



# PROTECTIVE EFFECT OF AN HERBAL EXTRACT IN AMLODIPINE INDUCED TESTICULAR DYSFUNCTION IN RATS

SHINI DOMINIC \* AND V.PADMAJA

College of Pharmaceutical Sciences, Govt. Medical College, Thiruvananthapuram

## Key words

Amlodipine, *Asteracantha longifolia*, testicular dysfunction

## Correspondence

Shini Dominic M.Pharm, Ph.D.  
College of Pharmaceutical Sciences,  
Govt. Medical College,  
Thiruvananthapuram, Kerala-695011

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## ABSTRACT

**Plan:** To verify the protective effects of the ethanolic extract of *Asteracantha longifolia* seeds in Amlodipine induced testicular dysfunction in rats.

**Preface:** The widely used Dihydropyridine type calcium channel blockers like Amlodipine have reported to be causing some reproductive toxicity in males. But no studies have been made so far to nullify the toxicity of this widely prescribed group of drugs. So, an attempt is done here to nullify the reproductive toxicity of Amlodipine.

**Methodology:** Biochemical and histological parameters of testes and different sperm parameters were analysed in ten different groups of young Wister albino rats treated with Amlodipine alone, and Amlodipine along with two different doses of ethanolic extract of *Asteracantha longifolia* seeds for three different periods of drug administration.

**Outcome:** Co administration of the ethanolic extract of *Asteracantha longifolia* seeds was found to be providing a significant protection ( $P < 0.05$ ) against Amlodipine induced testicular dysfunction in rats.

## 1. INTRODUCTION

Calcium channel blockers (CCBs) are a widely used class of drugs in human and veterinary diseases since their introduction in 1960's. They are used for the treatment of different cardiovascular disorders like hypertension, arrhythmia and angina pectoris. Besides they are also used in migraine headache and for their tocolytic and other smooth muscle relaxant properties. According to their chemical structures the different types of CCBs include dihydropyridine derivatives (DHPs) like Nifedipine and Amlodipine, phenylalkylamines like Verapamil, benzothiazepinones like Diltiazem etc.

Corresponding author email: [shinidm@gmail.com](mailto:shinidm@gmail.com)

Contact: 0091 9446304101

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DHPs have greater selectivity for vascular smooth muscles than myocardium because they block smooth muscle calcium channels at concentrations below than those required for significant cardiac effects. In other words, DHPs have much affinity for vascular calcium channels than for calcium channels in heart<sup>[1]</sup>. As contraction of vascular smooth muscles is dependent on the free intracellular concentration of calcium ions, inhibition of transmembrane movement of calcium ions through sensitive calcium channels can decrease the total amount of calcium that reaches intracellular sites. Thus all calcium channel blockers lower blood pressure mainly by relaxing arteriolar smooth muscles and thus decreasing peripheral vascular resistance.<sup>[2]</sup>

Though CCBs are widely used, many data accumulated over the past few years have revealed that these agents have the potential to cause infertility in males. Dihydropyridine (DHP) calcium channel blockers which are the established drugs for chronic stable angina and hypertension are commonly associated with infertility.<sup>[3, 4]</sup>

Following the clinical reports during the 1994 -1997 period regarding the infertility issues associated with the use of these drugs, a limited number of experimental studies done in “small laboratory animals” such as rats and mice also confirmed the anti-fertility potential of these drugs in males. But no attempts have been made so far to nullify the reproductive toxicity of this widely prescribed group of drugs.

With the hope of filling this gap, the present study was planned to find out whether a widely available herbal drug can revert the reproductive toxicity associated with the use of CCBs. For this study the widely available CCB Amlodipine was selected as the test drug and the ethanolic extract of a traditionally used fertility booster, namely, the seeds of *Asteracantha longifolia* (Vayalchulli) was taken as the herbal preparation.

In the toxicity evaluation of ethanolic extract of *Asteracantha longifolia* seeds, based on both acute and sub-acute toxicity studies in Wistar albino rats conducted in our laboratories it was found that the ethanolic extract of *Asteracantha longifolia* seeds is safe for oral administration at low (100mg/kg) and moderate (250 mg/kg) doses while high dose (500mg/kg) is not absolutely free from toxic effects.<sup>[5]</sup> So, in the present study the low (100mg/kg) and moderate (250 mg/kg) doses of the ethanolic extract of *Asteracantha longifolia* seeds were selected.

The maximum dose of Amlodipine for different cardiovascular ailments is 10 mg p.o daily. So the rat equivalent of this clinical dose of Amlodipine which is 0.9 mg/Kg body weight of rats was selected as the test dose here.<sup>[6]</sup>

The toxic effects of Amlodipine on the reproductive system of male Wistar albino rats, when administered orally in the therapeutic dose for different periods and its modifications, if any, by the co administration of the ethanolic extract of *Asteracantha longifolia* seeds, can be assessed by evaluating the oxidative stress and histopathology of testes, by evaluating the semen and by verifying the gonado somatic index etc.

Semen analysis is the initial and most essential step in the evaluation of infertility. It is considered as the cornerstone of the laboratory evaluation of the infertile male and it helps to define the severity of male factor infertility. A carefully performed sperm analysis is the primary source of information on sperm production and reproductive tract patency. The gonado somatic index or GSI is a tool for measuring sexual maturity of animals and it indicates gonadal mass as a proportion of total body mass. Similarly, drug-induced oxidative stress is implicated as a mechanism of toxicity in numerous tissues and organs. In the testes also oxidative stress is a powerful mechanism that can lead to sperm damage, deformity and eventually, male infertility. [7] A large proportion of infertile men have elevated levels of seminal reactive oxygen species. [8]. Similarly histological evaluation of test animal tissues has a prominent role in the assessment of male reproductive risk. It provides information on the severity of the toxicity and cellular site of the damage. Besides it gives an idea about the potential of the tissue to recover from the damage.

## **2. MATERIALS AND METHODS**

### *2.1. Sample Collection*

Pure sample of Amlodipine was collected from Dr.Reddy's Laboratories, Hyderabad.

### *2.2. Preparation of Herbal Extract*

The seeds of *Asteracantha longifolia* collected from Thiruvananthapuram and authenticated by Dr.M.A.Shajahan, M.D (Ayu), PhD, Professor & HOD, Govt. Ayurveda College and Hospital, Thiruvananthapuram were used for the study.

The dried seeds were coarsely powdered and extracted using rectified spirit by the method of Continuous hot extraction (Soxhlet extraction)

### *2.3. Ethical Clearance:*

The entire study protocol was approved by the Institutional Animal Ethical Committee, Medical College, Thiruvananthapuram. (IAEC No - 02/15/2010/MCT dated 08-06-2010)

#### *2.3.1. Animal Selection, Maintenance and Care*

Healthy male albino rats of 5-6 months of age (Wistar strain) weighing 180-220g were used for the study. They were obtained from animal house, Medical College, Thiruvananthapuram. These animals were fed on standard pellet diet (Manufactured by Nav Maharashtra Chakan Oil Mills Ltd; Pune and supplied by Sai Durga Feeds and Foods, Bangalore) and water ad libitum. The animals were maintained under standard conditions of relative humidity, 12 hours light-dark cycle, adequate ventilation and ambient room temperature.

