Adiantum capillus-veneris L. modulates reproductive dysfunctions linked to Furan induced oxidative stress and histopathological alteration in adult male Sprague Dawley rats

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DEPARTMENT OF ZOOLOGY

FACULTY OF BIOLOGICAL SCIENCES

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ISLAMABAD

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A dissertation submitted in the partial fulfillment of the requirements for the Degree of Master of Philosophy

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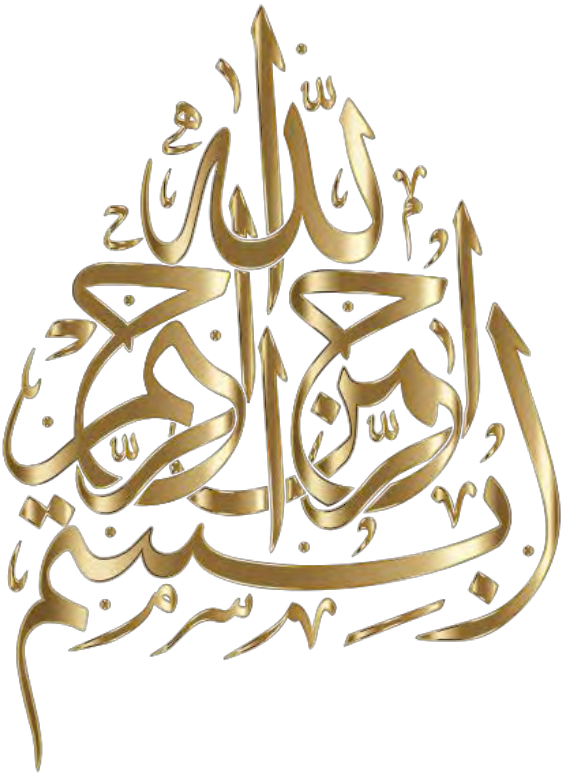
“In the Name of Allah, the most Beneficent, the most Merciful”



Dedicated to My Parents Who have given me the opportunity to Study from the best institutions and Supported throughout my life.

DECLARATION

I hereby declare that the work presented in the following thesis is my own effort and the material contained in this thesis is my original work. I have not previously presented this work elsewhere for any other degree.

Faiza Rafique

CERTIFICATE

This dissertation submitted "*Adiantum capillus-veneris* L. modulates reproductive dysfunctions linked to Furan induced oxidative stress and histopathological alteration in adult male Sprague Dawley rats." by **Faiza Rafique** is accepted in its present form by the Department of Zoology, Faculty of Biological Sciences, Quaid-I-Azam University, Islamabad as satisfying the thesis requirements for the degree of Master of Philosophy in Reproductive Physiology.

Supervisor: _____

Dr. Sarwat Jahan

External Examiner: _____

Chairman: _____

Dr. Amina Zuberi

Dated:

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I owe my gratitude to the One who is the Most Beneficent, Altruistic and Merciful, ***Almighty Allah****, Who puts the sun's seal on the tablets of the flowing waters and throws clouds to the skies, Who distills the waters of the clouds over the seas to conceive the pearl in the womb of the oyster, Who creates fire in every stone, color in the fire, radiance in the color, Who gives voices to the dust, word to the voices, and life to the world, Who created us as a Muslim and blessed us with the knowledge to differentiate between right and wrong. All prays to Him as He blessed us with the* ***Holy Prophet, Hazrat Muhammad*** *(SAW) for whom the whole universe is created and who enabled us to worship only one God. He (SAW) brought us out of darkness and enlightened the way of heaven.*

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Faiza Rafique

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LIST OF ABBREVIATIONS

BMI	Body Mass Index
CAT	Catalase
DEPPD	N, N-Diethyl-p-phenylenediamine sulphate salt
$FeSO_4$	Ferrous sulphate
H_2O_2	Hydrogen peroxide
kg	Kilogram
PBS	Phosphate Buffer Saline
NADH	Nicotine adenine dinucleotide
mM	millimolar
ROS	Reactive Oxygen Species
POD	Guaiacol Peroxidase
SOD	Super Oxide Dismutase
TBARs	Thiobarbituric Acid Reactive Substances Assay
WHO	World Health Organization
µm	Micromolar
µl	Microliter
ACV	*Adiantum capillus-veneris* L.
EDCs	Endocrine-disrupting chemicals
FDA	Food and Drug Administration
EFSA	European Food Safety Authority
PUFAS	polyunsaturated fatty acid
TNF-α	Tumor necrosis factor alpha
NO	Nitric oxide
ANOVA	One way analysis of variance
NADH	Nicotine adenine dinucleotide
Rpm	Revolution per minute
U/L	Unit per liter

DNA	Deoxyribonucleic Acid
mM	Milimolar
µm	Micrometer
ng	Nano gram
mg	Milli gram
Fe^{2+}	Ferrous ion
Cu^{2+}	Cuprous ion

Abstract

Furan is a volatile heterocyclic organic "high-production-volume" chemical with low molecular weight formed from numerous predecessor molecules naturally present in food during numerous steps of processing, which involve Maillard reaction. *Adiantum capillus-veneris* L**.** also known as southern maidenhair fern is an evergreen perennial fern which belongs to family Pteridaceae. It is a medicinal plant having antioxidant, antibacterial, antifungal, antialopecia. anti-urolithiasis, anti-inflammatory benefits. The current study was designed to investigate the ameliorative effects of methanolic leaf extract of *Adiantum capillus-veneris* L**.** against oxidative stress and histopathological alteration caused by furan in adult male Sprague Dawley rats. To conduct the present study, twenty adult male Sprague Dawley rats were divided into 4 groups; Group 1 was regarded as 'control group' and treated with 0.9% normal saline, Group 2 received 40mg/kg of furan dissolved in corn oil, Group 3 rats were provided with 250mg/kg of methanolic leaf extract of *Adiantum-capillus veneris* L. and group 4 animals were administered with 40 mg/kg of furan along with 250mg/kg leaf extract of plant for 28 consecutive days. On day 29, all the animals were weighed and sacrificed by decapitation. Blood samples were taken, followed by plasma extraction, and stored at -20˚C for biochemical analysis. For histological studies, epididymis and right testis were fixed in 10% formalin. The results showed that furan administration induced a notable ($p<0.01$) decline in body and testicular weight, decreased antioxidants concentrations of catalase ($p<0.01$), peroxidases ($p<0.05$) and sodium dismutase ($p<0.01$) in furan treatment group in comparison with control, whereas a noteworthy increase in number of reactive oxygen species ($p<0.05$) and thiobarbituric acid reactive species was evident. However, ACV treatment group showed conflicting effects for all these studied parameters. On the contrary, the findings of Furan+ACV were comparable to that of control, showing parallel results. Histopathological changes in the testis of rats were induced by furan as seen by decreased tubular diameter ($p<0.001$), lumen diameter ($p<0.001$), and increased epithelial height ($p<0.01$) in furan treated group, when comparison was made with control, while analysis of testes in Furan+ACV group showed restoration of disturbances caused by furan. The study concluded that furan decreases body weight and accessory organ weight, lowers antioxidants, increases oxidative stress, and brings histopathological

changes in testes, whereas ACV treated group showed contradictory results, further we found that ACV restored toxicological effects caused by furan in Furan+ACV group. Therefore, it is suggested that reproductive toxicity of furan in rats can be restored by ACV due to its antioxidant, antiobesity effects, and protective effects on reproductive system and thus, can be used to treat reproductive health problems.

Introduction

During recent decade, there has been a lot of concern regarding the negative effect of various environmental toxicants on human health and reproduction. Environmental factors such as exposure to environmental toxicants are one of the major causes of reproductive dysfunctions in humans. Numerous environmental toxicants are capable to induce pathophysiological changes in animals and thus, have been named as endocrine-disrupting chemicals (EDCs). In 1996, United States Environmental protection Agency (EPA) introduced term endocrine disruptors and was defined as exogenic agents that affect emission, synthesis, transportation, binding, action, of hormones in body which are responsible for keeping homeostasis, reproduction, development and behavior (Kavlock *et al*., 1996). Thus, it is suggested that several endocrine disruptors have been defined at different times depending on their discovery and activities.

More than 800 chemicals are known as EDCs because of their endocrine disruption properties. EDCs are extremely diverse and can be categorized based on their origin and occurrence; natural and synthetic (Grün & Blumberg, 2009)**.** The natural compounds are present in human and animal foods (e.g., phytoestrogen), while synthetic compounds have been further classified into plasticizers, pesticides, industrial solvents, polybrominated biphenyls, plastics, , fungicide, food source and pharmaceutical agents (Caliman *et al*., 2009; Diamanti-Kandarakis *et al*., 2009).

