SAFETY ASPECTS OF *ANDROGRAPHIS PANICULATA* AN INVESTIGATION INTO POSSIBLE MALE REPRODUCTIVE TOXICITY

by

DR. RENU AGARWAL

Thesis submitted in fulfillment of the requirements for the Degree of Master Of Science (PHARMACOLOGY)

Oct 2004

DISCLAIMER

I hereby certify that the work in thesis is my own except for the quotations and summaries which have been duly acknowledged.

Dated……. ……..

(Dr. Renu Agarwal)

P-UM-1098

ACKNOWLEDGEMENT

My sincere gratitude and deepest appreciation to my supervisor Prof. Dato' Mafauzy Mohamed for his continuous support and guidance. I wish to gratefully acknowledge Associate Prof. Dr. Siti Amrah Sulaiman (Pharmacology), Associate Prof. Dr. Hasnan Jafaar (Head of the Department of Pathology) who have contributed greatly with their invaluable advice and constructive criticism during the preparation of this thesis. I would also thank Prof. Chan Kit Lam, School of pharmacy, USM, Penang for sending us Andrographis paniculata extract along with its HPLC findings and Malaysian Ministry of Science and Technology for financial supports under Intensified Research in Priority Area (IRPA) research grants. My sincere thanks to head of Animal House Unit, Associate Prof. Dr. Afifi Sheikh Abu Bakar for providing necessary help whenever required.

I am also taking this opportunity to thank Dr. Mohd. Suhaimi Abd. Wahab, Head of Department, Pharmacology and all other lecturers and Professors in the Department of Pharmacology for their guidance and encouragement throughout my course. To my colleagues, thank you for invaluable assistance and encouragement.

Encik Maarof, Encik Ruzman Bin Mohd. Nor, Encik Nor Muhammad from animal house unit, Encik Abdul Razak, Puan Roslina Mat Zain and Puan Halizah Miran from Pharmacology Laboratory, Puan Rusidah Mat Yatim and Puan Jamaliah Lin from Pathology Laboratory and Cik Siti Fadilah Abdullah from Cranio-Facial Biology laboratory, thank you all for your great help and enormous contribution in the technical aspect of this study.

I would like to express my heartfelt gratitude to my beloved husband, Dr. Puneet Agarwal without whose help and encouragement I would never be able to accomplish this work and complete my post-graduate studies. I would also like to express sincere gratitude to my parents (late) Shri D.S. Agarwal , Smt. Sushila Agarwal for guiding me to this stage in life. No words can ever describe my heartfelt thanks; only God can repay them in kind. In the end many thanks to my sons Vishesh and Vipul for their love and patience.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

%	Percent
AP	*Andrographis paniculata*
APE	95% ethanol extract of *Andrographis paniculata*
AR	Androgen receptor
ASC	Animal studies committee
AST	Aspartate amino transferase
BW	Body weight
CMC	Carboxy Methyl Cellulose
DDA	14-deoxy-11, 12- didehydro andrographolide
DSP	Daily sperm production
FSH	Follicle Stimulating Hormone
GC-MS	Gas Chromatography- Mass Spectrometry
gm/kg	grams per kilogram
GOT	Glutamate oxaloacetate aminotransferase
H & E	Haematoxylin and Eosin
HPLC	High Performance Liquid Chromatography
ICH	International Conference of Harmonisation
L:D	Light:Dark
LH	Leutinizing Hormone
mg/kg	milligram/kilogram
NHMS	National Health and Morbidity Survey
NS	Normal saline
Rel. wt.	Relative weight

SHR	Spontaneously hypertensive rats
STZ	Streptozotocin
T	Testosterone
TDI	Tubular differentiation index
UV	Ultra violet
WHO	World Health Organization

ABSTRAK

PENGENALAN: *Andrographis paniculata* (AP) adalah salah satu bentuk terapi alternatif yang popular untuk penyakit diabetes di Malaysia. Kementerian Sains dan Teknologi Malaysia telah memilih untuk memajukan piawaian (95%) ekstrak etanol (APE) sebagai rawatan yang efektif dan selamat untuk penyakit diabetes. Memandangkan kajian terdahulu menunjukkan pertikaian keputusan mengenai kesan AP ke atas sistem reproduktif lelaki, kajian ini dijalankan untuk menyelidik kemungkinan terdapat ketoksikan APE ke atas sistem reproduktif lelaki.

OBJEKTIF: Mengkaji kesan 95% ekstrak etanol *Andrographis paniculata* (APE) ke atas kesihatan reproduktif jantan dalam tikus Sprague Dawley.

BAHAN DAN KAEDAH: 50 tikus jantan dewasa yang sihat dibahagikan secara rawak kepada 5 kumpulan dengan 10 tikus setiap satu. Kumpulan 1, 2 dan 3 menerima tiga dos APE (10, 100 & 1000 mg/kg), kumpulan 4 menerima glibenclamide (5 mg/kg) sementara kumpulan 5 menerima 2% CMC (vehikel). Berat badan direkodkan setiap minggu. Rawatan oral diberi sekali setiap hari selama 4 minggu dan kemudian setiap seekor tikus jantan dibiarkan mengawan dengan 3 ekor tikus betina sehinggalah kesemua tikus betina tersebut didapati positif dengan benih jantan atau di biarkan bersama maksima selama 2 minggu. Tikus jantan kemudiannya dikorbankan. Organ pembiakan ditimbang. Testis kiri digunakan untuk pengiraan jumlah spermatid, kauda kiri untuk pengiraan sperma dan kajian morfologi, sementara itu testis bahagian kanan digunakan untuk pemeriksaan histopatologi.

