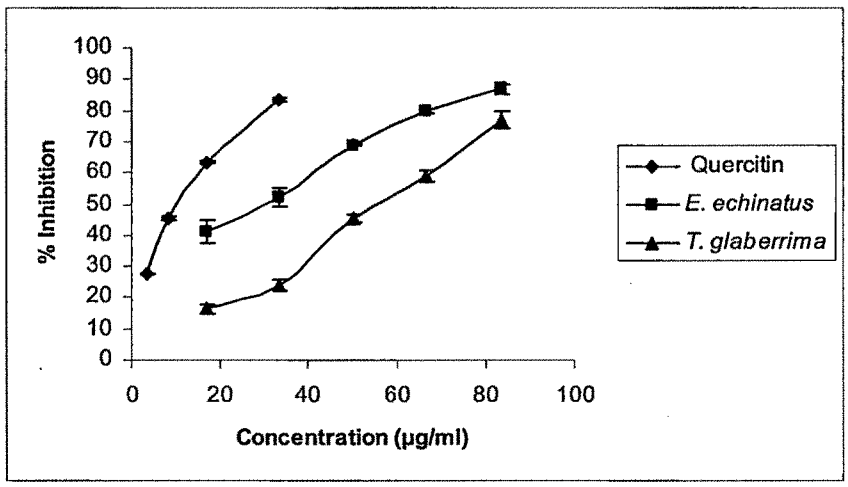

Figure 3.15. Superoxide scavenging activity of the methanol extract of *E. echinatus* and *T. glaberrima*

3.9. Studies on effect of terpenoidal fraction of *Echinops echinatus* roots on reproductive parameters of male rats

Considering the importance for search of male antifertility agents from traditional medicines, it was thought worthwhile in our studies to undertake further investigations on *E. echinatus* which in the preliminary studies showed the inhibition of regular male reproductive functions.

Preliminary phytochemical screening revealed the presence of terpenoids as the chief constituents in *E. echinatus* roots. Therefore, it was thought worthwhile to prepare a fraction containing terpenoids and investigate its effect on the male reproductive parameters.

3.9.1. Preparation of extract and terpenoidal fraction

A coarse powder (500 g) of the shade-dried roots was extracted with petroleum ether (60 – 80°) in a Soxhlet apparatus. The extract was concentrated on a rotary vacuum evaporator (BUCHI Rotavapor R 200) and dried under vacuum (yield = 4.96% w/w). Vacuum-dried petroleum ether extract was subjected to saponification, and the unsaponifiable material rich in terpenoids was separated out and dried under vacuum (25% w/w) (Indian pharmacopoeia, 1996; see chapter 4 and section 4.1). Vacuum-dried terpenoid rich fraction of petroleum ether extract (TRFPE) was taken for further studies.

3.9.2. Animals

Adult albino Wistar rats 90 days old and weighing between 210 and 270 g were obtained from M/s Zydus Cadilla Research Centre, Ahmedabad, India, and housed at a temperature of 24–28 °C with a relative humidity of 45– 55% with free access to food and water *ad libitum*. Before the experiment began, the local committee of ethics on animal experimentation approved all experimental procedures (no. 404/01/a/CPCSEA).

3.9.3. Acute toxicity studies

Healthy adult albino (Wistar) rats of either sex, starved overnight, were divided into four groups (n = 6) and were fed with increasing doses (50, 100, 200 and 300 mg/kg body weight) of the TRFPE. The animals were observed up to 14 days for toxicity and mortality. Finally, 1/10th of the highest tolerable dose was taken for further studies (Ghosh, 1984).

3.9.4. Treatment schedule

Male albino Wistar rats were used in the studies and randomly divided into three groups (n = 8): group 1 received the vehicle (1 ml/day of 1% tween-80 in water) orally for 8 days and served as control. Groups 2 and 3 were administered TRFPE suspended in 1% tween 80 at a dose of 30 and 60 mg/kg body weight, respectively, for a period of 8 days.