Human body is exposed to different type of endocrine disruptors through oral or intravenous route. Multiple studies have reported that ingestion of unhygienic food and contaminated water, inhalation of polluted air or direct contact with soil polluted with insecticides serve as the main contributor to exposure to EDCs. Newborns get contaminated with EDC by breast feeding and contact with baby products (A. Polyzos *et al*., 2011; Balaguer *et al*., 2017; Gore *et al*., 2015; Kabir *et al*., 2015) .

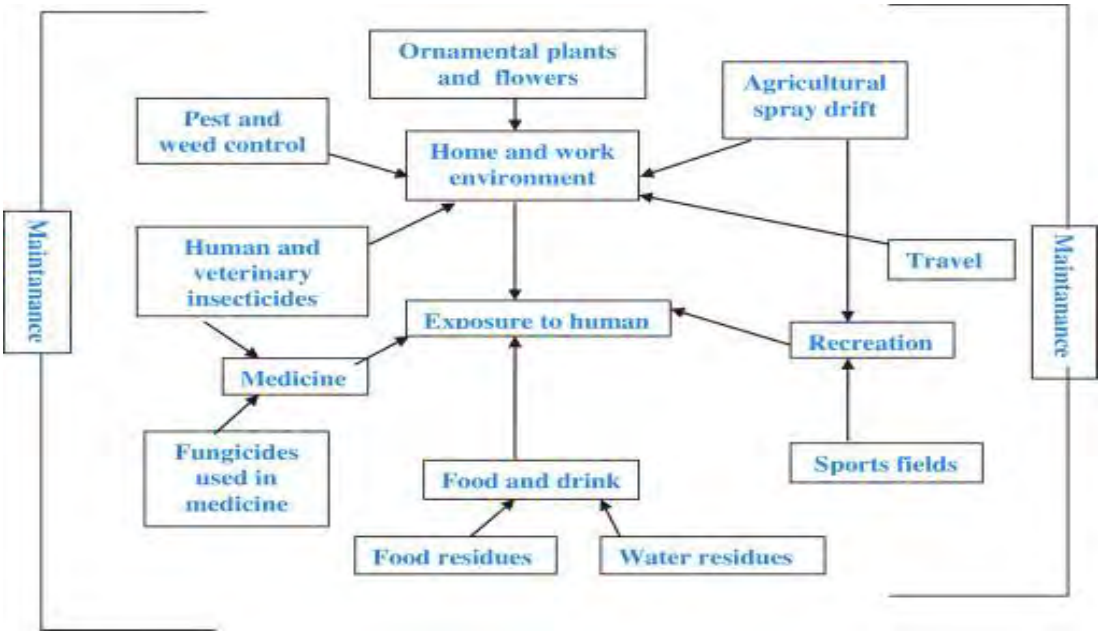


Fig. 1. Various routes of exposure of human to EDCs (Preda *et al*., 2012).

The actions of EDCs are mediated through direct contact with nuclear hormone receptors, or by binding and activating numerous hormone receptors (androgen receptor) and may mimic the natural activities of hormones (agonist action) (Heindel *et al*., 2015; Mnif *et al*., 2011; Monneret, 2017). They may induce the reduction of endogenic hormones concentrations by impeding formation, transport, break down, and removal of hormones (Mnif *et al*., 2011; Thomas Zoeller *et al*., 2012).EDCs impairs the body's ability to effectively regulate the endocrine system, resulting in induction of developmental, reproductive, and neurological consequences. Association of EDCs with other EDCs enhances the harmful effects of EDCs (Nohynek *et al*., 2013). EDCs regulate all the major body systems affecting endocrine axis, i.e., hypothalamus-pituitary gonadal, hypothalamus-pituitary adrenal, and hypothalamus-pituitary thyroid axis (HPG, HPA, and HPT axis respectively) (Thomas Zoeller *et al*., 2012). However, the reproductive system indicates the most susceptible endocrine axis to EDCs actions.

Heat induced food toxicants

Recently, heat-induced food contaminants have attracted the attention of both the scientists as well as general public (Velíšek *et al*., 1978). Some of the most prominent representatives of food contaminants are acrylamide, chloropropanols, and

furan. Acrylamide, chloropropanes, and furan are recognized as a thermal process contaminant in foods. Existence of chloropropanols and furan in food was first reported in 1970s, while acrylamide was identified in 2002 (Mogol & Gö, 2016). Due to presence of enriched amount of these substances in food consumed in high quantities, such as bread, and in baby food, these compounds have gain attention recently.

Furan

Furan (C_4H_4O,) is a low molecular weight heterocyclic aromatic compound containing one oxygen atom and is partly soluble in water. In 2004, US Food and Drug Administration (US FDA) and the European Food Safety Authority (EFSA) reported the occurrence of furan in foods, mainly heat-processed baby foods packaged in jars and cans (Heppner & Schlatter, 2007). It is also a primary component of wood smoke, cigarette smoke, and gases drain from engines in the environment (Barlow *et al*., 2009; Hazarika *et al*., 2004).

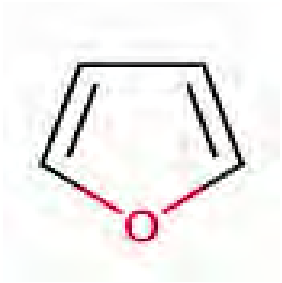


Fig 2: Chemical structure of Furan

Synthesis of Furan

Furan is synthesized during thermal breakdown of natural food ingredients that belongs to a group of dioxins. Furan found within food is synthesized by thermal breakdown of carbohydrates, amino acids, ascorbic acid, and its derivatives as well as by thermal oxidation of carotenoids and polyunsaturated fatty acid (PUFAS) (D. Crews *et al*., 2007; Yaylayan & Yaylayan, 2006). Formation of furan via oxidation of PUFAS, thermal breakdown of carbohydrates and amino acids involve Maillard reaction which is shown in figure 03.

According to literature review, vitamin C has the greatest capacity to produce furan (Perez Locas & Yaylayan, 2004). Different factors such as water ,temperature,

storage time, pH, and presence or absence of inhibitors and activators also affect furan formation and concentration in food (Nie *et al*., 2013; Owczarek-Fendor *et al*., 2012).

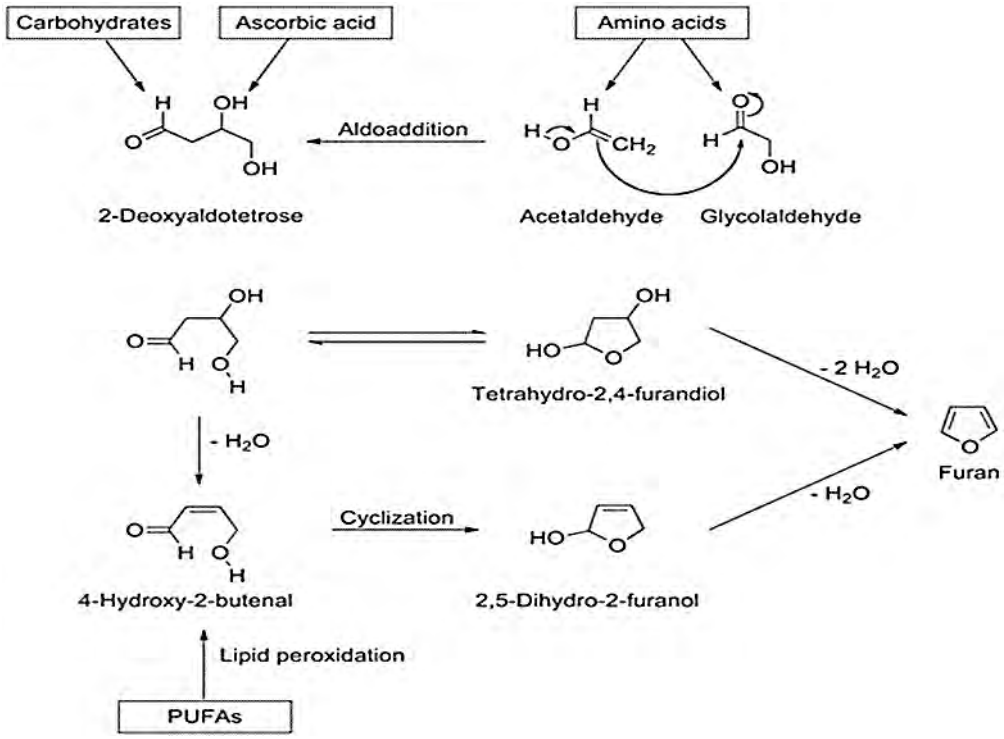


Fig 3: Distinct pathways for formation of furan from thermal breakdown of amino acids, carbohydrates, and oxidation of polyunsaturated fatty acids (Yaylayan & Yaylayan, 2006).