KEPUTUSAN: Histologi testis menunjukkan bahagian oedema interstitial dengan spermatogenesis yang masih sempurna dalam kumpulan 1; sel kuman yang tidak tersusun, pertukaran sel yang tidak matang dan diameter tubular yang amat kecil dalam kumpulan 2 dan 3. Sel Leydig menunjukkan tetapi terdapat hyperplasia yang sederhana dalam kumpulan yang dirawat dengan APE. Organ reproduktif menunjukkan hala dimana timbangan yang lebih berat dalam kumpulan 1 dan timbangan organ yang lebih ringan dalam kumpulan 2 dan 3 dibandingkan dengan kumpulan kawalan. Kiraan sperma: Kiraan spermatid dan pengeluaran sperma harian rendah secara signifikasi ($p<0.01$) dalam kumpulan 2 dan lebih rendah bagi kumpulan 3. Sperma abnormal lebih banyak di dapati di dalam kumpulan 2 dan 3. Tiada satu pun antara parameter yang dinyatakan di atas menunjukan perbezaan secara signifikan antara kumpulan 4 dan 5.

PERBINCANGAN: APE memberi kesan negatif terhadap spermatogenesis melalui 2 mekanism yang berbeza: 1) Peningkatan dalam peresapan kapilari 2) Kerosakan kepada fungsi sel Sertoli. Peningkatan peresapan kapilari menyebabkan peningkatan berat organ reproductif dalam kumpulan 1; sementara itu, kerosakan teruk spermatogenesis dalaam kumpulan 2 dan 3 yang disebabkan oleh kerosakan sel Sertoli menyebabkan organ yang lebih ringan dan kualiti sperma yang rendah.

KESIMPULANNYA: Kesimpulannya, keputusannya menunjukkan rawatan APE mempunyai kesan negatif terhadap fungsi reproduktif tikus jantan yang bergantung kepada kadar dos yang diberi kepada tikus Sprague Dawley.

ABSTRACT

INTRODUCTION: *Andrographis paniculata* (AP) is one popular form of alternative therapy for diabetes mellitus in Malaysia. It is chosen by Ministry of Science and Technology, Malaysia to develop its standardized 95% ethanol extract (APE) as an effective and safe treatment of diabetes mellitus. Since previous studies have shown conflicting results regarding effects of AP on male reproductive system, this study was undertaken to investigate possible male reproductive toxicity of APE.

OBJECTIVES: To study effects of 95% ethanol extract of *Andrographis paniculata* (APE) on male reproductive health in Sprague Dawley rats.

MATERIALS AND METHOD: Fifty adult healthy male rats were randomly divided into 5 groups of 10 animals each. Group 1, 2 and 3 received three different doses of APE (10, 100 & 1000 mg/kg), group 4 received glibenclamide (5mg/kg) while group 5 received 2% CMC (vehicle). Treatment was administered once daily for 4 weeks premating and a maximum of 2 weeks mating period. Body weight was recorded weekly. Four weeks premating treatment was followed by mating of each male with 3 female animals until all females were sperm positive or a maximum of 2 weeks. Male animals were then sacrificed. Reproductive organs were removed and weighed. Left testis was used for spermatid count, left cauda for sperm count and morphology while right testis was used for histopathological examination.

RESULTS: Testicular histology showed severe interstitial edema of testis with intact spermatogenesis in group 1; progressively severe disorganized arrangement of germ cells, sloughing of immature cells and significantly smaller tubular diameter in groups 2 and 3. However, Leydig cells showed mild hyperplasia in APE treated groups. Reproductive organ weight was high in group 1 and low in groups 2 and 3 when

compared to control. Sperm count, spermatid count and daily sperm production were significantly low ($p<0.01$) in group 2 and were further reduced in group 3. Abnormal sperms were seen in groups 2 and 3. None of the above mentioned parameters showed significant differences between groups 4 & 5.

DISCUSSION: APE adversely affects spermatogenesis by at least two different mechanisms: 1) Increase in capillary permeability 2) Damage to Sertoli cell functions. Increased capillary permeability has resulted into increased reproductive organ weight in group 1, while extensive damage to spermatogenesis in groups 2 and 3, caused primarily by damage to Sertoli cells has resulted into low organ weight and poor semen quality.

CONCLUSION: In conclusion, the results suggest that APE treatment adversely affects male reproductive functions in a dose-dependent manner in Sprague Dawley rats.

CHAPTER 1. INTRODUCTION

This chapter gives an overview of –

- *herbal medicines and their ever increasing use in chronic diseases such as diabetes mellitus*
- *diabetes mellitus, its prevalence and therapeutic options*
- *importance of research into safety profile of herbal medicines*
- *reproductive toxicity*
- *ICH and ICH guidelines for male reproductive toxicity studies*

1. INTRODUCTION

Herbs have been used for centuries to improve health and well being of mankind. Mainstream modern medicine is now beginning to re-focus on herbs and natural plant therapies as a primary means to deal with current-day health issues. Increasing use of herbal medicines in recent years is evidence of public interest in having alternatives to conventional medicine to treat various illnesses. Use of herbal medicine is very popular in South East Asia especially to treat chronic illnesses. In many developing countries, where access to hospitals and health care providers is limited, herbal medicines often are the only generally available form of medicine. Moreover, high cost and high incidence of adverse effects with these conventional drugs makes herbal medicines a more convenient choice in chronic illnesses such as diabetes mellitus.