3.9.5. Effect on body weight and weight of reproductive organs

Body weight and weight of the reproductive organs was recorded as per the method described in section 3.6.2

3.9.6. Epididymal sperm count

Homogenisation-resistant epididymal sperm were counted as described by Bustos-Obregon and Gonzalez-Hormazabal (2003) with some modifications. Homogenisation was performed in 5 ml of 0.9% saline, and the homogenized epididymal preparation was refrigerated at 4 °C for 24 hours to allow sperm to be released from the walls. Data are referred to as sperms (10^6) per epididymis.

3.9.7. Estimation of serum testosterone

It was carried out as per the method described in section 3.6.4

3.9.8. Testicular histomorphology

It was carried out as per the method described in section 3.6.5

3.9.9. Data analysis

To determine statistically significant differences among treatment groups, data were analyzed by using one-way analysis of variance (ANOVA) followed by Dunnet test. Values of $P < 0.05$ were considered statistically significant.

3.9.10. Results

Results of acute toxicity studies showed that TRFPE, when administered orally at the maximum dose of 300 mg/kg body weight, did not produce any evident sign of toxicity or mortality. The final body weights of animals in all groups were increased compared with their respective initial weights; however, the increase was found to be greater in the control group compared with the treated groups (Table 3.7). On the other hand, a decrease in relative weights of testis, cauda epididymides and seminal vesicles were observed in the treated group compared with the control group (Table 3.7). Treated animals exhibited a significant decrease in serum testosterone levels and cauda epididymal sperm concentration compared with animals in the control group (Table 3.8). Histology of the testis in the control group showed normal features with successive stages of transformation of the seminiferous epithelium into spermatozoa, whereas histological examination of the testis after acute treatment with two different doses of TRFPE at 30 and 60 mg/kg body weight showed the inhibitory effects on the growth of seminiferous epithelium and on spermatogenesis (Figure 3.16), with a significant reduction in the STD and GECT (Figure 3.17; Table 3.8).

Table 3.7. Change in the body weight and weight of reproductive organs after treatment with TRFPE in male rats.

Groups (n = 8)	Body weight (g)			Weight of male reproductive organs mg/100 g body weight (Mean ± SEM)			
	Initial	Final	Total increase (%)	Testis	Epididymis	Vas Deferens	Seminal vesicles
Control (Vehicle)	276.2 ± 15.6	286.9 ± 22.6	3.8	542.2 ± 25.3	235.8 ± 12.9	57.7 ± 5.3	252.9 ± 20.7
TRFPE (30 mg/kg body weight)	246.6 ± 11.9	252.9 ± 13.4	2.5	598.9 ± 24.1	217.2 ± 16.5	55.2 ± 5.5	179.6 ± 16.2*
TRFPE (60 mg/kg body weight)	218.6 ± 19.4	226.6 ± 22.7	3.6	526.7 ± 25.8	206.6 ± 09.3	60.1± 6.2	245.8 ± 15.6

Values are Mean ± SEM (n = 8)

* $P < 0.05$ versus control

Table 3.8. Effect of oral administration of TRFPE on testicular histomorphology and epididymal sperm concentration

Groups	Semniferous tubular diameter (μm)	Germinal epithelial cell thickness (μm)	Sperm count 10^6/ epididymis	Testosterone (ng/ml)
Control (Vehicle)	321.1 ± 8.8	87.4 ± 1.6	175.0 ± 11.5	3.4 ± 0.5
TRFPE (30 mg/kg body weight)	253.0 ± 4.5**	63.6 ± 2.0**	102.1 ± 5.1**	1.4 ± 0.2**
TRFPE (60 mg/kg body weight)	266.9 ± 4.2**	60.0 ± 2.3**	110.0 ± 10.0**	1.4 ± 0.3**

Values are Mean ± SEM (n = 8)