Furan content in food and human exposure

Human food contains thousands of structurally different chemical substances, mostly from natural origin and components added such as colorants, nutrients, and flavors. FDA scientists first recognized furan in several thermally processed food items and also reported that nutritional drinks, and bakery products have elevated concentration of furan (C. Crews & Castle, 2007).

Exposure of human to furan has been assessed based on feeding and the amount of furan content in different food substances. In developed countries, the average estimated exposure of adult human to furan through food is 0.34 to 1.23 g/kg bw/day (EFSA, 2009). At the age of 3-12 months, mean estimated exposure is 0.27 to 1.01 g/kg bw/day, while in infants, it is 0.23-1.77 g/kg bw/day. Highest source of

furan in adults is through consumption of coffee, that is about 1912 µg/kg in powdered ground coffee (Altaki *et al*., 2017). According to US and European report, roasted coffee contained excess amount of furan. Moreover, all thermally processed foods such as cereals products, meat products, baby food, sauces and soups have more than 100 µg furan/kg. Another source of furan transfer in infants is through breastfeeding (Lehmann *et al*., 2018; Wegener & López-Sánchez, 2010) **.** Recent findings have shown that bottled and canned drinks are also substantial source of furan for infants (Wegener & López-Sánchez, 2010)**.** 24–28 g/kg of furan was detected in jarred baby foods and 153 g/kg of furan was analyzed in mixtures of meat and vegetables. In contrast, no furan appeared in self-synthesized baby foods (Bianchi *et al*., 2006; Lachenmeier *et al*., 2012; Zoller *et al*., 2007).

Furan Toxicity

Furan is considered as "possible carcinogen of humans "and is known to be a cancer-causing agent in rats and other animal models by the International Agency for Research and Cancer and National Toxicology Program (Anon 2016, Anon 2016.; Seok *et al*. 2015). Numerous reports have revealed the adverse effects of furan on vital organs of rodents as well (Ldeniz *et al*., 2011; Webster *et al*., n.d.). It may cause DNA damage and necrosis of liver which results in high level of serum alanine aminotransferase in rats (Baş *et al*., 2016; Gill *et al*., 2010; McDaniel *et al*., 2012). It is reported in preceding studies that main cellular target of furan is mitochondria. It may affect mitochondrial energy production by altering gene expression involved in normal function of mitochondria (Hamadeh *et al*., 2004). Similarly, total antioxidant capacity, reactive oxygen species (ROS) and oxidative damage are used for determination of toxic impacts of furan on kidney and liver (Awad *et al*., 2018; de Conti *et al*., 2017). Previous reports suggest that levels of malondialdehyde rise, while superoxide dismutase, and catalase concentrations are reduced in rats exposed to furan (Baş *et al*., 2016).

Reproductive toxicity

The reproductive system is vulnerable to pollutants, such as food additives and pesticides (Karacaoğlu & Selmanoğlu, 2010). One of these pollutants is furan, whose exposure in male rats affect reproductive system; reproductive hormones such as LH

and testosterone levels decrease in rats exposed to furan due to its antiandrogenic activity (Cooke *et al*. 2012.; Karacaoğlu and Selmanoğlu 2010). Furan exposure also causes cell death in testis and bring histopathological changes in reproductive organs causing impaired spermatogenesis and stimulated programmed cell death in Leydig and germ cells (Karacaoğlu & Selmanoğlu, 2010b). In literature, inadequate data is available regarding toxic mechanism of furan on reproductive system of rats (Uçar & Pandir, 2017). However, furan generate reactive oxygen species in sperm and lymphocytes and results in immediate DNA damage (Pandır, 2015). Previous finding showed that exposure of furan via breastfeeding causes abnormalities in the fetus because such compound can pass through placental barrier (Krowke *et al*., 1990; Pluim *et al*., 1993).

Oxidative stress caused by furan:

Due to low polarity, furan can easily cross biological membranes (Bakhiya & Appel, 2010) and is quickly absorbed in body (D. Crews *et al*., 2007). Furan exposure in human is increasing day by day due to its production in overcooked food and baby products (Ghosh *et al*., 2015). Previous observations have suggested that the liver and kidney are the main acting sites for furan toxicity. As the main portion of defense mechanism of the living cell, CAT enzymes converts ROS to hydrogen peroxide and then, to water, which shield the cells from ROS toxicity (Syed *et al*., 2012). Furan is found to be toxic for hepatocytes, pancreas and renal cells, due to induction of oxidative stress (El-Habiby *et al*., 2017).

Herbal medicine is the oldest form of healthcare in the world. It has been used by all cultures throughout history. In the evolution of modern civilization, it was an important step. Herbal medications make up a large portion of today's pharmaceuticals. According to WHO, herbal medicine is used by 80 percent of the world's population for some aspect of primary health care. One of the most common species with possible therapeutic and nutritional value is *Adiantum capillus-veneris* (Al-Snafi & Al-Snafi, 2015).

***Adiantum capillus-veneris* L.**

Adiantum, a genus of the Pteridaceae family, can be found throughout the world from hot tropical zones to cool temperate zones and is endemic to Australasia,

the Levant in Western Asia, Eurasia, and southern part of the USA. The plant is also known as southern maidenhair fern, is an evergreen fern. (C. Singh *et al*., 2020). It has over 200 species spread across both Southern and Northern hemispheres (C. Singh *et al*., 2020) The plant is dispersed in America, Afghanistan, Africa, Ethiopia, Bhutan, India, Nepal, North America, Arabia, Southern, and Central Europe, Ireland, southwest England, Australia, and Pakistan.

Common names

Some of the most common names for *Adiantum capillus-veneris* L. are Maria's fern, Our Lady's hair, Maiden hair fern, Kazbaratul Ber**,** Unani, Persia-ushan, Barsioshan, Dumtuli.

Traditional uses:

Adiantum capillus-veneris L. is a medicinal plant having therapeutic benefits. It is an decorative fern used for fragrant purposes (Ahmed *et al*., 2013; Haider *et al*., 2011; Ibraheim *et al*., 2011). The entire plant is utilized as a source of fragrance and a cough demulcent. Antipyretics are made from leaves of this plant (Kanwal *et al*., 2018). It has been used to treat leprosy, animal bites, thyroid problems, and musculoskeletal problems. It has the potential to treat hypothyroidism, cold, bronchitis, pneumonia, severe phlegm, Cough, flu, menstrual problems as well as skin problems. It is also used as a blood cleanser (Abbasi *et al*., 2010; Ahmed *et al*., 2013; Haider *et al*., 2011; M. Singh *et al*., 2008). Adiantum leaves and honey have long been used in India as a treatment for seasonal cold fever. In the literature, there are numerous studies demonstrating its various health benefits, including antibacterial and antifungal, antioxidant, antialopecia, anti-inflammatory, anti-urolithiasis, antinociceptive and hypoglycemic properties (Ahmed *et al*., 2013; Haider *et al*., 2011; Ibraheim *et al*., 2011; Ishaq *et al*., 2014; Nilforoushzadeh *et al*., 2014; Noubarani *et al*., 2014; C. Singh *et al*., 2020). Species of Adiantum were used for dandruff, and antiparasitic. The fresh and dried leaves were used as sudorific, antidandruff, febrifuge, astringent, laxative, galactagogue, depurative, emollient, weak expectorant, antitussive, refrigerant, stimulant, demulcent, and tonic. The dried leaves of this plants were also used to make a tea (Al-Snafi & Al-Snafi, 2015).

During phytochemical examinations alkaloids, cardiac glycosides, steroids, reducing sugars, tannins, triterpenoids, flavonoids such as, quercetin, stigmasterol and capesterol were discovered as plant constituents (Haider *et al*., 2011; Ishaq *et al*., 2014).

Pharmacological potency of *Adiantum capillus-veneris* L.

Antiobesity effect

Extract of Aerial parts of ACV exhibited phospholipase inhibitory effect due to presence of one of its phytoconstituents, chlorogenic acid (Kasabri *et al*., 2017).

Antioxidant activity

Free radical scavenging molecules such as tannins, flavonoids, saponins terpenoids, and reducing sugar are present in leaves of *Adiantum capillus-veneris* L., thus, due to their occurrence in this plant, it exhibits antioxidant properties (Sinam *et al*., 2012).

Anti-inflammatory activity

Plant ethanolic extract has demonstrated considerable anti-inflammatory activity related to the inhibition of NO release and diminishing TNF-α level. Triterpenes may play a key part in the plant's anti-inflammatory that are mediated due to their inhibitory influence on the synthesis of inflammatory cytokines, which suppresses the activation of nuclear factor kappa B (Haider *et al*., 2011).

Anti-diabetic activity

A streptozocin-induced diabetic rat model showed the anti-diabetic ability of methanol leaf extracts of *Adiantum capillus-veneris L.*, that may be due to the presence of flavonoids and tannins. Improvement in the fasting blood sugar, demonstrated the anti-diabetic properties of this plant (Ranjan *et al*., 2014).