Diabetes mellitus is a serious, common and controllable chronic metabolic disorder that has a significant impact on health, quality of life, and life expectancy of patients. Due to its chronicity and life long treatment, it is a great burden on the health care systems of developed as well as developing nations. Prevalence of diabetes in adults worldwide was estimated to be 4.0% in 1995 and is expected to rise to 5.4% by the year 2025. The number of adults with diabetes in the world will rise from 135 million in 1995 to 300 million in the year 2025 (King et al., 1998). The aging of populations and the effects of modernization of lifestyle have led to a dramatic increase in the prevalence of diabetes globally with very high rates in developing nations, particularly in Asia and the Pacific (Amos et al., 1997). In Malaysia, high prevalence of diabetes mellitus is a growing concern. In Second National Health and Morbidity Survey

prevalence of diabetes mellitus in Malaysia was found to be 8.2%. The earlier survey done in 1986 had shown a prevalence of 6.3 % (NHMS, 1996).

Diabetes mellitus is a multisystem disorder caused by absolute or relative deficiency of pancreatic insulin. Insulin controls the amount of glucose/sugar in blood by controlling the rate at which glucose is metabolized. Deficiency of insulin gives rise to abnormally high levels of glucose in blood stream. Prolonged high levels of blood glucose leads to damaged blood vessels, which may cause eye disease, heart disease, nerve damage (neuropathy) in internal organs and extremities, kidney disease (nephropathy), and foot ulcers.

Conventional therapeutic options to treat diabetes mellitus include dietary control, physical exercise, oral antidiabetic drugs and insulin. Besides the conventional therapy, alternative therapies with antihyperglycaemic effects are increasingly sought by patients with diabetes. This comes as no surprise since alternative treatments have been widely used in chronic diseases, which may be only partially alleviated by conventional treatment. Alternative therapies with anti-diabetic activity have been researched extensively, particularly in Asia. One such popular form of alternative therapy for diabetes mellitus in Malaysia is *Andrographis paniculata*, which belongs to plant family *Acanthaceae* and is locally known as Hempedu Bumi. Due to its promising and high commercial potential, Ministry of Science and Technology, Malaysia has chosen Hempedu Bumi to develop its standardized 95% ethanol extract as an effective and safe treatment of diabetes mellitus. Advanced herbal preparations, often processed in a way that establishes relatively fixed chemical parameters are often called standardized extracts. Herbal medicines are commonly used by herbal

practitioners for medicinal effects in one of the three forms:

1. Tinctures – These are typically made using an alcohol and water mixture as the solvent. The herb is first soaked in the solvent for a specified amount of time depending on the herb. This soaking period is usually from several hours to several days in length, and some herbs may be soaked for much longer period of time. The resultant solution is then pressed out, yielding the tincture.

2. Fluid extracts – These are more concentrated than tinctures. Although they are most often made from hydro-alcoholic mixtures, other solvents may also be used such as vinegar, glycerine, propylene glycol, etc. Commercial fluid extracts usually are made by distilling off some of the alcohol, typically by using methods that do not require elevated temperature, such as vacuum distillation and counter-current filtration. However, some small manufacturers produce fluid extracts in a similar manner to tinctures via “cold percolation" process.

3. Solid extract - is produced by further concentration of the extract by the mechanisms described above as well as by other techniques such as thin layer evaporation. The solvent is completely removed from the solution, leaving a viscous extract which is soft and semisolid or a dry solid extract depending upon the plant, portion of the plant, or solvent used, or if a drying process was used. The dry solid extract, if not already in powdered form, can be ground into coarse granules or a fine powder.

Herbalists argue that their herbal therapies are cheap, available in remote areas, and above all, they are effective. However physicians want more evidence regarding safety and efficacy of traditional remedies. World Health Organization has recognized the importance of research in the field of herbal medicines. According to first ever document on global WHO traditional medicine strategy titled *WHO Traditional Medicine Strategy 2002 –2005* "The use of traditional medicine is increasing rapidly in developed countries. In many parts of the world, policy-makers, health professionals and the public are wrestling with questions about the safety, quality, availability, preservation and further development of this type of health care. If traditional medicine is to be promoted as a source of healthcare, efforts must be made to promote its rational use, and identification of the safest and most effective therapies will be crucial".

Over the past decade important safety issues associated with the use of herbal products have resulted in regulatory action world-wide in an effort to protect public health. To receive a marketing authorization, herbal medicines are required to meet safety, quality and efficacy criteria in a similar manner to any other licensed medicine. One of the important aspects of safety profile of a pharmaceutical preparation is that it should not cause reproductive toxicity.

Reproductive toxicity includes the toxic effects on the formation and maturation of male and female gametes, sexual function, the events surrounding the fusion of gametes and the development of the fertilized ovum. It also includes nourishment and transport of the conceptus within the genital tract, implantation, embryogenesis, intrauterine growth, placentation and placental function, parturition, lactation and

neonatal survival. Adverse reproductive effects in males are considered as significant as adverse effects occurring in females.

To ensure the safety, and to evaluate possible male reproductive health-related problems associated with the use of APE, we investigated its effects on male reproductive system in Sprague Dawley rats according to guidelines proposed by International Conference on Harmonisation (ICH guidelines 2000) of technical requirements for registration of pharmaceuticals for human use.

The ICH initiative, which started in 1990, is an interregional venture covering 17 high-income countries. It includes drug regulatory authorities of the European Union, Japan, and USA, assisted by the research-based pharmaceutical industry associations of those countries. ICH is a joint initiative involving both regulators and industry as equal partners in the scientific and technical discussions of the testing procedures, which are required to ensure and assess the safety, quality and efficacy of medicines.