* $p < 0.05$ versus control; ** $p < 0.01$ versus control

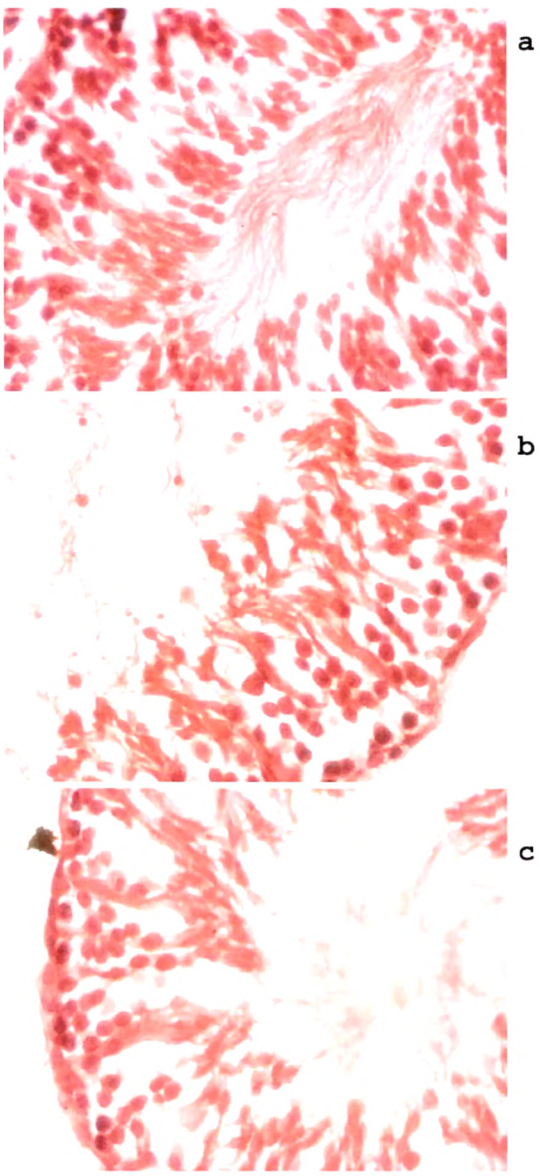

Figure 3.16. Histology of the testis in different groups. a Control. b Terpenoid-rich fraction of petroleum ether extract (TRFPE) 30 mg/kg. c TRFPE 60 mg/kg showing successive stages of transformation of the seminiferous epithelium into spermatozoa (X40)