Distribution in Pakistan

In Pakistan, the distribution of the plant is in different areas of Swat and Peshawar, Balakot, Changla gali, Abbottabad (altitude 2,350 m) Mansehra, Battagram (altitude 1,100 m), Murree, Rawalpindi (altitude 1,800 m) and District Shangla.

Topography

On the globe, District Shangla is situated on 34° 31' to 33° 08' N and 72° 33' to 73° 01' E. Districts Batagram and Kala Dhaka SpinGher is located on the east, district Swat is located on the west, district Buner is located on the south and district Kohistan is located on the north. Forest covers total area of 8,090 acres, average rainfall 1778mm while monthly is 150mm, maximum snowfall is up to 60inches from November to March, maximum temperature during June and July is above 25°C and minimum up to 0°C in December. The taxonomy of the plant according to National Plant Data Center, NRCS, USDA is as follows:

Kingdom: Plantae

Sub-kingdom: Traciobionta

Division: Pteridophyta

Class: Pteridopsida

Order: Pteridales

Family: Pteridaceae

Genus: Adiantum

Species: *Adiantum capillus- veneris* L.

Fig 4*: Adiantum capillus-veneris* L.

Many studies on the Adiantum plant have shown that it possesses antibacterial, anti-inflammatory, anti-obesity, and antioxidant properties, but no research on its anti-infertility characteristics has been done. The goal of this research is to determine the antifertility role of *Adiantum capillus-veneris* L. against furan.

Aims and Objectives

The aim of this experiment is to investigate the protective effect of *Adiantum capillus-veneris* L. against toxicity caused by furan in male rats. The objectives of this study are:

- To evaluate histopathological changes induced by furan.
- To determine the oxidative stress, induce by furan in testes of rats.

Materials and Methods

The current study was directed in Laboratory of Reproductive Physiology, Zoology Department, Quaid-i-Azam University, Islamabad. Animals were handled in accordance with guidelines authorized by the resident ethical committee of Zoology Department, which is research-specific in animal handling.

Chemical used

Furan (CAS 110-00-9) was bought from Sigma Aldrich, USA., H_2O_2, Phenazine methosulphate, Corn oil were purchased from Sigma-Aldrich.

Animals

Adult male Sprague Dawley rats (twenty) were selected from animal house of Zoology Department Quaid-i-Azam University Islamabad, Pakistan with an average weight of 150±10g. They were kept randomly in 4 groups and confined in separate stainless-steel cages (Ullah *et al*. 2016). During the experimentation, these animals were kept alive for 28 days in aeriform room having of 20- 26˚C temperature and 12 hours of dark/light cycle. Throughout experiment Food chaw and tap water was fed to the animals ad libitum.

Plant Material

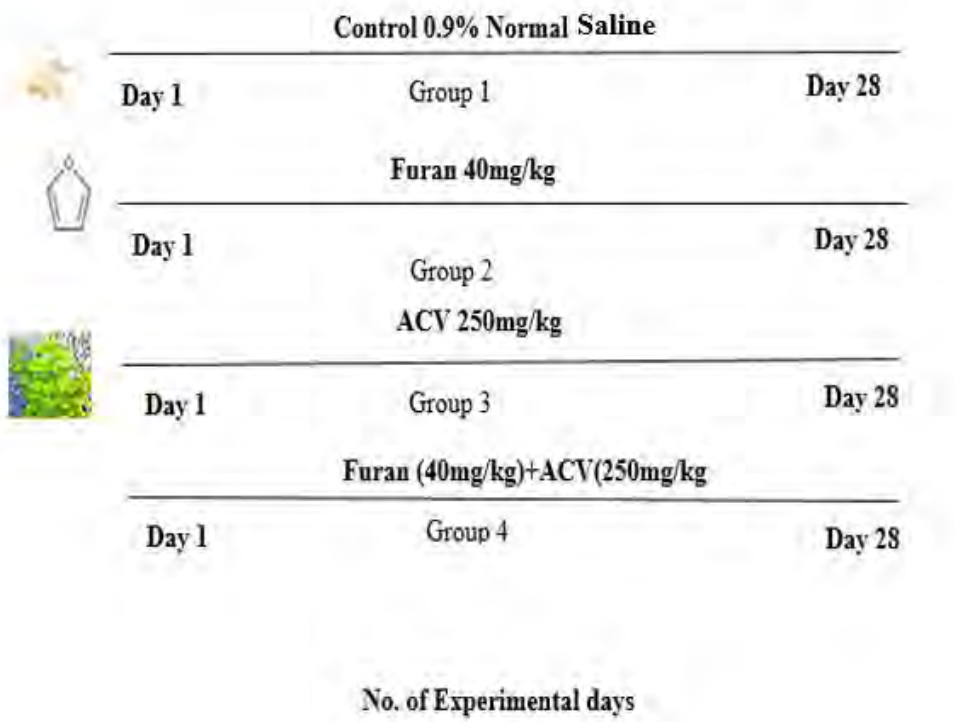
Dr. Mushtaq Ahmad at Plant Systematics and Biodiversity Lab and Herbarium of Pakistan Quaid-i-Azam University Islamabad. recognized the leaf sample of *Adiantum capillus- veneris* L. taken from cultivated and farming fields of Shangla Alpuria, Peshawar, Chakesar, Swat.

Plant Extract Preparation

Leaves of *Adiantum capillus- veneris* L. were dried out in air and then put in storage for the preparation of extract. Leaves were first crushed in waring blender and then seived, dried powder of leaves was isolated with methanol. The extract was strained by means of Whattman filter paper and purified on rotary evaporator (Gulfraz *et al*. 2007).

Experimental Design

For present study, the animals (n=5) were placed into four groups. All the doses were given orally between 10-11am. For about 28 consecutive days, as shown in figure given below.



Group I

This group was considered as 'control group' and treated with 0.9 % Normal saline.

Group II

Animals received 40 mg/kg of furan dissolved in corn oil.

Group III

Rats were provided with 250 mg/kg of methanolic leaf extract of *Adiantum-capillus veneris* L. dissolved in 0.9 % Normal saline.

Group IV

Animals were administered with 40 mg/kg of furan and 250 mg/kg methanolic leaf extract of *Adiantum capillus-veneris* L. dissolved in 0.9 % Normal saline.

Final body weights of all the animals were assessed and animals were sacrificed by decapitation on 29th day.

Blood and Tissue collection

For 28 days, the experiment was conducted. Animals were weighed and decapitated on 29th day. Following decapitation, heparinized syringes were used to collect trunk blood directly and kept in heparinized tubes. For 15 minutes, blood samples were centrifuged at 3000 rpm. Plasma was separated and stored at -20˚C till evaluated. From all animals both testicular and epididymal tissues were obtained. Histological studies were done on epididymis and testicular tissue (right) secure in 10 % formalin.

Biochemical Analysis

For biochemical parameters and protein estimation, blood plasma was separated and saved at 20˚C. The frozen samples were thawed and were used for antioxidant status and for protein estimation of control and all treatment groups.

Catalase (CAT) Activity

With minor alterations, (Chance and Maehly 1955) method was used to determine activities of Catalase.

Procedure:

In order to measure levels of CAT in testicular tissue, 0.1 ml plasma, phosphate buffer (pH 5.0), 2.5ml of 50 mM and 0.4ml of 5.9 mM H_2O_2 were added in a cuvette. Variation in absorbance of solution was noted at wavelength of 240nm after one minute. CAT activity was termed as change in absorbance of 0.01 units in one minute.

Superoxide Dismutase (SOD) Activity

Activity of SOD was determined by using procedure of (Kakkar, Das, and Viswanathan 1984).

Procedure:

For the analysis of SOD levels, 0.3 ml of sample was mixed with phenazine methosulphate (186 μM) (0.1ml), sodium pyrophosphate buffer (pH 7.0) (1.2 ml)

and 0.2 ml of NADH was added to initiate reaction. Lastly, after 1 minute reaction was halted by addition of 1 ml of glacial acetic acid and readings were noted at 560 nm . Results were clarified as units per milligram of protein.

Guaiacol Peroxidase (POD) Activity

Guaiacol peroxidase (POD) activity was evaluated through method suggested by (Chance and Maehly 1955).

Procedure:

Reaction was held out by addition of 2.5 ml of 50 mM phosphate buffer (pH= 5.0), 0.3 ml of 40 mM H_2O_2, 20 mM guaiacol (0.1 ml) into 0.1 ml of homogenate. After 1-minute Changes in absorbance were observed at 470 nm. POD activity was defined as Change in absorbance of 0.01 unit over a minute.

Estimation of Reactive Oxygen Species Assay (ROS)

The protocol of Nagasaka *et al*. 2007 was used for evaluation of ROS in testicular tissues.