The ICH guidelines are produced by groups of eminent specialists drawn from the regulatory authorities and pharmaceutical companies of the ICH countries. Their scientific level is high and they represent an up-to-date approach to technical requirements. The costs related to full implementation of the guidelines are considerable, but it is argued that they are offset by more rapid registration of new drugs in the ICH countries. In June 1993 an ICH Harmonised Tripartite Guideline on Detection of Toxicity to Reproduction for Medicinal Products was adopted by the ICH Steering Committee and has since been implemented in the three ICH regions. In March 1999 it was proposed that active dissemination of the guidelines and their

adoption by some non-ICH countries have led to a growing perception that they represent international standards.

Therefore in this study to investigate effects of APE on male reproductive system, we adopted ICH guidelines (*An Addendum to the ICH Guideline,* 2000) to ensure the reliability and safety of APE before it is recommended for human consumption.

CHAPTER 2. REVIEW OF LITERATURE

This chapter gives a summary of previously done research work related to

– Andrographis paniculata (AP)

- antihyperglycaemic and other therapeutic benefits of AP

- male reproductive system – its anatomy and physiology

- male reproductive toxicity parameters

- male reproductive toxicity of AP- an issue still unclear.

2. REVIEW OF LITERATURE

2.1 *ANDROGRAPHIS PANICULATA* (AP)

Andrographis paniculata (Figure 4.1) is among many local herbs in South East Asia, and is claimed to have medicinal properties. It is also known as “King of Bitters," and is a member of plant family *Acanthaceae.* Local name of the plant is “Hempedu Bumi”. It is cultivated quite easily since it grows in all types of soil. Moreover, it grows in soil types where almost no other plant can be cultivated, particularly "serpentine soil," which is relatively high in aluminium, copper and zinc. Such hardiness helps account for its wide distribution. It grows abundantly in south-eastern Asia: India, Pakistan and Indonesia and is cultivated extensively in China and Thailand, the East and West Indies, and Mauritius (Gupta et al., 1990). AP is an annual branched, erect - running 1/2 to 1 meter in height. Its stem is dark green, 2 - 6 mm in diameter, quadrangular with longitudinal furrows and wings at angles of the younger parts. The leaves are opposite, decussate, lanceolate, up to 8 cm long and broad, glabrous, margin entire, and venation pinnate; the petiole is very short. The flowers are small with bilabial corollas. The fruits are small 2-celled odorless capsules which taste intensely bitter. The aerial parts of the plant (leaves and stems) are used to extract the active phytochemicals.

Four well known diterpene lactones found in the plant AP include andrographolide, neoandrographolide, 14-deoxy-11, 12-didehydroandrographolide and 14- deoxy andrographolide. All of these have been isolated from the leaves (Basak et al., 1999).

Jain et al., (2000) used high pressure liquid chromatography to determine the amount of three major diterpenoids, andrographolide and neoandrographolide and 14-deoxy-11, 12- didehydro andrographolide in AP leaf extract. The primary medicinal component of AP is andrographolide. It has a very bitter taste and is colorless crystalline in appearance. Other active components include homoandrographolide, andrographan, andrographon, andrographosterin, and stigmasterol. Both growing region and seasonality play a role to determine the concentration of these diterpene lactones. The highest concentration of the active components is found just before the plant blooms. The HPLC method developed by Jain et al., (2000) is simple, rapid and precise and therefore can be used to determine concentration of active components in various extracts of AP.

2.2 PHARMACOKINETIC AND ORAL BIOAVAILABILITY OF ANDROGRAPHOLIDE

Panossian et al., (2000) determined the amount of andrographolide, the most active phytochemical of the plant AP in the blood plasma of rats and human volunteers following the oral administration of AP extract and AP fixed combination Kan Jang tablets using validated analytical methods (HPLC and GC-MS). Results of their study showed that andrographolide was quickly and almost completely absorbed into the blood following the oral administration of AP at a dose of 20 mg/kg body weight in rats. Its bio-availability, however, decreased four-fold when a 10-times-higher dose was used. A large part (55 %) of andrographolide is bound to plasma proteins. Renal excretion is not the main route for eliminating andrographolide. Maximum plasma levels were reached after 1.5-2 hours, as quantified by using a UV diode-array detection method. Half-life and

mean residence times were 6.6 and 10.0 hours, respectively.

2.3 ANTIHYPERGLYCAEMIC ACTIVITY OF AP

AP is used as a folk medicine remedy for diabetes mellitus in Malaysia. Borhanuddin et al., (1994) investigated hypoglycaemic effects of AP on non-diabetic rabbits. Their study showed that water extract of AP in a dose of 10 mg/kg body weight can prevent induction of hyperglycaemia ($P < 0.001$) induced by oral administration of glucose 2 mg/kg body weight. However it failed to do so in adrenaline induced hyperglycaemia. It also failed to demonstrate any "fasting blood sugar lowering effect" upon chronic administration (6 weeks) of AP.