These rats were divided into different groups such as

- Group I - Control-0.3% CMC in a dose of 1ml/ 100gm body weight
- Group II - Treated with Amlodipine 0.9 mg/kg once daily for 28 days.
- Group III - Treated with Amlodipine 0.9 mg/kg and *Asteracantha longifolia* seed extract 100mg/kg once daily for 28 days.
- Group IV - Treated with Amlodipine 0.9 mg/kg and *Asteracantha longifolia* seed extract 250mg/kg once daily for 28 days
- Group V - Treated with Amlodipine 0.9 mg/kg once daily for 42 days.
- Group VI - Treated with Amlodipine 0.9 mg/kg and *Asteracantha longifolia* seed extract 100mg/kg once daily for 42 days.
- Group VII - Treated with Amlodipine 0.9 mg/kg and *Asteracantha longifolia* seed extract 250mg/kg once daily for 42 days.
- Group VIII - Treated with Amlodipine 0.9 mg/kg once daily for 65 days.
- Group IX - Treated with Amlodipine 0.9 mg/kg and *Asteracantha longifolia* seed extract 100mg/kg once daily for 65 days.
- Group X - Treated with Amlodipine 0.9 mg/kg and *Asteracantha longifolia* seed extract 250mg/kg once daily for 65 days.

(In each group 6 numbers of rats were present and the drugs were administered orally)

After completing the specified treatment period, the rats were weighed and euthanized by cervical dislocation, the peritoneal cavity was opened through a lower transverse abdominal incision and the testes were removed along with the epididymis for determining the following.

- *Sperm parameters like sperm count, sperm motility and GSI*
- *Biochemical parameters of testes including the level of*
  - *Testicular protein*
  - *Malondialdehyde*
  - *Superoxide dismutase*
  - *Catalase*
- *Histological examination of testes*

## 2.4. Sperm Parameters

### 2.4.1. Sperm Count

For sperm count, the cauda epididymal plasma was suspended in phosphate buffer saline (PBS) taken in a watch glass, cleared the tissue debris and the clear solution was drawn up to the 0.5 mark of WBC pipette. The semen diluting fluid (prepared by dissolving 5 g of Sodium bicarbonate ( $\text{NaHCO}_3$ ) and 1 ml of 40% Formaldehyde in 100 ml of Normal saline) was drawn up to the 11 mark of the pipette and mixed thoroughly. After discarding the first 1-2 drops, one drop each was added to both sides of Neubauer's haemocytometer. The spermatozoa were allowed to settle down in the haemocytometer by keeping the loaded haemocytometer in a humid chamber for 5 minutes. The sperm count was done with the high power objective (40X) as in RBC counting. The total numbers of sperms were counted in the 5 major squares on each side of the haemocytometer and determined the average for calculating sperm count. [9]

The haemocytometer is 0.1mm deep and the 25 large squares represent an area of 1 square millimeter with a volume of 0.1  $\mu\text{l}$ . When 5 squares are counted, the sperms which are settled out of 0.02  $\mu\text{l}$  only are counted. Therefore count in 5 squares must be multiplied by 50000, in order to determine the total sperm count in 1.0 ml semen.

To get the sperm concentration of the original cauda epididymal semen sample the dilution factor was also accounted and the sperm count was calculated as

$$\text{Sperm count/ml} = (\text{dilution factor}) (\text{count in five squares}) (0.05 \times 10^6)$$

#### 2.4.2. Sperm Motility

The sperm motility was determined microscopically within 5 minutes of their isolation from epididymis. The epididymal plasma was suspended in PBS, cleared the tissue debris and a clear solution was used for the assessment of motility of sperms. The total number of motile sperms was counted similarly as in sperm count. Motility was expressed as percentage of motile sperms compared to the total sperm count<sup>[10]</sup>

#### 2.4.3. Gonado-Somatic Index (GSI)

Immediately after the isolation weights of the right and left testes were noted separately and Gonado-somatic index was determined with the help of formula<sup>[11]</sup>

$$\text{GSI} = (\text{Gonad weight/total body weight}) \times 100$$

$$\text{Gonad weight} = (\text{weight of the right testis} + \text{weight of the left testis})/2$$

#### 2.5. Biochemical Parameters

##### 2.5.1. Estimation of Oxidative Stress and Anti-oxidant Enzyme Assay

##### 2.5.2. Preparation of Tissue Homogenate

At the end of the different durations of treatment with Amlodipine, rats were sacrificed by cervical dislocation and the right testis was used for determining the biochemical parameters.

Right testis was weighed, sliced and added to the definite volume of a solution of Tris hydrochloride buffer (pH 7.2) in order to get a 10% (w/v) tissue homogenate. The mixture was then taken in a clean sterile centrifuge tube and homogenized to get a 10% tissue homogenate. The homogenate was centrifuged at 15,000 rpm for 15 minutes and the supernatant was used for antioxidant assay.<sup>[12,13]</sup>

##### 2.5.3. Estimation of Protein Level in Testes

The amount of soluble protein present in the tissue extract can be determined by the standard method proposed by Biuret et al using bovine serum albumin as the standard. .<sup>[14]</sup>

To 20µl of the prepared testicular homogenate added 1ml of Biuret reagent and the absorbance was noted at 540 nm (using JascoV-560 double beam spectrophotometer) at room temperature. The protein content present in unit quantity of the testicular homogenate was calculated using the following equation.

$$\text{Protein (mg/g)} = \frac{\text{Optical density of test} \times \text{con of standard (mg \%)}}{\text{Optical density standard} \times 10}$$

#### 2.5.4. Lipid Peroxidation Assay

Mammalian spermatozoa are rich in poly unsaturated fatty acids (PUFA) which upon lipid peroxidation form PUFA peroxides. Malondialdehyde (MDA) is one of the several low-molecular-weight end products formed via the decomposition of PUFA peroxides. MDA is a marker for oxidative stress and the variations in the level of MDA in biological samples indicate the extent of oxidative stress or lipid peroxidation in these samples.<sup>[15]</sup>

To 0.2 ml of the testicular homogenate added 0.2ml 8.1% Sodium dodecyl sulphonate (SDS), 1.5 ml of 20% acetic acid solution having a pH of 3.5 (adjusted with NaOH), and 1.5ml of 0.8% TBA. The mixture was made up to 4.0 ml with distilled water and heated in a water bath at 95<sup>0</sup> C for 60 minutes.

Then the mixture was cooled under tap water and 5.0 ml of a mixture of n-butanol and pyridine in the ratio 15:1 v/v was added to it and shaken vigorously.

The mixture was centrifuged at 4000 rpm for 10 minutes, the organic layer was separated and its absorbance was taken at 532 nm. (using JascoV-560 double beam spectrophotometer) Pure sample of butanol-pyridine mixture was used as blank.