Procedure:

Preparation of sodium acetate (0.1 M) buffer was done by adding 4.1 g of sodium acetate in 500 ml of distilled water, and their pH was held at 4.8. Afterward by adding 50 mg of ($FeSO_4$) in 10 ml of sodium acetate buffer a second solution was prepared. Both solutions were combined in 1:25 and incubated for 20 minutes at room temperature in dark. Then solutions mixture (20 µl), buffer (1.2 ml) and homogenate (20µl) were taken in a cuvette and by using Smart Spec TM plus spectrophotometer absorbance was patterned at 505 nm. After every 15 seconds for every sample three readings were achieved.

Thiobarbituric Acid Reactive Substances (TBARS)

For the determination of lipid peroxidation (Wright, Colby, and Miles 1981) method was followed.

Procedure:

A reaction solution with a total volume of 1 ml was prepared by adding 0.2 ml of sample, 0.02 mM $Fecl_3$ of 100mM, ascorbic acid (0.2 ml) of 100mM and 0.58 ml

phosphate buffer (0.1 M) having pH 7.4. Finishing mixture was incubated for 1 hour at 37˚C in water bath, and 10% trichloroacetic acid (1 ml) was added to stop reaction. Then put 0.67 % thiobarbituric acid (1ml) and, for 20 minutes all the tubes were first placed in water bath of boiling water and then moved to crushed ice. Followed by centrifugation at 535 nm and for 15 minutes at 25000 rpm readings were observed from spectrophotometer. Results were described as µmol of TBARS/min/mg tissue.

Total protein

For the evaluation of total protein in testicular tissues a protein kit was used bought from AMEDA Labordiagnostik GmbH, Krenngasse, Graz, Austria (Cat # BR5202-S). By plotting absorbance of kit standards against absorbance of samples total protein was assessed. Total protein contents were expressed in mg g^{-1} of tissue.

Procedure

- Samples, standard and blank were added into respective wells.
- Then added given reagent in each well one by one .
- After addition mixed gently and incubated for 10 minutes at 37℃
- Read absorbance of standard and sample within one hour at 540nm.
- Finally, concentration was calculated by using the formula given below.
- $\mathbf{A_{sample}/A_{standard} \times C_{standard} = g/dL\ Total\ protein}$

Tissue Histology

Histology of testicular and epididymal tissues were conducted to measure antifertility effects of furan on testis and epididymis. After collection of testicular and epididymal tissues, following steps were performed.

Fixation

Placed tissue in Sera ---------- 4-6 hours

Placed tissue in 10% formalin ----------24-48 hours

Dehydration

After fixation, tissues were dehydrated at room temperature in ascending grades of alcohol. 70% Ethanol--60 min, 80% Ethanol ---60 min, 90% Ethanol --60 min, 95% Ethanol--60 min, 100% Ethanol I--90 min, 100% Ethanol II--90 min, 100% Ethanol III--90 min.

Embedding

For embedding following steps were taken. Tissues were placed in xylene and fixed in paraffin in following order. Xylene I--60 min, Xylene II---60 min, Paraffin I (58°C)--120 min. After that, tissues were moved in molten wax within a boat. After removing bubbles from wax, it was allowed to solidify. Paraffin wax blocks with tissues in them were trimmed and mounted on wooden blocks for section cutting by using knife or scalpel.

Albumen Slides preparation

Preparation of Albumin

Two egg whites were added to 1200mL of deionized water, followed by stirring for 5 minutes on magnetic stirrer. Then, concentrated ammonium hydroxide (4mL) was added. Again, stirred for 5 minutes, followed by filtering across a low-grade filter (coffee filter). At the end, stored albumin at 40˚C in the dark in a screw-top glass bottle.

Coating Slides

1. For coating slides, at a low setting on slide warmer slides were individually laid out.
2. A thin layer of albumin was painted onto each slide using a small clean paint brush. The process is repeated at least three times.
3. At a low setting, slides were allowed to dry on the heating plate overnight.
4. At room temperature slides coated with albumin were stored in the original packaging until needed.

Microtomy

These tissues embedded in paraffin were then mounted on wooden blocks using melted wax after embedding. Using microtome (in which wooden blocks were

placed) seven µm thin sections were cut. Tissue-filled long ribbons were stretched, followed by fixation in previously prepared clean albumenized glass slides. These slides were kept on Fischer slide warmer at 60˚C. After that, for the completion of stretching glass slides were placed in incubator for a night.

Staining

Slides were stained in different grades as follows: Xylene I – 3 min, Xylene II – 3 min, 100% alcohol I - 3 min, 100% alcohol II – 3 min, 90% alcohol – 3 min, 70% alcohol – 3 min, Washed with water 3 min, Hematoxylin – 8 min, Washed with water – 2 min, Acidified alcohol – 1 min, Washed with water – 2 min, Bluing solution – 2 min, Washed with water – 2 min, 90% alcohol – 10 dips, Eosin – 2 min, Washed with water – 2 min, 90% alcohol -5 min, 100% alcohol I – 5 min, 100% alcohol II – 5 min, 100% alcohol III – 5 min, Xylene I - 5 min, Xylene II – 5 min, 3 small drops of DPX on each slide were put to permount, then placed cover slip gently before permount dried.

Light Microscopic Study

Tissue sections (7 µm thick) were examined at 40 magnifications using light microscope (Nikon, 187842, Japan). Slides of all experimental groups were analyzed. Following parameters were studied for histomorphometric analysis by means of image J2x software, package program, such as testicular luminal and tubular diameter, epithelial height, as well as diameter (tubular and lumen) and epithelial height of epididymal tissue.

Microphotography:

Using Leica LB microscope (Germany) fitted with cannon digital camera (Japan) microphotography was done.

Statistical Analysis

SPSS software was used to perform one way analysis of variance (ANOVA) followed by post-hoc Tukey's test for comparison of various groups. All the results were presented as Mean ± SEM. The level of significance was set at $p<0.05$.

Results

Body weight

There was a highly significant decrease (P<0.001) in body weight gain in furan treated group as compared to the control while a non-signific decrease in body weight gain was observed among ACV (p=0.901) and F+ACV (p=0.411) groups. A highly significant increase (p<0.01) in body weight gain was seen among ACV and F+ACV groups when compared with furan. No significant change in body weight gain was noted in the F+ACV group in comparison with ACV treated group (p<0.483).

Table 1: Mean ± SEM body weight gain after 28 days of exposure among adult male rats.

Parameters	Control	Furan	ACV	Furan+ ACV	P-value statistics
Body Weight gain (g)	41.17±4.38	21.09±3.02^{a***}	43.56±1.08^{b***}	42.06±4.21^{b**}	0.002

Values are specified as Mean ±SEM. *, **, *** showing significance variance at p< 0.05, p<0.01, p<0.001 respectively. **a** represents Value vs control, **b** indicates Value vs furan and **c** signifies Value vs ACV.

Testis weights

A highly remarkable decline in testicular weight was seen in group treated with furan (p<0.001) in comparison with control while a non-significant decrease in testicular tissue was noticed among ACV (p=0.70) and F+ACV (p=0.70) treated groups. There was a significant increase in both testicular weights among ACV and F+ACV groups (p<0.05) when compared with furan treatment group. When comparison was made with ACV treatment group insignificant decrease was assessed in both testicular weights in case of F+ACV group (p=0.79).

Epididymis weights

There was a highly significant increase in both right and left epididymal weight seen among furan, ACV, and F+ACV treatment groups in comparison with control (p<0.001). A negligible increase in both right and left epididymal weight was observed among ACV and F+ACV groups (p=0.60) when compared with furan. A non-significant increase in epididymal weight was seen in group treated with F+ACV (P=0.709) in comparison with ACV.

Accessory Organ weight

A noteworthy reduction ($p<0.001$) was seen in weight of accessory organ in furan treatment group when comparison made9o with control while a non-significant decline ($p=0.59$) in accessory organ weight was seen among ACV and F+ACV treatment groups as compared with control. A highly remarkable rise in weight of prostate and seminal vesicle was detected in ACV and F+ACV groups ($p<0.001$) as compared with furan.

Table 2: Mean ± SEM testicular, Epididymal, and Accessory Organ weights after 28 days of exposure among adult male rats.