Zhang & Tan, (2000) investigated antihyperglycaemic effects of 80% ethanolic extract of the aerial parts of AP in normal and streptozotocin (STZ)-induced diabetic rats. The extract was administered orally at 3 dose levels (0.1, 0.2, and 0.4 g/ kg body weight). At the end of the 14 day period, the extract significantly increased body weight ($P < 0.01$) and reduced fasting serum glucose in diabetic rats ($P < 0.001$) when compared with vehicle. Liver and kidney thiobarbituric acid-reactive substances (TBARS) levels were significantly lower ($P < 0.0001$, $P < 0.005$, respectively), whereas liver glutathione concentrations were significantly higher ($P < 0.05$) in extract treated diabetic rats compared with vehicle-treated diabetic rats. The extract significantly increased the activity of superoxide dismutase and catalase, but had no significant effect on glutathione peroxidase activity in diabetic rats. The results of the study showed that ethanolic extract of AP significantly lowered the blood glucose levels in diabetic rats and this effect was dose dependent. This experiment also

demonstrated reduced oxidative stress in diabetic rats. The authors postulated that anti hyperglycaemic effect of extract was possibly due to potentiation of insulin action and direct glucose lowering effect. Increased glucose metabolism may also partially be responsible for its hypoglycaemic effect. Moreover, the additional hypotryglyceridemic effect seen in the study is also beneficial in diabetic state. However, other possible mechanism(s) remain unclear.

In another study conducted by Kartini, et al., (2000) antihyperglycaemic effects of ethanol extract of AP were investigated in STZ induced diabetic rats. After 3 weeks of treatment, treated rats had not only significant fall in blood glucose level but also had increased survival rate.

2.4 OTHER THERAPEUTIC BENEFITS OF AP

2.4.1 Hepatoprotective

AP is claimed to possess hepatoprotective properties. Hepatoprotective activity of andrographolide was studied by Handa & Sharma, (1990). Acute hepatitis was induced in rats by single dose of galactosamine (800 mg/kg, intraperitoneal)/paracetamol (3g/kg, orally). Hepatoprotective activity was monitored by estimating liver enzymes (GOT and AST), alkaline phosphatase and bilirubin in serum, hepatic triglycerides, and by histopathological changes in the livers of experimental rats. Treatment with AP led to complete normalization of toxin-induced increase in the levels of all the five biochemical parameters, and significantly ameliorated toxin-induced histopathological changes in the livers of experimental rats.

Rana & Avadhoot, (1991) studied hepatoprotective effects of AP against carbon tetrachloride-induced liver damage. Alcoholic extract of the leaves of AP was obtained by cold maceration and a dose of 300 mg/kg of the extract was selected for the study. The extract was found to be effective in preventing liver damage that was evident by morphological, biochemical and functional parameters.

In another study by Visen et al., (1993) andrographolide, the active constituent isolated from the plant AP, showed a significant dose dependent (0.75-12 mg/kg body weight orally for 7 days) protective activity against paracetamol-induced toxicity on *ex vivo* preparation of isolated rat hepatocytes. It completely antagonized the toxic effects of paracetamol on certain enzymes (GOT, AST and alkaline phosphatase) in serum as well as in isolated hepatic cells. Andrographolide was found to be more potent than silymarin, a standard hepatoprotective agent.

2.4.2 Cardioprotective

Effects of AP extracts on cardiovascular system have been investigated by many researchers. Zhao & Fang, (1991) investigated antithrombotic and myocardial protective properties of AP. In this study the endothelium of the left anterior descending coronary artery of 16 dogs was injured mechanically. In the group pretreated with AP extract there was no elevation of the ST segment but plasma 6-k-prostaglandin F_1 alpha and platelet cyclic adenosine monophosphate were increased. The production of thromboxane and aggregation of platelets were inhibited, and no thrombus or myocardial infarction was induced when compared with control group.

The hypotensive activity of an aqueous extract of AP was studied using chronic intraperitoneal infusions by osmotic pumps (Zhang & Tan, 1996). The extract exhibited a dose-dependent hypotensive effect on the systolic blood pressure of spontaneously hypertensive rats (SHR). Plasma, but not lung acetylcholine esterase activity and kidney thiobarbituric acid level were significantly lower in extract-treated SHR when compared with vehicle-treated SHR controls. This experiment showed that the aqueous extract of AP lowers systolic blood pressure in the SHR possibly by reducing circulating acetylcholine in the plasma as well as by reducing free radical levels in the kidneys.

In the study by Zhang et al., (1998) the cardiovascular activity of 14-deoxy-11, 12-didehydro andrographolide (DDA) from AP was elucidated in anaesthetized Sprague-Dawley rats and isolated rat right atria. DDA produced significant fall in mean arterial blood pressure and heart rate in a dose-dependent manner. In the isolated right atria, DDA caused negative chronotropic action and antagonized isoproterenol-induced positive chronotropic action in a non-competitive and dose-dependent manner. These results further supported the bradycardia-inducing and beta-adrenoceptor antagonistic properties of DDA *in vivo*.

Burgos et al., (2000) investigated effects of 70% ethanol extract of AP leaves on calcium homeostasis in rat vas deferens. The possible blockade of voltage operated calcium channels by AP extract was compared with that of verapamil. The results of their experiment indicate that AP blocks the uptake of calcium by selectively blocking voltage operated calcium channels in a dose dependent and non competitive manner thereby causing smooth muscle relaxation. Based on these findings authors suggested

the possible utility of AP extracts in the treatment of diseases such as arterial hypertension.

2.4.3 Anti-inflammatory and Antipyretic activities

AP is known to have anti-inflammatory activity and is used in many countries to relieve pain, sore throat and fever. In one randomized double blind study by Thamlikitkul et al., (1991) to assess the efficacy of AP, patients received either paracetamol or 3 g/day of AP or 6 g/day of AP for 7 days. Efficacy of paracetamol or high dose AP was significantly more than that of low dose AP at day 3 in terms of the relief of fever and sore throat. However, the clinical effects were not different at day 7. Minimal and self limiting side effects were found in about 20 per cent of patients in each group.