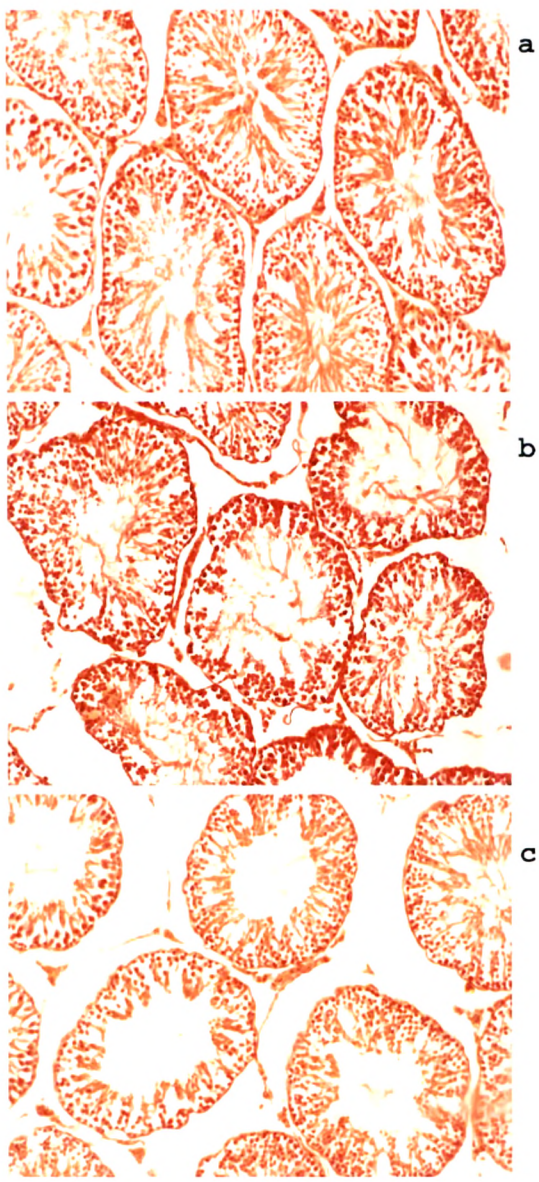

Figure 3.17. Effect of terpenoid-rich fraction of petroleum ether extract (TRFPE) on seminiferous tubular diameter and germinal epithelial cell thickness of different groups. a Control. b Treated with TRFPE 30 mg/kg. c treated with TRFPE 60 mg/kg (X10)

3.10. Discussion

It has been reported that a number of Indian medicinal plants used in Ayurvedic and other traditional systems medicine are classified under the controversial category. This may be due to their unresolved identity, unverified properties or other such reasons (see chapter 1; section 1.3). Brahmadandi is one such controversial drug in Indian system of medicine. The survey of literature revealed that the drug, Brahmadandi, used in folklore medicine as nervine tonic, aphrodisiac, in the treatment of seminal debility and as sex tonic, comprise of two plants i,e *E. echinatus* and *T. glaberrima*. The methanol extract of the roots of *E. echinatus* and aerial parts of *T. glaberrima* were therefore screened for reported activity of these plants.

The data obtained reveal that oral administration methanol extract of the roots of *E. echinatus* exhibits inhibitory effects on the functions of male reproductive system and may act as an antifertility agent. Whereas, oral administration of the crude methanol extract of the aerial parts of *T. glaberrima* effectively facilitated several components of the copulatory behavior and thus promoting the sexual activity.

Body weight of the animals suggests that the treatment of rats with methanol extract of *E. echinatus* roots did not induce any excessive toxicity to the animals. Hence animal malnutrition could not interfere with the functional activity and structure of the male reproductive system. Results of the sexual behavior studies suggest that the treatment with methanol extract of *E. echinatus* did not reduce the sexual interest of the animals as evidenced by the orientational behavior. A marked decrease in the testicular weight was observed in the animals treated with *E. echinatus* compared to that in the control group, which is known to be related to the number of spermatids and spermatozoa in the tissue (Gupta et al., 2000). Observation of the testicular histomorphology showed that the extract did not affect the diameter of semniferous tubules, but was found to inhibit the normal

spermatogenesis. It is probable that the observed effects on testis induced by the *E. echinatus* extract were due to the significant decrease in testosterone level, which was responsible for the diminished spermatogenesis, and hence reduction in sperm counts (Toney and Danzo, 1989; Shan and Hardy, 1992). Circulatory levels of testosterone are required for the maintenance of normal structure and function of the accessory sex organs, though the threshold levels may vary with organs. Reduced epididymal sperm concentration and weight of the accessory sex organs further supports the suppressed concentration of testosterone in the circulation (Gupta et al., 1993; Lohiya and Ansari, 1999). Also there is considerable evidence that the 50% ethanol extract of the roots of *E. echinatus* shows anti-spermatogenic and anti-androgenic effects in male rats (Chaturvedi et al., 1995).

The results are further supported by the fact that plants containing triterpenoids especially those of lupane group including pure lupeol acetate exhibits antifertility effects in male rats (Topcu, 2006; Gupta et al., 2005). Preliminary phytochemical tests revealed the presence of terpenoids as the chief constituents in the extract of *E. echinatus*. Further phytochemical investigations on the chemical constituents of the roots, at the later stage, lead us to the isolation of lupeol. Also the plant has been reported to contain taraxasterol acetate (Singh et al., 1991), a pentacyclic triterpenoid of lupane skeleton. Therefore the observed activity may thus be due to rich content of pentacyclic terpenoids mainly those of lupane type in the plant.