Parameters	control	Furan	ACV	Furan+ ACV	
Testis weights (R)	1.43±0.02	$1.32\pm0.05^{a***}$	$1.45\pm0.02^{b*}$	$1.44\pm0.02^{b*}$	P=0.009
Testis weights (L)	1.42±0.02	$1.30\pm0.03^{a***}$	$1.43\pm0.02^{b*}$	$1.42\pm0.05^{b*}$	0.02
Epididymis weights (R)	0.12±0.07	$0.66\pm0.10^{a***}$	$0.71\pm0.03^{a***}$	$0.62\pm0.06^{a***}$	0.001
Epididymis weights (L)	0.56±0.12	$0.64\pm0.10^{a***}$	$0.70\pm0.02^{a***}$	$0.60\pm0.05^{a***}$	0.68
Prostate weight (g)	0.65±0.01	$0.50\pm0.01^{a***}$	$0.67\pm0.01^{b***}$	$0.66\pm0.01^{b***}$	0.001
Seminal vesicle Weight (g)	0.74±0.01	$0.40\pm0.03^{a***}$	$0.78\pm0.01^{b***}$	$0.77\pm0.02^{b***}$	0.001

Values are specified as Mean ±SEM. *, **, *** showing significance variance at $p< 0.05$, $p<0.01$, $p<0.001$ respectively. **a** represents Value vs control, **b** indicates Value vs furan and **c** signifies Value vs ACV.

Catalase (CAT)

A highly remarkable decrease in CAT levels was observed in the furan treated group ($p<0.01$) while CAT levels were significantly rise in the ACV group ($p<0.05$) when a comparison was made with control. A non-significant increase in CAT levels in the F+ACV group was seen ($p=0.086$) in comparison with control group. A highly remarkable increase in CAT levels was assessed among ACV and F+ACV groups ($p<0.01$) when compared with the furan group. A highly remarkable decrease in CAT

levels was detected in the F+ACV treated group (P<0.01) when a comparison was made with the ACV group (Figure 5).

Sodium dismutase (SOD)

A highly significant decline (p<0.01) was observed in SOD levels in furan group as compared with the control. There was a highly remarkable increase in SOD levels in ACV group (p<0.01) when a comparison was made with the control. No remarkable change was noticed in SOD levels in F+ACV treated group (p=0.786) when comparing to control. A remarkable increase was observed in SOD levels among ACV group and F+ACV group (p<0.01) in comparison with furan. A notable decrease (p<0.01) was observed in SOD levels among F+ACV as compared to ACV treatment group (Figure 6).

Peroxidases (POD)

A remarkable decrease in POD levels was seen in the furan treated group (p<0.05) in comparison with control while a noteworthy increase in levels of POD was observed in ACV (p<0.01). No remarkable change in POD levels was noticed in F+ACV group when compared with control group (p=0.601). A noteworthy increase in POD levels in ACV group (p<0.01) was noticed in comparison with furan treatment group. A non-significant rise in POD levels was detected in the F+ACV group (p=0.164) as compared with furan. A highly significant decline in POD levels was noticed in F+ACV (p<0.01) in comparison with ACV treatment group (Figure 7).

Reactive oxygen species (ROS)

A remarkable rise in ROS level (p<0.05) was noticed in furan treatment group when comparison was made with control. Nonsignificant reduction in ROS levels was seen in group treated with ACV (p=0.162) when compared with control while a non-significant rise in ROS levels was seen in F+ACV treatment group (p=0.353) in comparison with control. A highly significant decrease in ROS levels was detected in group treated with ACV (p<0.01) as compared to furan. Levels of ROS were considerably increased in F+ACV group (p<0.01) as compared to furan. A negligible rise in ROS levels was seen in F+ACV when compared with the ACV group (Figure 8).

Thiobarbituric Acid Reactive Substances (TBARs)

A negligible increase in TBARs level was seen in furan group (p=0.139) as compared with control. A non-significant reduction in TBARs levels was seen among ACV and F+ACV groups when compared with control. A remarkable decline ($p< 0.05$) in TBARs levels were observed among the ACV group and F+ACV group as compared to furan treatment group. A non-significant rise was noticed in TBARs levels in F+ACV group (P=0.475) as compared to ACV treated group (Figure 9).

Protein content

A highly significant decrease was observed in protein content among furan, ACV, and F+ACV groups ($p<0.01$)when a comparison was made with control group. There was a considerable rise in protein content among ACV and F+ACV groups ($p<0.01$) when compared with furan. A highly significant decrease was seen in protein content in F+ACV group when compared with ACV group ($p<0.01$). (Figure 10).

Table 3: Mean ± SEM biochemical parameters after 28 days of exposure among adult male rats.

Antioxidants/oxidants	Control	Furan	ACV	F +ACV	P-value statistics
Catalase (U mg^{-1} protein)	23.77±1.77	11.42±0.69^{a***}	27.01±0.88^{a*b***}	21.03±0.66 ^{bc***}	0.000
Sodium oxide dismutase (nmol min^{-1} mg^{-1} protein)	22.46±0.85	11.67±1.92^{a***}	30.67±0.94^{ab***}	21.99±0.72 ^{bc***}	0.000
Peroxidases (nmol min^{-1} mg^{-1} protein)	15.89±2.39	11.57±1.05 ^{a*}	24.51±0.78 ^{ab***}	14.72±1.57 ^{c***}	0.000
Reactive oxygen species (U mg^{-1} tissue)	1.26±0.19	1.85±0.28 ^{a*}	0.92±0.01^{b***}	1.03±0.04 ^{b*}	0.002
Thiobarbituric Acid Reactive Substances (nM TBAR min^{-1} mg^{-1})	0.76±0.18	1.12±0.25	0.39±0.11 ^{b*}	0.56±0.07 ^{b*}	0.025
Protein content	55.58±2.52	33.69±0.31^{a***}	52.96±0.08^{ab***}	43.26±0.10^{abc***}	0.000

Values are specified as Mean ±SEM. *, **, *** showing significance variance at $p< 0.05$, $p<0.01$, $p<0.001$ respectively. **a** represents Value vs control, **b** indicates Value vs furan and **c** signifies Value vs ACV.

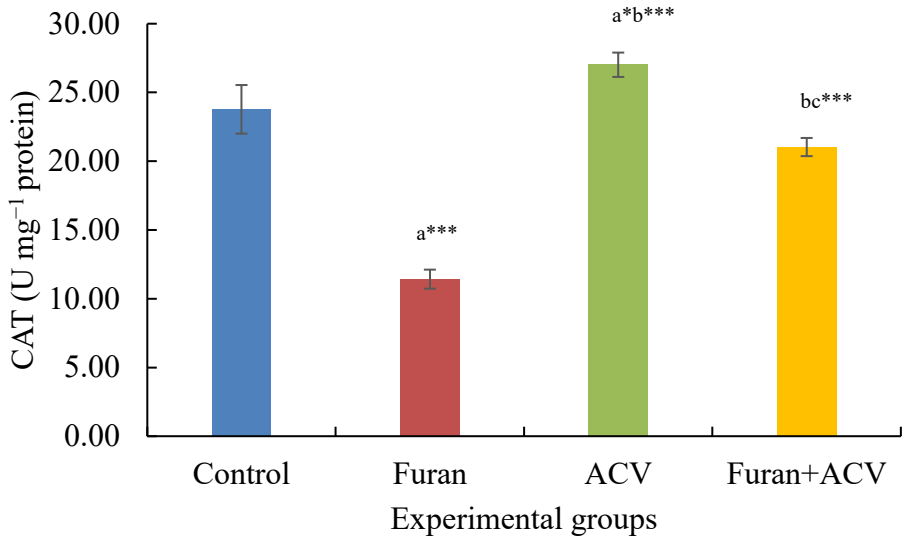


Figure 5: Comparison of blood plasma concentration of Catalase (U mg^{-1} protein) in control and treated groups.

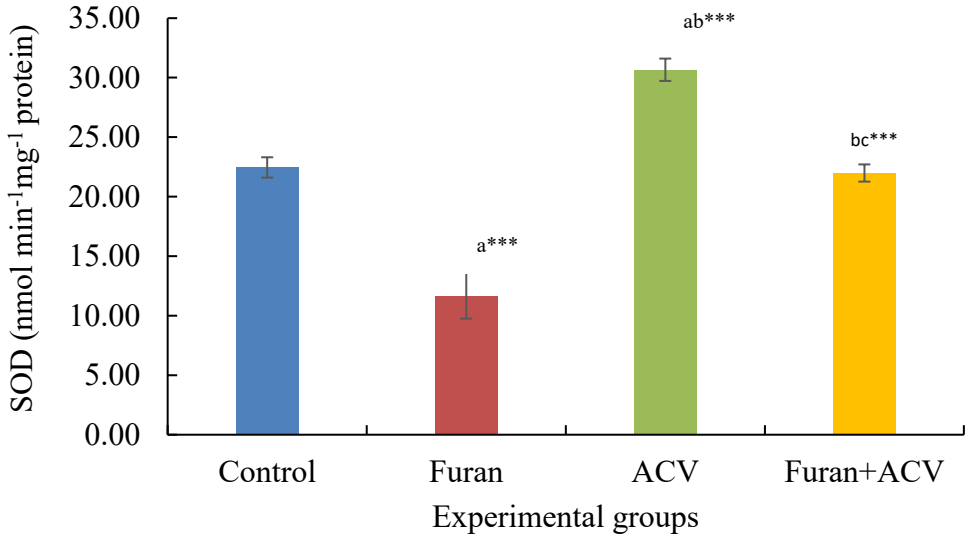


Figure 6: Comparison of blood plasma concentration of Superoxide dismutase (nmol $min^{-1}mg^{-1}$ protein) in control and treated groups.