Another study was conducted to measure the effectiveness of standardized extract of AP in reducing the prevalence and intensity of symptoms and signs of common cold as compared with a placebo (Caceres et al., 1999). One hundred and fifty eight patients were divided in two equal size groups, one of which received AP dried extract (1200 mg/day) and the other a placebo for a period of 5 days. It was concluded that AP had a high degree of effectiveness in reducing the prevalence and intensity of the symptoms in uncomplicated common cold beginning at day two of treatment. No adverse effects were observed or reported.

Two randomized double-blind, placebo-controlled parallel group clinical trials were conducted by Melchior et al., (2000), a pilot study and a phase III study. They investigated the effect of a standardized extract (SHA-10) of AP fixed combination (Kan jang) in the treatment of uncomplicated upper-respiratory tract infections.

Medication was taken three times daily for a minimum of 3 days and a maximum of 8 days for the pilot study, and for exactly three days in the phase III study. The primary outcome measures were patient's self-evaluation of pain in the muscles, cough, throat symptoms, headache, nasal symptoms, eye symptoms and temperature. The physician's fixed score diagnosis was based mainly on sign/symptoms related to ear, nose, oral cavity, lymph glands-tonsils and eyes. The total symptom score showed a tendency toward improvement in the pilot study ($p = 0.08$), while both the total symptom score and total diagnosis score showed highly significant improvement ($p < 0.0006$, $p < 0.003$) in the treatment group as compared with the placebo. In both studies, throat symptoms/signs showed the most significant improvement.

2.4.4 Anti-parasitic

Dutta & Sukul, (1982) observed that water decoction of the leaves of AP killed the microfilaria of *Dipetalonema reconditum* in 40 min (*in vitro*). Three subcutaneous injections of the extract in infected dogs at 0.06 ml per kg body-weight reduced the number of microfilariae in blood by more than 85%. The larvae were not totally eliminated with more injections but the reduced microfilarial level persisted. No toxic effect of the extract was observed.

In vitro and *in vivo* studies done by Najib et al., (1999) revealed that Malaysian medicinal plants, *Piper sarmentosum*, AP and *Tinospora crispa* produced considerable antimalarial effects. Chloroform extract showed better effects than the methanol extract *in vitro*. The chloroform extract of AP showed complete parasite growth inhibition with a dose as low as 0.05 mg/ml within 24 h incubation period. Methanol extract of AP was effective at the dose of 2.5 mg/ml and under incubation time of 48 hr. *In vivo* activity of

AP also demonstrated higher antimalarial effect than other two plant species.

Zaridah et al., (2001) tested five aqueous extracts from three plant species, i.e., dried husks, dried seeds and dried leaves of *Xylocarpus granatum* (*Meliaceae*), dried stems of *Tinospora crispa* (*Menispermaceae*) and dried leaves of AP (*Acanthacea*e), *in vitro*, against adult worms of subperiodic *Brugia malayi*. The relative movability value of the adult worms over the 24-h observation period was used as a measure of the antifilarial activity of the aqueous extracts. Dried seeds extract of *X. granatum* demonstrated the strongest activity, followed by the dried leaves extract of AP, dried stems extract of *T. crispa*, dried husks extract and dried leaves extract of *X. granatum*.

2.4.5 Immunostimulant and Anti-HIV

Immunostimulant properties of AP have also been investigated by some researchers. Ethanolic extract and purified diterpene andrographolides of AP induced significant stimulation of antibody and delayed type hypersensitivity response to sheep red blood cells in mice (Puri et al., 1993). The plant preparations also stimulated non-specific immune response in the animals, measured in terms of macrophage migration index, phagocytosis of 14C-leucine labelled *Escherichia coli* and proliferation of splenic lymphocytes. The stimulation of both antigen specific and non-specific immune response was, however, of lower order with andrographolide than with the ethanolic extract, suggesting thereby that substance(s) other than andrographolide present in the extract might also be contributing towards immunostimulation.

Basak et al., (1999) investigated the effects of diterpenes from AP and their succinoyl esters on clinically important proprotein convertases -1, -7 and furin. These convertases

are a group of enzymes involved in post-translational modification of a variety of proteins. These proteins include a wide range of hormones, neuropeptides, growth factors, coagulation factors, cell-surface receptor proteins as well as surface viral glycoproteins. Among the individual components of AP, neoandrographolide exhibited the highest inhibitory action with an IC_{50} of 53.5 micromol against furin. Andrographolide showed significant inhibitory activity against above mentioned enzymes only after succinoylation. Authors attributed this strong inhibitory action to structural modification of andrographolide molecule and suggested possibility of development of a new class of non-peptide inhibitors of proprotein convertases. Authors also suggested that this inhibitory action might be responsible for reported anti-HIV property of 14-deoxyandrographolide succinic acid monoester.

A phase I dose-escalating clinical trial of andrographolide from AP was conducted in 13 HIV positive patients and five HIV negative, healthy volunteers (Calabrese et al., 2000). The objectives were primarily to assess safety, tolerability, effects on plasma virion HIV-1 RNA levels and CD4 (+) lymphocyte levels. No subjects used antiretroviral medications during the trial. Those with liver or renal abnormalities were excluded. The planned regimen was 5 mg/kg body weight for 3 weeks, escalating to 10 mg/kg body weight for 3 weeks, and to 20 mg/kg body weight for a final 3 weeks. The trial was interrupted at 6 weeks due to adverse events including an anaphylactic reaction in one patient. All adverse events had resolved by the end of observation. A significant rise in the mean CD4 (+) lymphocyte level of HIV subjects occurred after administration of 10 mg/kg andrographolide (from a baseline of 405 cells/mm^3 to 501 cells/mm^3; $p = 0.002$). There were no statistically significant changes in mean plasma HIV-1 RNA levels throughout the trial.