Thus the oral administration of methanol extract of the roots of *E. echinatus* to male rats inhibited regular functions of reproductive organs. Acute treatment with TRFPE of the roots of *E. echinatus* to male rats exhibited reduction in serum testosterone levels, sperm count and reduction of STD and GECT values. TRFPE showed high levels of lupeol content when subjected for isolation and separation of chemical constituents. Lupeol acetate was reported to reduce the epididymal sperm count (Gupta et al.,

2005). Hence the activity of the methanol extract of the roots of *E. echinatus* and its fraction TRFPE may be endowed upon the presence of terpenoids and lupeol.

Studies on the methanol extract of *T. glaberrima* provide evidence on the ability of the extract to enhance male sexual behavior in sexually active rats. The data obtained reveal that when administered orally, methanol extract of *T. glaberrima* effectively facilitates several components of the copulatory behavior. The plant is reported to be used as nervine tonic (Kirtikar and Basu, 1975) and such drugs enhance blood circulation and produce general well being by toning up the mental and physical functions. Therefore the observed activity could be due to either drug induced changes in neurotransmitter levels or their action in the cells. The increase in the body weight of *T. glaberrima* treated rats could be due to the androgenic properties of this plant since androgens possess anabolic activity (Johnson and Everitt, 1988). Though there was no significant change in the serum levels of testosterone, there is a possibility that a steroid like effect of the extract might be responsible for the prosexual effects reported here.

Studies on the testicular histomorphology showed that mean STD and GECT were significantly higher in the animals treated with *T. glaberrima* compared to that in the control group. Increase in these values is an indication of better proliferation of the testicular tissues and thereby representing better spermatozoal maturation within the semniferous tubules leading to the healthier spermatogenesis.

The chemical constituents and the mechanism of action responsible for these activities are not known. However the plant has been reported to contain phytosterols (spinasterol, stigmasterol), terpenoids, flavonoids, (Chawla et al., 1976; Manerikar et al., 1978) etc. The flavonoid content of the plant may also contribute to its sex stimulating activity as flavonoids were shown to alter the androgen levels (Ageel et al., 1994) which play an

important role in sexual stimulation and also it is well known that these compounds increase significantly SOD and catalase activities (Toyokuni et al., 2003) thereby imparting a indirect potentiating effect on the observed activity. Preliminary phytochemical screening revealed the presence of alkaloids in the plant and it is also reported that a number of plants containing alkaloids possess aphrodisiac activity (Singh & Mukherjee, 1998). Therefore the observed aphrodisiac studies may be due to the alkaloid content of the plant.

In Ayurvedic texts plants that show aphrodisiac properties have been described into five categories (Singh & Mukherjee, 1998). 1. Drugs which stimulate the production of semen e.g., *Microstylis wallichii* Lindl (Orchideaceae), *Mucuna pruriens* Bak (Leguminosae), *Asparagus recemosus* Willd (Liliaceae), etc., 2. Drugs which improve and purify the quality of semen e.g., *Saussurea lappa* Clarke (Compositae), *Vetivera ziznoides* Stapf (Gramineae) etc., 3. Drugs which help sexually and in ejaculation e.g., *Strychnos nuxvomica* Linn (Loganiaceae), *Myristica fragrans* Houtt (Myristicaceae), etc. 4. Drugs delaying the time of ejaculation e.g., *Sida cordifolia* Linn (Malvaceae), Opium, Musk, etc., and 5. Drugs arousing sexual desire e.g., *Withania somnifera* Dunal (Solanaceae), *Datura stramonium* Linn (Solanaceae), *Asparagus recemosus*, etc. From the results it was observed that treatment with *T. glaberrima* showed a marked increase in the epididymal sperm concentration and thereby acting on the production of semen.

The results indicate the potential of *T. glaberrima* as therapeutic agent in treating male infertility. The investigation shows that the methanol extract of *T. glaberrima* can enhance the sexual activity in normal rats and favors the spermatogenesis. Plants shows aphrodisiac properties by various mechanisms and from the data obtained in the present study, the plant *T. glaberrima* was found to fall under the category of aphrodisiacs that increases sexual desire and those which improve the quality and stimulate

the production of semen. Generally the sexual behaviors are enhanced by elevated neurotransmitter levels or by direct action on the target tissues which may be due to one of the factors discussed here.