The intensity of lipid peroxidation was expressed as nano moles of MDA per mg of protein using molar absorptivity as  $1.56 \times 10^5 \text{ M cm}^{-1}$ .

$$\text{MDA} = \frac{\text{Absorbance} \times \text{D.F} \times 10^9}{\text{Molar absorptivity}}$$

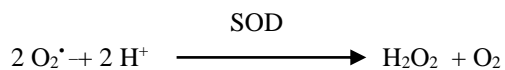
\*Where D.F is dilution factor

#### 2.5.5. Assay of Enzymatic Antioxidants

The different enzymatic and non-enzymatic antioxidants present in animal tissues protect them from the damaging effects of reactive oxygen species (ROS). Superoxide dismutase (SOD) and Catalase are two of the enzymatic antioxidants present in the sperm and seminal fluid. By determining their level in the testicular homogenate the antioxidant status of the testes can be assessed.

#### 2.5.6. Superoxide Dismutase Assay

Superoxide dismutase is a group of metal containing enzymes that catalyse the dismutation of highly reactive superoxide anion  $\text{O}_2^{\bullet -}$  to molecular oxygen and to the less reactive hydrogen peroxide species.



It is an important antioxidant defence in nearly all cells exposed to oxygen. The SOD activity was expressed as units per milligram of testicular protein.<sup>[16]</sup>

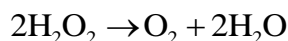
The assay was carried out in the testicular homogenate prepared. The reaction mixture of control, light control, blank and test solution in four different test tubes were prepared by adding 0.1ml of 1.5 M sodium carbonate, 0.3ml of 0.13M methionine, 0.3ml of 10mM EDTA, 0.3ml of 0.63mM fresh NBT (absent in blank), 0.3ml of 13µM riboflavin and 0.1ml homogenate. (Absent in blank and in light control) The mixture in each tube was made up to a final volume of 3ml using 50mM phosphate buffer, the control tubes were kept in darkness and light control tubes were kept under an incandescent lamp. After 30 minutes of incubation, the optical density of test, light control and control solution were read at 560nm using JascoV-560 double beam spectrophotometer. Superoxide dismutase activity of the testicular homogenate in units/mg protein was determined using the equation

$$\text{Percentage inhibition} = \frac{\text{absorbance of light control} - \text{sample absorbance}}{\text{absorbance of light control}}$$

\*Sample absorbance = absorbance of test- absorbance of control

### 2.5.7. Catalase Assay

Catalase is an intracellular antioxidant enzyme mainly located in cellular peroxisomes and to some extent in the cytosol of mammalian cells. If the concentration of H<sub>2</sub>O<sub>2</sub> is high, Catalase catalyses the decomposition of hydrogen peroxide to water and oxygen.



Catalase activity was estimated by the method of Aebi (1984) where one unit of Catalase decomposes 1.0 µmole of H<sub>2</sub>O<sub>2</sub> per minute. The rate of disappearance of H<sub>2</sub>O<sub>2</sub> is followed by observing the rate of decrease in the absorbance at 240 nm. (Using JascoV-560 double beam spectrophotometer)

50µl of the prepared testicular tissue homogenate was added to a 3ml cuvette containing 1.95 ml of 50mM phosphate buffer. (pH 7.0) To this mixture 1.0 ml of 30mM H<sub>2</sub>O<sub>2</sub> was added and the change in absorbance was followed for 75 seconds at 240 nm at 15 sec intervals using JascoV-560 double beam spectrophotometer. Pure sample of 50mM phosphate buffer was taken as blank.

$$\text{Catalase activity} = \frac{A}{\text{Extinction coefficient} \times \text{volume of sample in ml} \times \text{mg of protein}}$$

\*A= difference in absorbance/ minute, Molar extinction coefficient of hydrogen peroxide = 0.071mmol/cm

The catalase activity of homogenate was expressed in terms of micro moles of hydrogen peroxide decomposed/min/mg protein.<sup>[17]</sup>

### 2.6. Histological Parameters

At the end of treatment with Amlodipine alone and with the two different doses of the extract for 42 and 65 days, animals were sacrificed by cervical dislocation and the left testis was used for histological studies.

For the histological studies the testis was fixed in Bouin's fixative which is a saturated aqueous solution of picric acid prepared by putting an excess amount of picric acid in distilled water for three days and taking out the supernatant. After fixing the tissue it was dehydrated in various grades of ethanol, embedded in paraffin wax and the sections were stained with Haematoxylin and Eosin. The slides were examined through a trinocular microscope (Leica ATC 2000) attached with a video camera. <sup>[18]</sup>

### 3. Statistical Analysis of the Data

At the end of studies all the data were analysed using the software SPSS, version 16. (One way ANOVA and post Hoc Turkey's)

All the values were expressed as mean  $\pm$  SEM, taking  $p < 0.05$  as significant. <sup>[19]</sup>

## 4. RESULTS AND DISCUSSION

### 4.1. Sperm Parameters

The effects of the co administration of two different doses of the ethanolic extract of *Asteracantha longifolia* seeds p.o daily, along with Amlodipine for three different durations of 28 days, 42 days and 65 days, on different sperm parameters like sperm count, sperm motility and Gonado somatic index etc. of Wistar albino rats are summarized in the table 1

Table 1. Effects of extract treatment on Amlodipine induced variations in sperm parameters of Wistar albino rats.

Group	Sperm count (million/ml)	Sperm motility (%)	GSI
I-Control	55.67 $\pm$ 1.584	82.13 $\pm$ 1.891	0.612 $\pm$ 0.034
II-Aml 28 days	51.00 $\pm$ 0.966	69.50 $\pm$ 1.746	0.549 $\pm$ 0.013
III-Aml 28+ext100mg	54.33 $\pm$ 2.170	80.83 $\pm$ 3.092	0.602 $\pm$ 0.017
IV-Aml28+ext 250mg	56.16 $\pm$ 2.088	84.16 $\pm$ 3.102#	0.616 $\pm$ 0.023
V-Aml 42 days	45.50 $\pm$ 1.087	52.33 $\pm$ 1.891	0.415 $\pm$ 0.020
VI-Aml 42+ext100mg	55.66 $\pm$ 2.123	81.83 $\pm$ 3.360^	0.541 $\pm$ 0.021^
VII-Aml42+ext 250mg	57.33 $\pm$ 2.962^	85.16 $\pm$ 3.198^	0.571 $\pm$ 0.024^
VIII-Aml 65 days	44.17 $\pm$ 2.948	50.33 $\pm$ 2.108	0.528 $\pm$ 0.026
IX-Aml 65+ext 100mg	56.83 $\pm$ 2.663^^	83.66 $\pm$ 3.441^^	0.621 $\pm$ 0.022
X-Aml 65+ext 250mg	60.50 $\pm$ 2.526^^	86.16 $\pm$ 2.700^^	0.631 $\pm$ 0.017
F value	F <sub>(9,50)</sub> =5.625	F <sub>(9,50)</sub> =24.909	F <sub>(9,50)</sub> =8.089

All values are mean  $\pm$  SEM of (n=6). Statistical analysis is by one-way ANOVA followed by post Hoc Turkey's. # denotes  $p$  value  $< 0.05$ , compared to the Amlodipine treatment group of 28 days, ^ denotes  $p$  value  $< 0.05$ , compared to the Amlodipine treatment group of 42 days, ^^ denotes  $p$  value  $< 0.05$ , compared to the Amlodipine treatment group of 65 days.