As is evident from the above description, AP has a wide range of therapeutic applications. Therefore investigation into its possible toxic effects is extremely important before it can be recommended for human consumption. As stated in the previous chapter, reproductive toxicity forms an important aspect of safety profile of any pharmaceutical preparation. The present study was designed to investigate possible toxic effects of AP on male reproductive system.

2.5 MALE REPRODUCTIVE SYSTEM AND MALE REPRODUCTIVE TOXICITY PARAMETERS.

The male reproductive system consists of a number of individual organs acting together to produce functional spermatozoa, and to deliver these spermatozoa to the female reproductive tract. These organs include the testicles and epididymides (suspended in the scrotal sac), the vas deferens that leads to penile urethra and the accessory sex glands. The testis is central to the male reproductive system. It is the organ which generates the haploid germ cell by the process of spermatogenesis and it is the site of androgen production. Testicular spermatozoa are non-motile and incapable of fertilization. The function of the epididymis is to bring testicular spermatozoa to maturity. Mature sperm are stored in the lower portion, or tail, of the epididymis, cauda epididymis. The vas deference begins at the cauda epididymis then turns sharply upward along the posterior margin of the testes. Each vas deferens joins the duct from the adjacent seminal vesicle (one of the accessory glands) to form a short ejaculatory duct. Each ejaculatory duct passes through the prostate gland and empties into the urethra. The accessory glands of the male reproductive system are the seminal vesicles, prostate gland, and the bulbourethral glands. These glands secrete

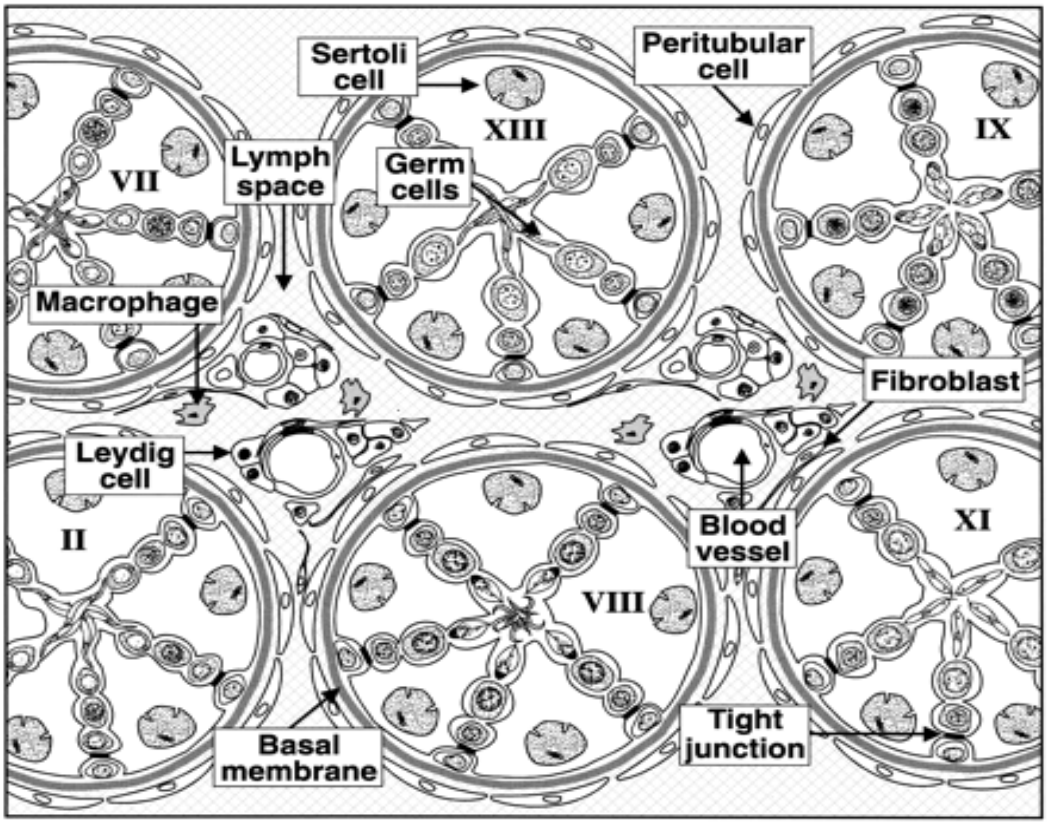
fluids that enter the urethra. Thus semen consists of sperms in fluid secreted by accessory glands (De Kretser et al., 1982).

The testicular tissue is organized into two compartments: the tubular compartment and the interstitium (Figure 2.1). The seminiferous tubules are the functional units of testis and are lined by seminiferous epithelium, which consists of Sertoli cells and germinal cells. The tubules are surrounded by the peritubular myoid cells. The interstitium is composed of Leydig cells, macrophages, fibroblasts, and blood vessels. These structures are embedded in the extracellular matrix. Spermatogenesis is a biological process within the seminiferous epithelium by which precursors termed spermatogonia undergo a complex series of divisions to give rise to spermatozoa (Gnessi et al., 1997).