Thus it may be substantiated that although the two plants, *E. echinatus* and *T. glaberrima*, are known by common trade name 'Brahmadandi' in the Indian market, these were found to exhibit opposite pharmacological activities when administered internally, and hence this may not be considered as to substitute of one for another source.

Reactive oxygen metabolites, such as hydrogen peroxide, superoxide radical, nitric oxide radical etc., appear to play many diverse roles in the maintenance and disruption of cell physiology. On the one hand they have been found to perform cell signalling functions in different cell types, including spermatozoa, and on the other hand they have been linked to ageing, disease and apoptosis (Koshio et al., 1988; Sundquivst, 1991; Yaki, 1993; Aitken et al., 1995). The role of oxidative stress in the aetiology of male infertility has been clearly established by a series of studies (Twigg et al., 1998; Aitken et al., 1998; Sanocka et al., 1996). High concentration of these reactive oxygen species (ROS) is associated with loss of sperm motility and decreased capacity for sperm oocyte fusion (Aitken et al., 1989), thus making the health of the sperms to depend upon the antioxidants. From the results it is observed that there is a significant increase in the activity of superoxide dismutase and catalase, the two powerful antioxidant enzymes of the body, in the testicular homogenate of the animals treated with *E. echinatus* and *T. glaberrima* when compared to that in control animals. In case of *T. glaberrima* such activity may be of immense therapeutic value since these enzymes may help to retain the motility and viability of the sperm by effectively neutralizing the deleterious effects of the ROS and thereby may help in improving the success rate of treating male infertility.

A series of reports have been published on plants as antioxidants under various diseased conditions such as diabetes, cancer, atherosclerosis, arthritis, etc., proving that the mechanism involved in curing these diseases may be from antioxidants and many of them show this property because of their phenolic and flavonoid content. The plants *E. echinatus* and *T. glaberrima* were found to possess anti-radical activity against different free radicals when screened in an in vitro system. The observed anti-radical activity may be due to their content of phenolic compounds, and thus these plants can act both as an active and effective remedy when used in therapeutics by effectively neutralizing the deleterious effects of oxygen radicals.

REFERENCES

1. Aebi HE, 1983. In Methods of Enzymatic Analysis. 3rd ed. Vol. 3, Verlag Chemie, Deerfield Beach, FL. pp. 273-286.
2. Ageel AM, Islam MW, Ginawi OT, Al-Yahya MA, 1994. Evaluation of the Aphrodisiac activity of *Listea chinensis* (Lauraceae) and *Orchis malculata* (Orcidaceae) extracts in rats. *Phytotherapy Research* 3: 103-105.
3. Aitken JR, Clarkson JS, Fishel S, 1989. Generation of reactive oxygen species, lipid peroxidation and human sperm function. *Biology of Reproduction* 40: 183-197.
4. Aitken RJ, Buckingham D, Harkiss D, 1993. Use of a xanthine oxidase free radical generating system to investigate the cytotoxic effects of reactive oxygen species on human spermatozoa. *Journal of Reproduction and Fertility* 97: 441–450
5. Aitken RJ, Emma G, Diana H, Jeremy PT, Philip M, Zoe J, Irvine SD, 1998. Relative Impact of Oxidative Stress on the Functional Competence and Genomic Integrity of Human Spermatozoa. *Biology of Reproduction* 59: 1037–1046.
6. Aitken RJ, Paterson M, Fisher H, Buckingham DW, Duin MV, 1995. Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. *Journal of cell science* 108: 2017–2025.
7. Ananthakumar KV, Srinivasan KK, Shanbhag T, Rao SG, 1994. Aphrodisiac activity of the seeds of *Mucuna pruriens*. *Indian Drugs 31*: 321-327.
8. Angrist B, Gershon S, 1976. Clinical effects of amphetamine and L-dopa on sexuality and aggression. *Comprehensive Psychiatry* 17: 715-722.
9. Anonymous, 1999. National committee for Clinical Laboratory Standards. Procedures for the handling and processing of blood specimens (2nd edn). Approved guideline. NCCLS document M29-A42; Wayne (PA): NCCLS; October, p. 40.
10. Beuchamp C, Fridovich I, 1971. Superoxide dismutase: Improved assays and assay applicable to acrylamide gels. *Analytical Biochemestry* 44: 276-277.