#### 4.1.1. Sperm Count

Table 1 and Fig.1 show the effects of co-administration of two different doses of the ethanolic extract of *Asteracantha longifolia* seeds p.o daily for three different periods, on Amlodipine induced decrease in sperm count, in Wistar albino rats,.

Amlodipine treatment for 28 days in a dose of 0.9mg/ kg p.o daily decreased the sperm count from the control value of  $(55.67 \pm 1.584)10^6$  /ml to  $(51.00 \pm 0.966)10^6$  /ml. When the ethanolic extract of *Asteracantha longifolia* seeds in a dose of 100 mg/kg p.o was co administered with Amlodipine in the dose of 0.9mg/ kg p.o, daily for 28 days, the sperm count was increased to  $(54.33 \pm 2.170)10^6$ /ml and the co administration of the extract in the dose of 250 mg/kg p.o per day for 28 days improved the sperm count to  $(56.16 \pm 2.088)10^6$ /ml.

When Amlodipine was administered for 42 days in a dose of 0.9mg/ kg p.o per day the sperm count was reduced to  $(45.50 \pm 1.087) 10^6$  /ml from the control value of  $(55.67 \pm 1.584)10^6$  /ml. But when the ethanolic extract of *Asteracantha longifolia* seeds in a dose of 100 mg/kg p.o per day was co administered with Amlodipine for 42 days the sperm count was increased to  $(55.66 \pm 2.123)10^6$  /ml and the co administration of the extract in the dose of 250 mg/kg p.o per day for 42 days significantly ( $p < 0.05$ ) improved the sperm count to  $(57.33 \pm 2.962)10^6$  /ml, compared to the Amlodipine treatment group of 42 days.

Similarly Amlodipine treatment for 65 days in a dose of 0.9mg/ kg p.o daily decreased the sperm count from the control value of  $(55.67 \pm 1.584)10^6$  /ml to  $(44.17 \pm 2.948)10^6$  /ml. But the co administration of the ethanolic extract of *Asteracantha longifolia* seeds in the doses of 100 mg/kg p.o per day and 250 mg/kg p.o per day for these 65 days significantly improved the sperm count to  $(56.83 \pm 2.663)10^6$  /ml and to  $(60.50 \pm 2.526)10^6$  /ml respectively, compared to the Amlodipine treatment group of 65 days. ( $p < 0.05$ )

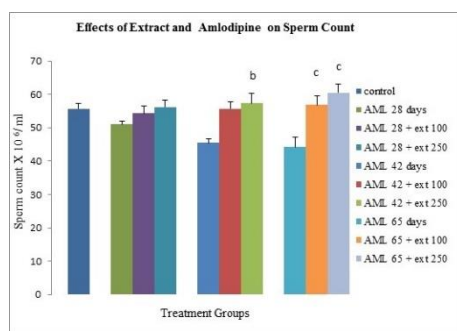


Fig.1.Effects of *Asteracantha longifolia* seed extract and Amlodipine on sperm count of Wistar albino rats.

(All values are mean ± SEM of (n=6), 'b' denotes p value < 0.05, compared to the Amlodipine treated group of 42 days, 'c' denotes p value < 0.05, compared to the Amlodipine treated group of 65 day

#### 4.1.2. Sperm Motility

Table 1 and Fig.2 show the effects of co-administration of two different doses of the ethanolic extract of *Asteracantha longifolia* seeds p.o daily for three different periods, on Amlodipine induced decrease in sperm motility, in Wistar albino rats.

Amlodipine treatment for 28 days in a dose of 0.9mg/ kg p.o daily decreased the sperm motility from the control value of (82.13±1.891) % to (69.50±1.746) %. When the ethanolic extract of *Asteracantha longifolia* seeds in a dose of 100 mg/kg p.o per day was co administered with Amlodipine for 28 days the sperm motility was increased to (80.83±3.092) % and the co administration of the extract in the dose of 250 mg/kg p.o per day for 28 days significantly (p<0.05) improved the sperm motility to (84.16±3.102) %, compared to the Amlodipine treatment group of 28 days.

When Amlodipine was administered for 42 days in a dose of 0.9mg/ kg p.o daily the sperm motility was reduced to (52.33±1.891) % from the control value of (82.13±1.891) %. But when the ethanolic extract of *Asteracantha longifolia* seeds in a dose of 100 mg/kg p.o per day was co administered with Amlodipine for these 42 days the sperm motility was increased significantly to (81.83±3.360) % and the co administration of the extract in the dose of 250 mg/kg p.o per day for 42 days also significantly improved the sperm motility to (85.16±3.198) %, both compared to the Amlodipine treatment group of 42 days. (p< 0.05)

Similarly Amlodipine treatment for 65 days in a dose of 0.9mg/ kg p.o per day decreased the sperm motility from the control value of (82.13±1.891) % to (50.33±2.108) %. But the co administration of the ethanolic extract of *Asteracantha longifolia* seeds in the doses of 100 mg/kg p.o per day and 250 mg/kg p.o per day for these 65 days significantly improved the sperm motility to (83.66±3.441) % and to (86.16±2.700) % respectively, compared to the Amlodipine treatment group of 65 days. (p< 0.05)

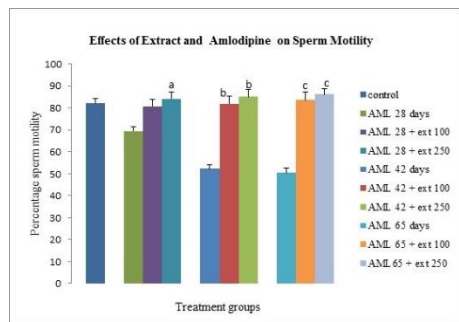


Fig.2.Effects of *Asteracantha longifolia* seed extract and Amlodipine on sperm motility of Wistar albino rats.

(All values are mean ± SEM of (n=6), 'a' denotes p value < 0.05, compared to the Amlodipine treated group of 28 days, 'b' denotes p value < 0.05, compared to the Amlodipine treated group of 42 days, 'c' denotes p value < 0.05, compared to the Amlodipine treated group of 65 days)

#### 4.1.3. Gonado-Somatic Index (GSI)

Table 1 and Fig.3 show the effects of co-administration of two different doses of the ethanolic extract of *Asteracantha longifolia* seeds p.o daily for three different periods, on Amlodipine induced decrease in GSI, in Wistar albino rats.

When Amlodipine treatment for 28 days in a dose of 0.9mg/kg p.o per day decreased the GSI from the control value of 0.612±0.034 to 0.549±0.013, the co-administration of the ethanolic extract of *Asteracantha longifolia* seeds in the doses of 100mg/kg p.o per day and 250 mg/kg p.o per day for these 28 days improved the GSI to 0.602±0.017 and to 0.616±0.023 respectively.

Similarly Amlodipine treatment for 42 days in a dose of 0.9mg/kg p.o per day decreased the GSI from the control value of  $0.612 \pm 0.034$  to  $0.415 \pm 0.020$ .