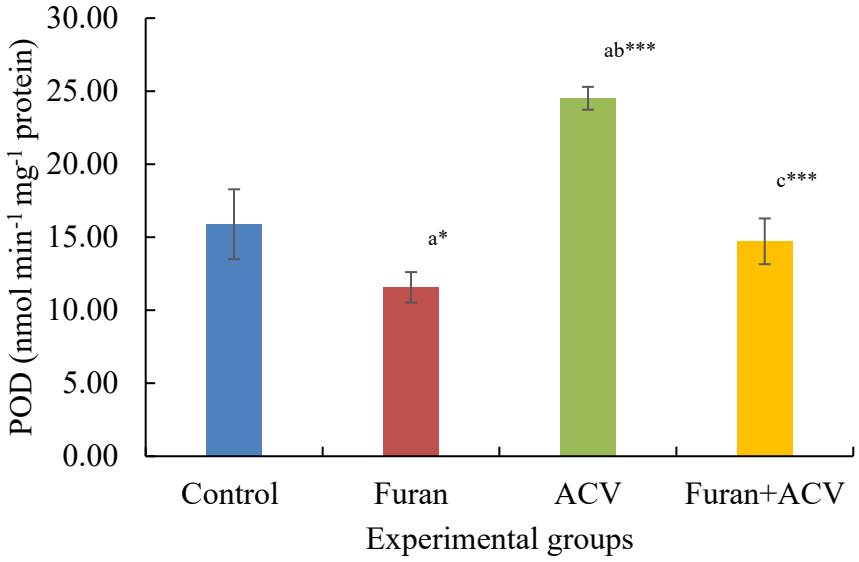


Figure 7: Comparison of blood plasma concentration of Peroxidase (nmol min^{-1} mg^{-1} protein) in control and treated groups.

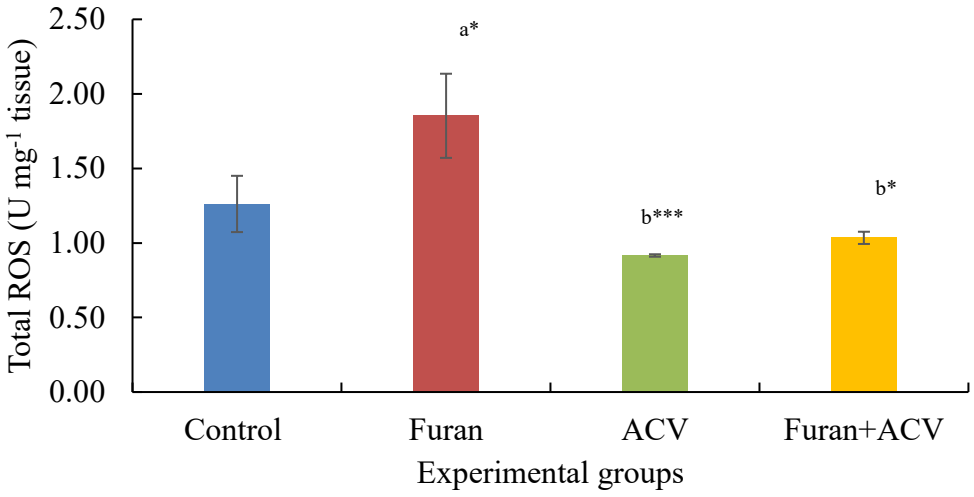


Figure 8: Comparison of blood plasma concentration of Reactive oxygen species (U mg^{-1} tissue) in control and treated groups.

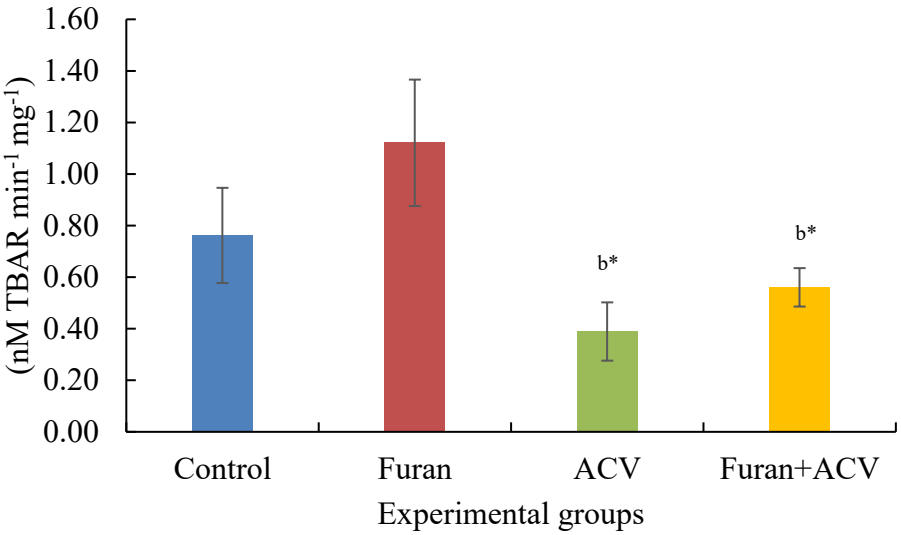


Figure 9: Comparison of blood plasma concentration TBARS in control and treated groups.

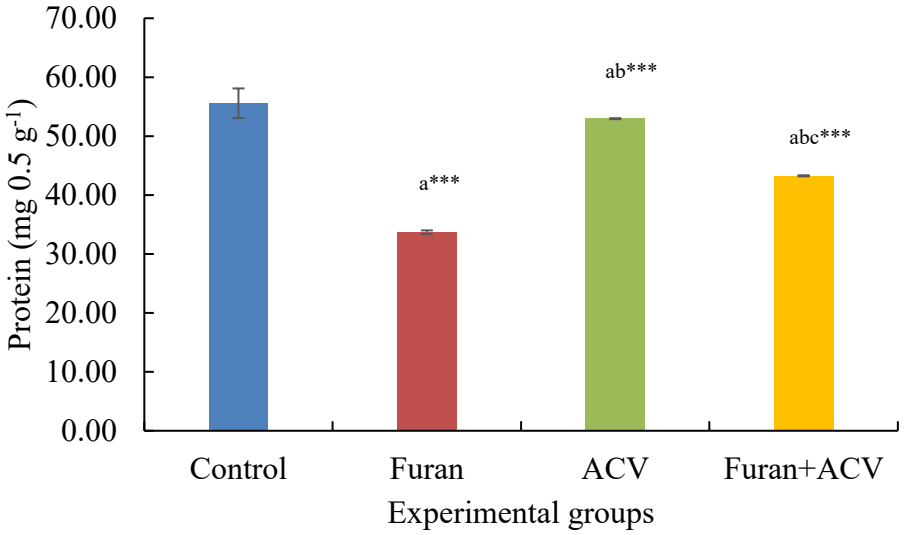


Figure 10: Comparison of blood plasma concentration of Protein content (mg 0.5 g^{-1}) in control and treated groups.

Histopathological results

In control group, seminiferous tubules were closely placed to each other, and held tightly with the presence of intact cellular organization. The luminal compartment was heavily flushed with mature spermatozoa and Sertoli cells were closely bound to each other. The interstitium was enriched with rounded and oval shaped Leydig cells as well as large blood vessels were seen around seminiferous tubules.

In furan treated group (40mg/kg), testicular organization of seminiferous tubules was adversely affected; Seminiferous tubules were deranged, and normal organization was absent, where large interstitial spaces were prominent with the occurrence of an insufficient number of Leydig cells. Basement membrane was also distorted and sertoli cells were present apart due to disruption of blood-testis barrier, the lumen was devoid of or contained limited number of mature spermatozoa. These outcomes are in agreement with a previous study by (Rehman et al. 2019) where testicular organization of seminiferous tubules was adversely affected due to furan exposure. ACV restored the testicular and epididymal disorganization caused by furan.