11.Blois MS, 1958. Antioxidant determinations by the use of a stable free radical. *Nature* 26: 1199-1200.

12.Bustos-Obregon E, Gonzalez-Hormazabal P, 2003. Effect of a single dose of malathion on spermatogenesis in mice. *Asian Journal of Andrology* 5:105-107.

13.Carro-Juarez M, Cervantes E, Cervantes-Mendez M, Rodriguez-Manzo G, 2004. Aphrodisiac properties of *Montanoa tomentosa* aqueous crude extract in male rats. *Pharmacology Biochemestry and Behavior* 78: 129-134.

14.Chaturvedi M, Mali PC, Dixit VP, 1995. Fertility regulation in male rats with the help of *Echinops echinatus* (Roxb) root extract. *Journal of Phytological Research* 8: 115-118.

15.Chawla AS, Kapoor VK, Sangal PK, Gupta AK, Evans FJ, 1976. Chemical constituents of Tricholepis glaberrima. *Planta medica* 30: 151-153.

16.Gauthaman K, Adaikan PG, Prasad RNV, 2002. Aphrodisiac properties of *Tribulus terrestris* extract (Protodioscin) in normal and castrated rats. *Life sciences* 71: 1385-1396.

17.Ghosh MN, 1984. Fundamental of experimental pharmacology, Scientific Book Agency, Culcutta, pp 84-88.

18.Griveau JF, Le Lannou D, 1994. Effects of antioxidants on human sperm preparation techniques. *International Journal of Andrology* 17: 225–231.

19.Gupta G, Srivastava AK, Setty BS, 1993. Androgenic regulation of glycolytic and HMP pathway in epididymides and vas deferens of rhesus monkey. *Indian Journal of Experimental Biology* 31: 305-311.

20.Gupta RS, Bhatnager AK, Joshi YC, Sharma MC, Khushalani V, Kachhawa JBS, 2005. Induction of antifertility with Lupeol Acetate in Male Albino Rats. *Pharmacology* 75: 57-62.

21.Gupta RS, Pramod Kumar, Dixit VP, Dobhal MP, 2000. Antifertility studies of the root extract of the *Barleria prionitis* Linn in male albino rats with special reference to testicular cell population dynamics. *Journal of Ethnopharmacology* 70: 111–117.

22.Halliwell B, Gutteridge JMC, 1984. Oxygen toxicity, oxygen radicals, transition metals and diseases. *Biochem J* 219: 1-14.

23.Indian Pharmacopoeia, 1996. Vol. 2. The Controller of Publications, Government of India, Delhi. pp A53-A90.

24.Johnson MH, Everitt BJ, 1988. Essential reproduction. Great Britain: Black Well Scientific Publication, p. 134.

25.Kirtikar KR, Basu BD, 1975. Indian Medicinal Plants vol. 2. Periodical experts, Delhi, pp. 1415-1426.

26.Koshio O, Akanuma Y, Kasuga M, 1988. Hydrogen peroxide stimulates tyrosine phosphorylation of the insulin receptor and its tyrosine kinase activity in intact cells. *Biochemical Journal* 250: 95–101.

27.Lohiya NK, Ansari AS, 1999. Male contraceptive agents. In: Joy, K.P., Krishna, A., Haldar, C. (Eds.), Comparative Endocrinology and Reproduction. New Delhi, Narosa Publishing House, pp. 260- 277.

28.Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, 1951. Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry* 193: 265-275.