But, when the ethanolic extract of *Asteracantha longifolia* seeds in a dose of 100 mg/kg p.o per day was co administered with Amlodipine for 42 days the GSI was increased significantly to  $0.541 \pm 0.021$ . ( $p < 0.05$ ) and the co administration of the extract in the dose of 250 mg/kg p.o per day for 42 days also significantly improved the GSI to  $0.571 \pm 0.024$ , ( $p < 0.05$ ) both compared to the Amlodipine treatment group of 42 days.

At the same time, when Amlodipine treatment for 65 days in a dose of 0.9mg/ kg p.o per day decreased the GSI from the control value of  $0.612 \pm 0.034$  to  $0.528 \pm 0.026$ , the co-administration of the ethanolic extract of *Asteracantha longifolia* seeds in the doses of 100 mg/kg p.o per day and 250 mg/kg p.o per day for these 65 days improved the GSI only to  $0.621 \pm 0.022$  and to  $0.631 \pm 0.017$  respectively which were not significant.

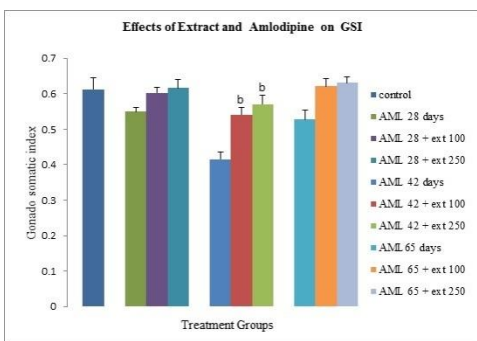


Fig.3. Effects of *Asteracantha longifolia* seed extract and Amlodipine on GSI of Wistar albino rats.

(All values are mean  $\pm$  SEM of (n=6), ‘b’ denotes p value  $< 0.05$ , compared to the Amlodipine treated group of 42 days.)

## 4.2. Biochemical Parameters

The effects of the co administration of two different doses of the ethanolic extract of *Asteracantha longifolia* seeds p.o daily, along with Amlodipine for three different durations of 28 days, 42 days and 65 days, on different testicular biochemical parameters including the level of protein, MDA, SOD, Catalase etc. of Wistar albino rats are summarized in the table 2.

### 4.2.1. Protein

Table 2 and Fig.4 show the effects of co-administration of two different doses of the ethanolic extract of *Asteracantha longifolia* seeds p.o daily for three different periods, on Amlodipine induced decrease in the level of protein, in the testicular homogenate of Wistar albino rats.

Amlodipine treatment for 28 days in a dose of 0.9mg/ kg p.o per day decreased the level of protein in the testicular homogenate from the control value of  $(0.0466 \pm 0.0036)$ mg/g to  $(0.0431 \pm 0.0023)$ mg/g.

When the ethanolic extract of *Asteracantha longifolia* seeds in a dose of 100 mg/kg p.o per day was co administered with Amlodipine for these 28 days the level of protein in the testicular homogenate was increased to (0.0460±0.0024)mg/g and the co administration of the extract in the dose of 250 mg/kg p.o per day for these 28 days improved the level of protein in the testicular homogenate to (0.0481±0.0003)mg/g.

Amlodipine treatment for 42 days in a dose of 0.9mg/ kg p.o per day decreased the level of protein in the testicular homogenate from the control value of (0.0466±0.0036) mg/g to (0.0394±0.0027)mg/g. When the ethanolic extract of *Asteracantha longifolia* seeds in a dose of 100 mg/kg p.o per day was co administered with Amlodipine for these 42 days the level of protein in the testicular homogenate was increased to (0.0483±0.0003) mg/g and the co administration of the extract in the dose of 250 mg/kg p.o per day for these 42 days significantly improved the level of protein in the testicular homogenate to (0.0513±0.0021)mg/g, compared to the Amlodipine treatment group of 42 days. (p< 0.05)

Table.2. Effects of extract treatment on Amlodipine induced variations in testicular biochemical parameters of Wistar albino rats

Group	Protein (mg/g)	MDA (nmole/mg protein)	SOD (unit/mg protein)	Catalase ( $\mu$ molesH <sub>2</sub> O <sub>2</sub> decomposed/min/mg protein)
I-Control	0.0466±0.0036	5.706±0.181	5.826±0.523	48.988±1.647
II-Aml 28 days	0.0431±0.0023	7.129±0.400	4.251±0.173	36.437±1.473
III-Aml 28+ ext 100mg	0.0460±0.0024	5.667±0.223#	5.860±0.276#	46.173±2.885
IV-Aml 28+ ext.250mg	0.0481±0.0003	5.118±0.275#	5.926±0.319#	50.067±2.917#
V-Aml 42 days	0.0394±0.0027	9.058±0.326	3.467±0.177	25.341±1.803
VI-Aml 42+ ext100mg	0.0483±0.0003	5.080±0.287^	5.974±0.292^	51.482±3.057^
VII-Aml 42+ ext 250mg	0.0513±0.0021^	4.008±0.174^	6.409±0.314^	57.584±3.434^
VIII-Aml 65 days	0.0389±0.0007	12.439±0.299	2.909±0.170	20.242±1.499
IX-Aml 65+ ext 100mg	0.0522±0.0019^^	4.889±0.269^^	6.117±0.210^^	58.671±2.908^^
X-Aml 65+ ext 250mg	0.0611±0.0025^^	2.945±0.252^^	8.735±0.365^^	83.457±2.913^^
F value	F <sub>(9,50)</sub> = 8.913	F <sub>(9,50)</sub> = 98.817	F <sub>(9,50)</sub> = 30.282	F <sub>(9,50)</sub> = 49.352

All values are mean ± SEM of (n=6). Statistical analysis is by one-way ANOVA followed by post Hoc Turkey's. # denotes p value < 0.05, compared to the Amlodipine treatment group of 28 days, ^ denotes p value < 0.05, compared to the Amlodipine treatment group of 42 days, ^^ denotes p value < 0.05, compared to the Amlodipine treatment group of 65 days.

Similarly, when Amlodipine treatment for 65 days in a dose of 0.9mg/ kg p.o per day decreased the level of protein in the testicular homogenate from the control value of (0.0466±0.0036)mg/g to (0.0389±0.0007)mg/g, the co-administration of the ethanolic extract of *Asteracantha longifolia* seeds in the doses of 100 mg/kg p.o per day and 250 mg/kg p.o per day for these 65 days significantly improved the level of protein in the testicular homogenate to (0.0522±0.0019) mg/g and (0.0611±0.0025) mg/g respectively, compared to the Amlodipine treatment group of 65 days. (p< 0.05).

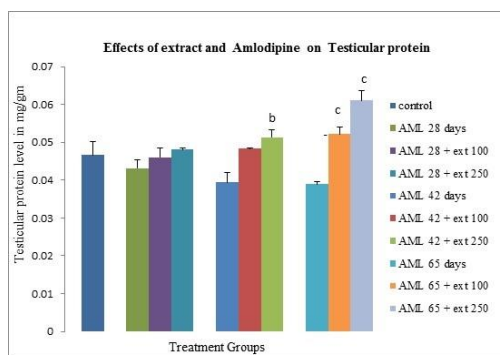


Fig.4. Effects of *Asteracantha longifolia* seed extract and Amlodipine on testicular protein of Wistar albino rats.