During the process of spermatogenesis the germ cells undergo a three distinct phases of development. 1) Mitosis- Proliferation of spermatogonia type A to leptotene spermatocytes. 2) Meiosis -Proliferation of leptotene spermatocyte to haploid spermatids. 3) Spermeiogenesis – Maturation of spermatids into spermatozoa. In the rat these stages follow one another giving rise to wave of spermatogenesis along the length of seminiferous tubules. By using periodic acid-Schiff staining, 14 stages of spermatogenesis can be identified in the seminiferous epithelium of rat. Spermatogenesis cycle takes 12-14 days to complete in rats. The germ cells in different stages of development are found at different levels from base of the tubule to lumen and are surrounded by the cytoplasm of somatic cells; the Sertoli cells (Cheng & Mruk, 2002).

The Sertoli cell cytoplasm extends the entire height of seminiferous epithelium because these cells nurture the germ cells through their cycles of development. The Sertoli cells cytoplasm surround the adjacent germ cells in an arboreal pattern and controls the radially directed organization of cell associations. The cytoplasm of Sertoli cells consists of a framework of microtubules (Fawcett, 1975) and intermediate filaments known as vimentin (Amlani & Vogl, 1988). As the germ cells divide and mature they move from basement membrane through tight junctional complexes of adjacent Sertoli cells until they reach the adluminal surface. The Sertoli-Sertoli cell junctions form the blood-testis barrier, which helps to protect the germ cells from potentially harmful blood borne chemicals (Hess, 1999). Besides Sertoli-Sertoli cells junctions, Russell, (1977) observed desmosome like junctions between Sertoli cells and germ cells (spermatogonia, spermatocytes and non-elongate spermatids) in the adult rat testis. As a result of this morphological intimacy between Sertoli and germ cells extensive interactions and communications take place between these cells throughout spermatogenesis both at biochemical and molecular level and germ cells largely rely on Sertoli cells not only for structural and nutritional support but also to coordinate various events of spermatogenesis (Cheng & Mruk, 2002).

Figure 2.1 Schematic representation of the anatomical arrangement of the adult rat testis.



It is well known that the essential prerequisite for normal testicular development and maintenance of spermatogenesis is the controlled secretion of LH, FSH, and testicular androgen during fetal and postnatal life (Gnessi et al., 1997). LH stimulates Leydig cells to secrete testosterone (testicular androgen), which is an absolute requirement for normal spermatogenesis. There are no receptors for FSH or testosterone on germ cells but Sertoli cell contains receptors for both FSH and testosterone, therefore these two hormones act through Sertoli cells (McLachlan et al., 1995). Thus Sertoli cells play the key role in spermatogenesis by not only controlling the radial arrangement of germ cells, surrounding environment of germ cells and by expressing receptors for FSH and testosterone but also by their ability to produce locally, a range of proteins, the paracrine factors, which are essential for spermatogenesis (Griswold, 1995).

The complexity of the whole process of spermatogenesis explains its marked sensitivity to toxic substances. Toxicity to male reproductive system in rats can be assessed by a number of parameters.

1. Fertile capability of male animals (rats) can be assessed by male reproductive performance study. This allows calculation of some reproductive indices, which are good indicators of fertile capability of male. These indices include: index of libido= (number mated/number paired) ×100, quantal pregnancy= (number pregnant/number mated) ×100, fertility index= (number pregnant/number paired) ×100 (Ratnasooriya & Dharmasiri, 2001).

2. The second parameter of importance is quality of semen. Researchers investigate the quality of semen by sperm count, sperm motility and sperm morphology assay. The sperms from cauda epididymis are most frequently used for analysis, as fully mature sperms are stored in cauda (De Kretser et al., 1982). Various methods for semen analysis are used. In the study conducted by Ratnasooriya & Dharmasiri (2001), the spermatozoa in the right cauda epididymis were extruded into a definite amount of isotonic saline and the number of spermatozoa was counted on a hemocytometer under the microscope. Total number was expressed as number of sperms per cauda epididymis. The gross morphology of spermatozoa can be observed using the above suspension. Ali et al., (2004) used above suspension for quantitation of percentage of morphologically abnormal sperms. They first stained it with 1% eosin and then evaluated microscopically (40× power). 200 sperms per animal were counted and divided into normal, abnormal head and abnormal tail types.

3. To investigate damaging effect on spermatogenesis and sperm maturation, changes in reproductive organ weight, homogenization resistant spermatid head count from testis and histopathology of testis are most often used parameters. Damaging effects on spermatogenesis invariably affect the reproductive organ weight therefore significant changes in this parameter can be a good indicator of reproductive toxicity (Ratnasooriya & Dharmasiri, 2001). Step 17–19 spermatids survive homogenization and can be counted in a hemocytometer. In the rat, developing spermatids spend about 6.3 days in these steps (Clermont & Harvey, 1965). Thus, daily sperm count can be calculated by dividing the number of spermatids determined, on a per-testis basis, by 6.3 (Crissman et al., 2000, Ali et al, 2004). Histopathological evaluation of testis allows direct examination of the site of spermatogenesis and detection of damaging effects. Qualitative examination gives an overall view of presence or absence of any abnormalities in testicular tissue whereas quantitative assessment of histopathology allows statistical comparison between groups. A number of parameters have been used by researchers for quantitative assessment of changes in histopathology of testis. Hessl & Nakai, (2000) used changes in testicular weight and percentage of tubules showing sloughing of germ cells to assess damage to testicular tissue caused by fungicide benomyl in male rats. The same parameters were also used by Sheri et al., (2001) while studying effects of Indenopyridine CDB-4022 on seminiferous tubules and D'Souza, (2003) for study of effects of tamoxifen on seminiferous tubules. Tubular differentiation index (TDI) is another parameter which gives a quantitative assessment of damage to spermatogenesis and was defined by Meistrich & Van Beek, (1993) as the