29.Manerikar SV, Kulkarni AB, 1978. Chemical investigation of *Tricholepis glaberrima. Indian Journal of Chemistry, Section B* 16: 439-440.

30.Misra HP, Fridovich I, 1972. The generation of superoxide radical during auto oxidation of Hemoglobin. *The journal of biological chemistry* 247 (21): pp 6960-6962.

31.Mukherjee KL, 1997. Medical laboratory technology; a procedure manual for routine diagnostic test, vol 2, Tata McGraw Hill Publication Company Ltd., New Delhi, pp 874-876.

32.Munasinghe TCJ, Seneviratne CK, Thabrew MI, Abeysekera AM, 2001. Antiradical and antilipoperoxidative effects of some plant extracts used by Sri Lankan traditional medical practitioners for cardioprotection. *Phytotherapy Research* 15: 519–523.

33.Oyaizu M, 1986. Studies on product of browning reaction prepared from glucose amine. *Japan Journal of Nutrition* 44: 307-315.

34.Ramachandran S, Sridhar Y, Kishore Gnana Sam S, Saravanan M, Thomas Leonard J, Anbalagan N, Sridhar SK, 2004. Aphrodisiac activity of *Butea frondosa* Koen. ex Roxb. extract in male rats. *Phytomedicine* 11:165-168.

35.Sanocka D, Miesel R, Jedrzejczak P, Kurpisz MK, 1996. Oxidative stress and male infertility. *Journal of Andrology* 17: 449-454.

36.Shan LX, Hardy MP, 1992. Developmental changes in levels of luteinizing hormone receptor and androgen receptor in rat Leydig cells. *Endocrinology.* 131: 1107-1114.

37.Singh B, Ram SN, Pandey VB, Joshi VK, Gambhir SS, 1991. Studies on anti-inflammatory activities of Taraxasterol acetate from Echinops echinatus in rats and mice. *Phytotherapy Research* 5: 103-106.

38.Singh G, Mukherjee T, 1998. Herbal Aphrodisiacs: A Review. *Indian Drugs* 35: 175-182.

39.Soares JR, Dinis TCP, Cunha AP, Almeida LM, 1997. Antioxidant activities of some extracts of Thymus zygis. *Free Radical Research* 26: 469–478.

40.Sundquivst T, 1991. Bovine aortic endothelial cells release hydrogen peroxide. *Journal of Cellular Physiology* 148: 152–156.

41.Taglimonte A, Fratta W, Gessa GL, 1974. Aphrodisiac effect of L-dopa and apomorphine in male sexually sluggish rats. *Experentia* 30: 381-382.

42.Toney TW, Danzo BJ, 1989. Estrogen and androgen regulation of protein synthesis by the immature rat epididymis. *Endocrinology.* 125: 231-242.

43.Topcu G, 2006. Bioactive Triterpenoids from *Salvia* Species. *Journal of Natural Products* 69: 482-487.

44.Toyokuni S, Tanaka T, Kawaguchi W, Fang NR, Ozeki M, Akatsuka S, Hiai H, Okezie I, Bahorun T, 2003. Effect of the Phenolic contents of Mauritian endemic plant extract on promoter activities of antioxidant enzymes. *Free Radical Research* 37: 1215-1224.

45.Twigg J, Fulton N, Gomez E, Irvine DS, Aitken RJ, 1998. Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. *Human Reproduction* 13: 1429–1436.

46.Ulusoy E, Cayan S, Yilmaz N, Aktas S, Acar D, Doruk E, 2004. Interferon α-2B may impair testicular histology including spermatogenesis in a rat model. *Archives of Andrology* 50:379–385.

47.Vani T, Rajani M, Sarkar S, Shishoo CJ, 1997. Antioxidant properties of the Ayurvedic formulation Triphala and its constituents. *International Journal of Pharmacognosy* 35(5): 313-317.

48.Yaki K, 1993. Lipid peroxides free radicals and disease. In: Yaki K. *Active Oxygens, Lipid Peroxides and Antioxidants*. Boca Raton: CRC Press, pp. 1–38.