(All values are mean ± SEM of (n=6), 'b' denotes p value < 0.05, compared to the Amlodipine treated group of 42 days, 'c' denotes p value < 0.05, compared to the Amlodipine treated group of 65 days)

#### 4.2.2. Malondialdehyde

Table 2 and Fig.5 show the effects of co-administration of two different doses of the ethanolic extract of *Asteracantha longifolia* seeds p.o daily for three different periods, on Amlodipine induced increase in the level of Malondialdehyde, in the testicular homogenate of Wistar albino rats.

Amlodipine treatment for 28 days in a dose of 0.9mg/ kg p.o per day increased the level of Malondialdehyde in the testicular homogenate from the control value of (5.706±0.181) units (nmole/mg protein) to (7.129±0.400) units. When the ethanolic extract of *Asteracantha longifolia* seeds in a dose of 100 mg/kg p.o per day was co administered with Amlodipine for 28 days the level of Malondialdehyde in the testicular homogenate was decreased significantly to (5.667±0.223) units and the co administration of the extract in the dose of 250 mg/kg p.o per day for these 28 days significantly reduced the level of Malondialdehyde in the testicular homogenate to (5.118±0.275) units, both compared to the Amlodipine treatment group of 28 days. (p< 0.05)

Similarly Amlodipine treatment for 42 days in a dose of 0.9mg/kg p.o per day increased the level of Malondialdehyde in the testicular homogenate from the control value of (5.706±0.181) units (nmole/mg protein) to (9.058±0.326) units. When the ethanolic extract of *Asteracantha longifolia* seeds in a dose of 100 mg/kg p.o per day was co administered with Amlodipine for 42 days the level of Malondialdehyde in the testicular homogenate was decreased significantly to (5.080±0.287) units and the co administration of the extract in the dose of 250 mg/kg p.o per day for these 42days significantly reduced the level of Malondialdehyde in the testicular homogenate to (4.008±0.174) units, both compared to the Amlodipine treatment group of 42 days. (p< 0.05)

Amlodipine treatment for 65 days in a dose of 0.9mg/ kg p.o per day increased the level of Malondialdehyde in the testicular homogenate from the control value of (5.706±0.181) units (nmole/mg protein) to 12.439±0.299 units. But the co administration of the ethanolic extract of *Asteracantha longifolia* seeds in the doses of 100 mg/kg and 250 mg/kg p.o per day for these 65 days significantly ( $p<0.05$ ) reduced the level of Malondialdehyde in the testicular homogenate to (4.889±0.269) units and (2.945±0.2520 units respectively, compared to the Amlodipine treatment group of 65 days. Besides these values were less than even the control values.

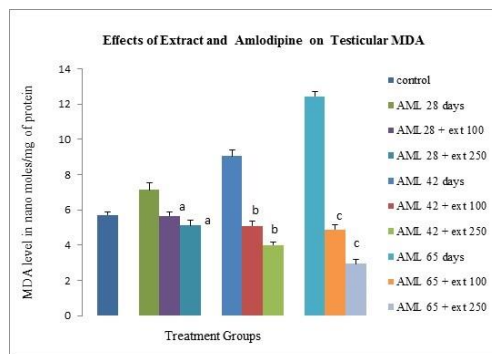


Fig.5. Effects of *Asteracantha longifolia* seed extract and Amlodipine on testicular MDA of Wistar albino rats.

(All values are mean ± SEM of (n=6), 'a' denotes p value < 0.05, compared to the Amlodipine treated group of 28 days, 'b' denotes p value < 0.05, compared to the Amlodipine treated group of 42 days, 'c' denotes p value < 0.05, compared to the Amlodipine treated group of 65 days)

#### 4.2.3. Superoxide Dismutase

Table 2 and Fig.6 show the effects of co-administration of two different doses of the ethanolic extract of *Asteracantha longifolia* seeds p.o daily for three different periods, on Amlodipine induced decrease in the level of Superoxide dismutase, in the testicular homogenate of Wistar albino rats.

Amlodipine treatment for 28 days in a dose of 0.9mg/kg p.o per day decreased the level of Superoxide dismutase in the testicular homogenate from the control value of (5.826±0.523) units (units /mg protein) to (4.251±0.173) units. When the ethanolic extract of *Asteracantha longifolia* seeds in a dose of 100 mg/kg p.o per day was co administered with Amlodipine for 28 days the level of Superoxide dismutase in the testicular homogenate was increased significantly to (5.860±0.276) units and the co administration of the extract in the dose of 250 mg/kg p.o per day for these 28 days significantly ( $p< 0.05$ ) improved the level of Superoxide dismutase in the testicular homogenate to (5.926±0.319)units, both compared to the Amlodipine treatment group of 28 days and both of them were higher than that in the control group.

Similarly Amlodipine treatment for 42 days in a dose of 0.9mg/kg p.o per day decreased the level of Superoxide dismutase in the testicular homogenate from the control value of (5.826±0.5230) units (units/mg protein) to (3.467±0.177) units. When the ethanolic extract of *Asteracantha longifolia* seeds in a dose of 100 mg/kg p.o per day was co administered with Amlodipine for 42 days the level of Superoxide dismutase in the testicular homogenate was increased significantly (5.974±0.292) units and the co administration of the extract in the dose of 250 mg/kg p.o per day for these 42 days significantly improved the level of Superoxide dismutase in the testicular homogenate to 96.409±0.3140 units, both compared to the Amlodipine treatment group of 42 days ( $p< 0.05$ ) and both of them were higher than the control value.

Amlodipine treatment for 65 days in a dose of 0.9mg/kg p.o per day decreased the level of Superoxide dismutase in the testicular homogenate from the control value of (5.826±0.523) units (units/mg protein) to (2.909±0.170) units.

But the co administration of the ethanolic extract of *Asteracantha longifolia* seeds in the doses of 100 mg/kg and 250 mg/kg p.o per day for these 65 days significantly improved the level of Superoxide dismutase in the testicular homogenate to (6.117±0.210) units and (8.735±0.365) units respectively, both compared to the Amlodipine treatment group of 65 days (p< 0.05) and both of them were higher than the control value.

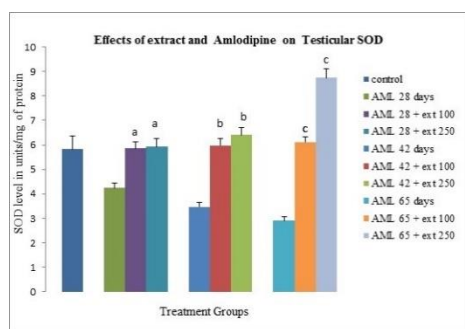


Fig.6.Effects of *Asteracantha longifolia* seed extract and Amlodipine on testicular SOD of Wistar albino rats.

(All values are mean ± SEM of (n=6), 'a' denotes p value < 0.05, compared to the Amlodipine treated group of 28 days, 'b' denotes p value < 0.05, compared to the Amlodipine treated group of 42 days, 'c' denotes p value < 0.05, compared to the Amlodipine treated group of 65 days)

#### 4.2.4. Catalase

Table 2 and Fig.7 show the effects of co-administration of two different doses of the ethanolic extract of *Asteracantha longifolia* seeds p.o daily for three different periods, on Amlodipine induced decrease in the level of Catalase, in the testicular homogenate of Wistar albino rats.

Amlodipine treatment for 28 days in a dose of 0.9mg/kg p.o per day decreased the level of Catalase in the testicular homogenate from the control value equivalent to (48.988±1.647) units (μ moles of H<sub>2</sub>O<sub>2</sub> decomposed/min/mg protein) to the value equivalent to (36.437±1.473) units. But the co administration of the ethanolic extract of *Asteracantha longifolia* seeds in the doses of 100 mg/kg and 250 mg/kg p.o per day for these 28 days improved the level of Catalase in the testicular homogenate mildly to the value equivalent to (46.173±2.885) units and significantly, compared to the Amlodipine treatment group of 28 days, (p< 0.05) to the value equivalent to (50.067±2.917) units respectively. With 250 mg/kg extract the Catalase level became higher than the control value.

Similarly Amlodipine treatment for 42 days in a dose of 0.9mg / kg p.o per day decreased the level of Catalase in the testicular homogenate from the control value equivalent to (48.988±1.647) units (μ moles of H<sub>2</sub>O<sub>2</sub> decomposed/min/mg protein) to the value equivalent to (25.341±1.803) units. When the ethanolic extract of *Asteracantha longifolia* seeds in a dose of 100 mg/kg p.o per day was co administered with Amlodipine for 42days the level of Catalase in the testicular homogenate increased significantly to the value equivalent to (51.482±3.057) units and the co administration of the extract in the dose of 250 mg/kg p.o per day significantly improved the level of Catalase in the testicular homogenate to the value equivalent to (57.584±3.434) units, both compared to the Amlodipine treatment group of 42 days. (p< 0.05)

In both the extract treated groups Catalase level became more than the control values. Amlodipine treatment for 65 days in a dose of 0.9mg/kg p.o per day decreased the level of Catalase in the testicular homogenate from the control value equivalent to (48.988±1.647) units ( $\mu$  moles of H<sub>2</sub>O<sub>2</sub> decomposed/min/mg protein) to the value equivalent to (20.242±1.499) units.

But the co administration of the ethanolic extract of *Asteracantha longifolia* seeds in the doses of 100 mg/kg and 250 mg/kg p.o per day for these 65 days improved the level of Catalase in the testicular homogenate significantly to the value equivalent to (58.671±2.908) units and to the value equivalent to (83.457±2.913) units respectively, compared to the Amlodipine treatment group of 65 days. (p< 0.05) In both the extract treated groups Catalase level became more than the control value.

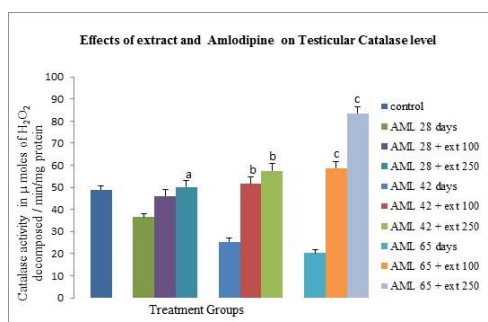


Fig.7. Effects of *Asteracantha longifolia* seed extract and Amlodipine on testicular Catalase of Wistar albino rats.

(All values are mean ± SEM of (n=6), 'a' denotes p value <0.05, compared to the Amlodipine treated group of 28 days, 'b' denotes p value < 0.05, compared to the Amlodipine treated group of 42 days, 'c' denotes p value < 0.05, compared to the Amlodipine treated group of 65 days)

### 4.3. Histological Parameters

#### 4.3.1. Control Testis

The testis of untreated control Wistar albino rats is shown in figures 8. In the testis of control group seminiferous tubules were of uniform size with thin intact basement membrane. Germ cells of various stages covering the complete process of spermatogenesis were present. Germ cells showed normal meiotic cell division and maturation. Numerous sperms were present in the tubules. Normal clusters of Leydig cells were present.

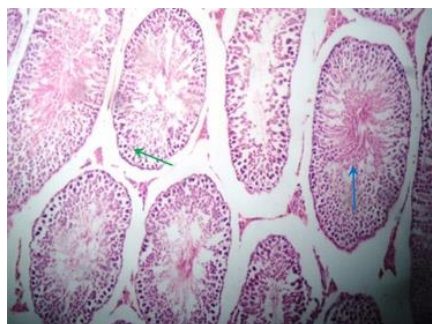


Fig.8. Testis of Wistar albino rats - control (H and E × 100)

- Germ cells of various stages of development
- Lumen full of sperms

#### 4.3.2. Amlodipine 42 Days

The testis of Wistar albino rats treated with Amlodipine alone in a dose of 0.9 mg / kg p.o daily for 42 days is shown in figures 9.



When compared to the seminiferous tubules of control rats, seminiferous tubules in this group were smaller in size and some tubules showed no sperms. Germinal epithelium was mildly thickened with reduced sperm maturation rate. Mild interstitial edema was seen. Leydig cells clusters were normal.

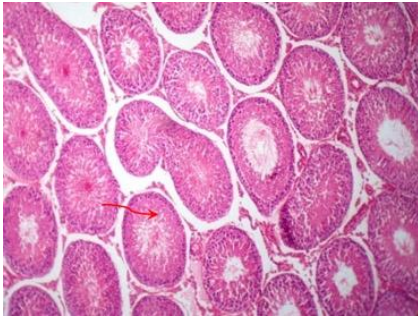


Fig.9: Testis of Wistar albino rats treated with Amlodipine for 42 days (H and E  $\times$  100)  
→ Small seminiferous tubules

#### 4.3.3. Amlodipine and Extract 100mg/kg for 42 Days

The testis of Wistar albino rats treated with Amlodipine in a dose of 0.9mg/kg p.o along with the ethanolic extract of *Asteracantha longifolia* seeds in a dose of 100 mg/kg, p.o, once daily for 42 days is shown in figures 10.

When the testes of rats which were treated with Amlodipine alone in a dose of 0.9 mg/kg p.o once daily for 42 days showed some smaller seminiferous tubules with no sperms, mildly thickened germinal epithelium and mild interstitial edema, co administration of the ethanolic extract of *Asteracantha longifolia* seeds in a dose of 100 mg/kg p.o once daily for these 42 days showed seminiferous tubules with thin and intact basement membrane. Though sperm maturation was slightly reduced in this group compared to that in the control group, more sperms could be seen in the seminiferous tubules and Leydig cell clusters were normal.

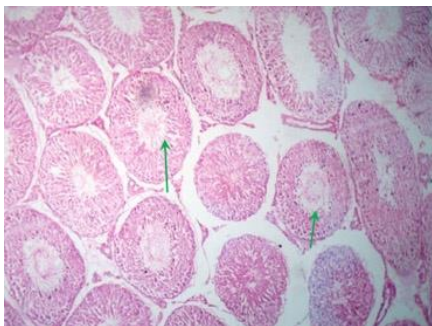


Fig.10. Testis treated with Amlodipine and extract 100mg/ kg for 42 days (H and E  $\times$ 100)  
→ More sperms in lumen compared to those in Group V

#### 4.3.4. Amlodipine and Extract 250mg/kg for 42 Days

The testis of Wistar albino rats treated with Amlodipine in a dose of 0.9 mg/kg p.o, along with the ethanolic extract of *Asteracantha longifolia* seeds in a dose of 250 mg/kg p.o, once daily for 42 days is shown in figures 11.

When the testes of rats which were treated with Amlodipine alone in a dose of 0.9 mg/kg p.o once daily for 42 days showed some smaller seminiferous tubules with no sperms, mildly thickened germinal epithelium and mild interstitial edema, co administration of the ethanolic extract of *Asteracantha longifolia* seeds in a dose of 250mg/kg p.o once daily for these 42 days showed seminiferous tubules with thin intact basement membrane. Besides germ cells showed normal meiotic cell division and maturation. Numerous sperm were present in the seminiferous tubules.

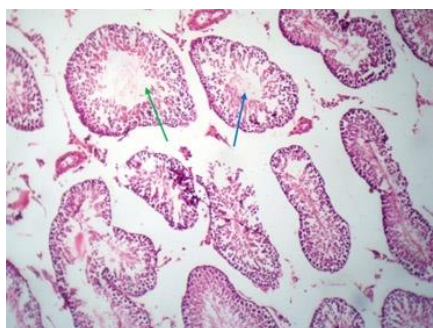


Fig.11. Testis treated with Amlodipine and extract 250mg/ kg for 42 days (H and E  $\times 100$ )

- More sperms in lumen compared to those in Group V
- Normal germ cells of different stages of development

#### 4.3.5. Amlodipine 65 Days

The testis of Wistar albino rats treated with Amlodipine alone in a dose of 0.9 mg / kg p.o daily for 65 days is shown in figures 12.

When compared to the seminiferous tubules of control rats, seminiferous tubules in this group showed much variation in their size and reduced sperm count. Besides, in sperms, maturation arrest, though incomplete was evident. In some seminiferous tubules though lumen was present there were no sperms but the basement membrane was thickened. Interstitial space was much widened. Leydig cell clusters were normal.

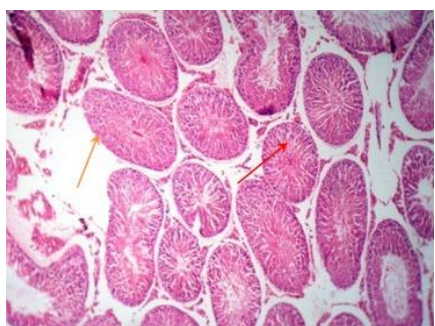


Fig.12. Testis of Wistar albino rats treated with Amlodipine for 65 days (H and E  $\times 100$ )

- Interstitial space widening
- Small seminiferous tubule compared to that in the untreated group

#### 4.3.6. Amlodipine and Extract 100mg/kg for 65 Days

The testis of Wistar albino rats treated with Amlodipine in a dose of 0.9 mg/kg p.o along with the ethanolic extract of *Asteracantha longifolia* seeds in a dose of 100 mg/kg p.o, once daily for 65 days is shown in figures 13.

When the testes of rats which were treated with Amlodipine alone, in a dose of 0.9 mg/kg p.o for 65 days showed tubules with much size variation, thickened basement membrane, reduced or nil sperm count and incomplete maturation arrest, co administration of the ethanolic extract of *Asteracantha longifolia* seeds in a dose of 100mg/kg p.o daily for these 65 days showed seminiferous tubules with wide tubular lumen and much increased sperm count. Besides interstitial space widening was less.

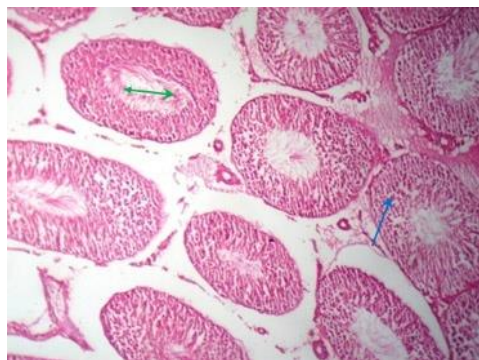


Fig.13. Testis treated with Amlodipine and extract 100 mg/ kg for 65 days (H and E  $\times$ 100)

- Wide lumen with more sperms
- Normal germ cells of different stages of development

#### 4.3.7. Amlodipine and Extract 250mg/kg for 65 Days

The testis of Wistar albino rats treated with Amlodipine in a dose of 0.9 mg/kg p.o along with the ethanolic extract of *Asteracantha longifolia* seeds in a dose of 250 mg/kg p.o, once daily for 65 days is shown in figures 14. Compared to the group of rats treated with Amlodipine alone in a dose of 0.9 mg/kg p.o for 65 days which showed tubules with much size variation, thickened basement membrane, reduced or nil sperm count and incomplete maturation arrest, co administration of the ethanolic extract of *Asteracantha longifolia* seeds in a dose of 250mg/kg p.o daily for these 65 days showed seminiferous tubules with thin and intact basement membrane, wide tubular lumen and highly increased sperm count. In addition there was minimum interstitial space among the tubules.

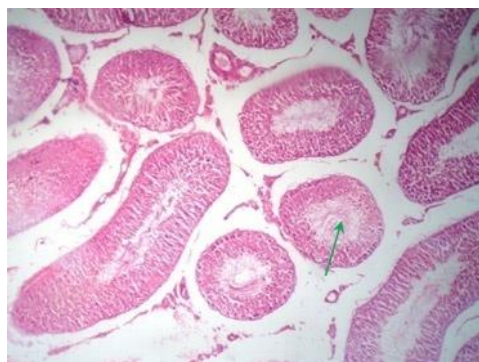


Fig. 14. Testis treated with Amlodipine and extract 250 mg/ kg for 65 days (H and E  $\times$ 100)

- Wide lumen with more sperms

## 5. CONCLUSION

The testicular dysfunction in Wistar albino rats produced by the continuous administration of Amlodipine was found to be reversed in a dose and duration dependent manner by the co administration of the ethanolic extract of the seeds of *Asteracantha longifolia* Lin. The testicular protective effects of this widely available plant may be a boon for those young men in their reproductive age who have to depend on dihydropyridine calcium channel blockers like Amlodipine for long periods in order to treat different cardiovascular problems including hypertension.

In order to develop the crude ethanolic extract of the seeds of this plant into a clinical dosage form, suitable for its co administration along with the DHP type calcium channel blockers like Amlodipine, different pharmacokinetic and toxicity studies, have to be done in the preclinical phase of its development itself. Further research is warranted in order to identify the exact mechanism of the active compounds present in the herbal extract for amlodipine induced testicular dysfunction in rats .

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