There was a remarkable ($p<0.001$) reduction in diameter (tubular and lumen) of testis in rats treated with furan while epithelial height presented a noteable increase ($p<0.01$) as compared to control. Testicular tubular diameter showed a significant increase ($p<0.05$) while diameter of testicular lumen ($p=0.104$) and epithelial height (0.392) indicated a non-significant increase in ACV group when compared to control. There was a highly remarkable decline ($p<0.01$) in tubular diameter, a noteworthy increase ($p<0.01$) in lumen diameter and when a comparison was made with control, a insignificant decrease in epithelial height in F+ACV treatment group was seen. A considerable ($p<0.01$) rise in tubular diameter and epithelial height was noticed in ACV group while lumen diameter showed a significant reduction ($p<0.05$) in ACV-treated rats in comparison with furan. A remarkable rise ($P<0.01$) was observed in testicular tubular diameter while a nonsignificant diminution ($p=0.856$) occurred in the lumen diameter of rats exposed to F+ACV as compared to furan. Epithelial height showed a nonsignificant rise ($p=0.256$) in F+ACV group when compared with furan treated group. A remarkable diminution ($p<0.01$) in tubular diameter, while a considerable rise

($p<0.05$) in lumen diameter was noticed in F+ACV group in comparison with ACV. A non-significant reduction in epithelial height was evident ($p<0.097$) in F+ACV group when comparison was made with ACV.

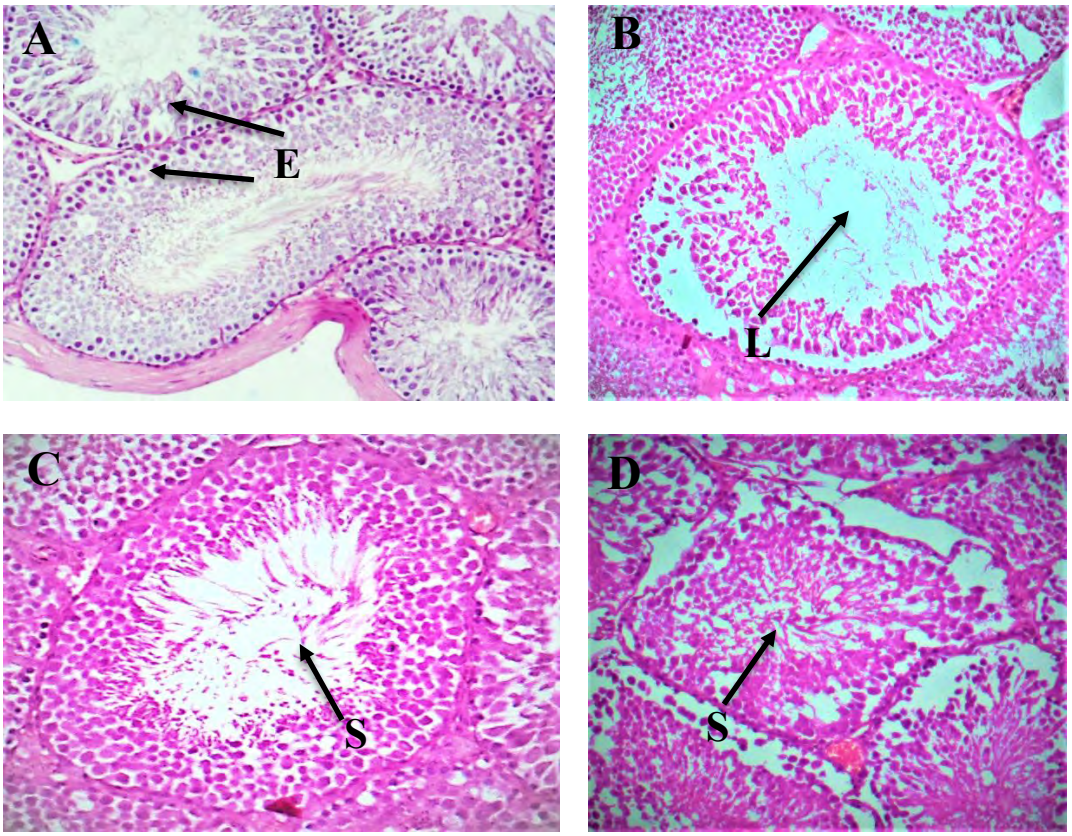


Figure 11. Photomicrograph of seminiferous tubules of adult male rats. (A) Control; exhibiting closely arranged seminiferous tubules, lumen flushed with mature spermatozoa and interstitium enriched with Leydig cells (B) Furan; showing seminiferous tubules were deranged, large interstitial spaces were prominent, insufficient number of Leydig cells and absence of mature sperms in tubules (C) ACV ; demonstrating Seminiferous tubules were visibly normal and sheathed with germinal epithelium, Interstitial spaces contained the Leydig cells and restored sperm number in tubules (D) F+ACV; exhibiting complete series of spermatogenic cells and nearly normal seminiferous tubules .

Table 4: (Mean ± SEM) Evaluation of seminiferous tubule diameter (tubular +lumen), and epithelial height of testis after 28 days of exposure among adult male rats.

Parameters	Experimental groups				P-value statistics
	Control	Furan	ACV	Furan+ACV	
Tubular diameter(µm)	200.26±3.32	147.52±3.16^{a***}	210.19±2.34^{a*b***}	179.20±3.71^{abc***}	0.0001
Lumen diameter(µm)	40.24±0.56	45.08±1.13^{a***}	42.07±0.63^{b*}	44.84±0.84^{a***c*}	0.000
Epithelial height(µm)	86.85±3.48	72.37±3.25^{a*}	95.99±1.60^{b**}	83.19±3.61	0.037

Values are specified as Mean ±SEM. *, **, *** showing significance variance at p< 0.05, p<0.01, p<0.001 respectively. **a** represents Value vs control, **b** indicates Value vs furan and **c** signifies Value vs ACV.

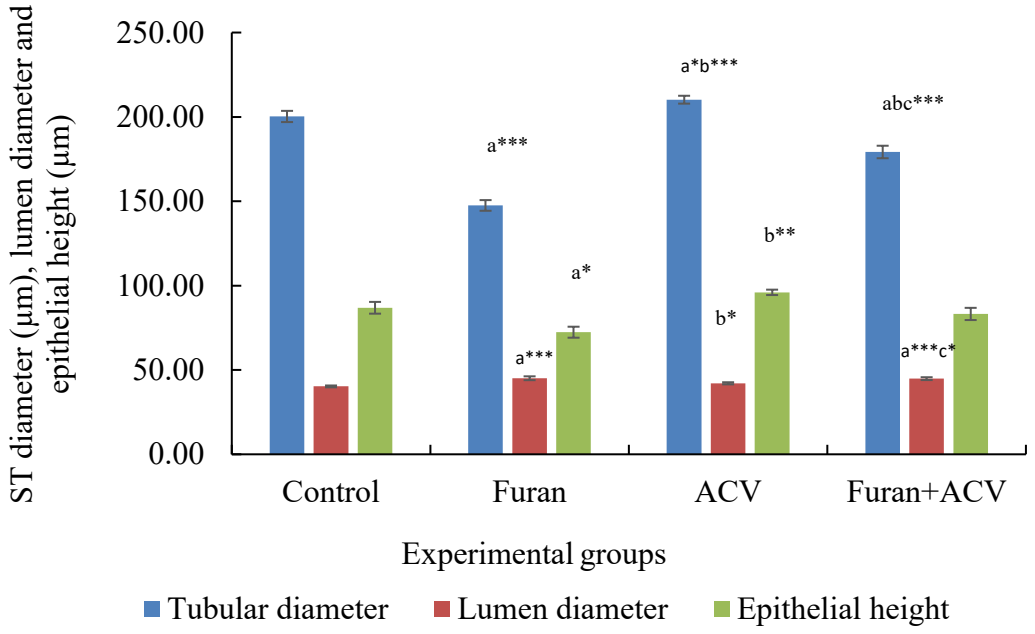


Fig 12: Mean ± SEM seminiferous tubule diameter (µm), lumen diameter and epithelial height (µm) after 28 days of exposure among adult male rats.

Caput epididymis

In the control group Histomorphological analysis of caput showed larger diameter, wider lumen, and thin pseudostratified epithelium .The epididymal tubules were closely organized and enclosed by stroma. A large number of spermatozoa were present in lumen.

Caput tubular diameter exhibited a remarkable ($p<0.001$) reduction while caput epithelial heights and lumen diameters showed no noticeable changes in group exposed to furan in comparison with control. In ACV treated group caput tubular diameter showed a noteworthy increase ($p<0.001$) while lumen diameter and epithelial height showed a nonsignificant increase as compared to furan treated group. There was a considerable decrease ($p<0.001$) in tubular diameter while a non-significant decrease in lumen diameter and epithelial height in F+ACV group in comparison with control . Caput tubular diameter presented a notable increase ($p<0.001$) whereas caput lumen diameter ($p=.328$) and epithelial height ($p=0.094$) showed an insignificant rise in group exposed to ACV when compared with furan. A substantial ($p<0.001$) reduction in tubular diameter while a negligible decrease in lumen diameter ($p=.169$) and epithelial height ($p=.957$) was seen in F+ACV treatment group as compared with furan treated group. A remarkable ($p<0.001$) decline in case of tubular diameter while insignificant decrease in lumen diameter ($p=.690$) and epithelial height ($p=.084$) was noticed in F+ACV group with respect to ACV treated group.

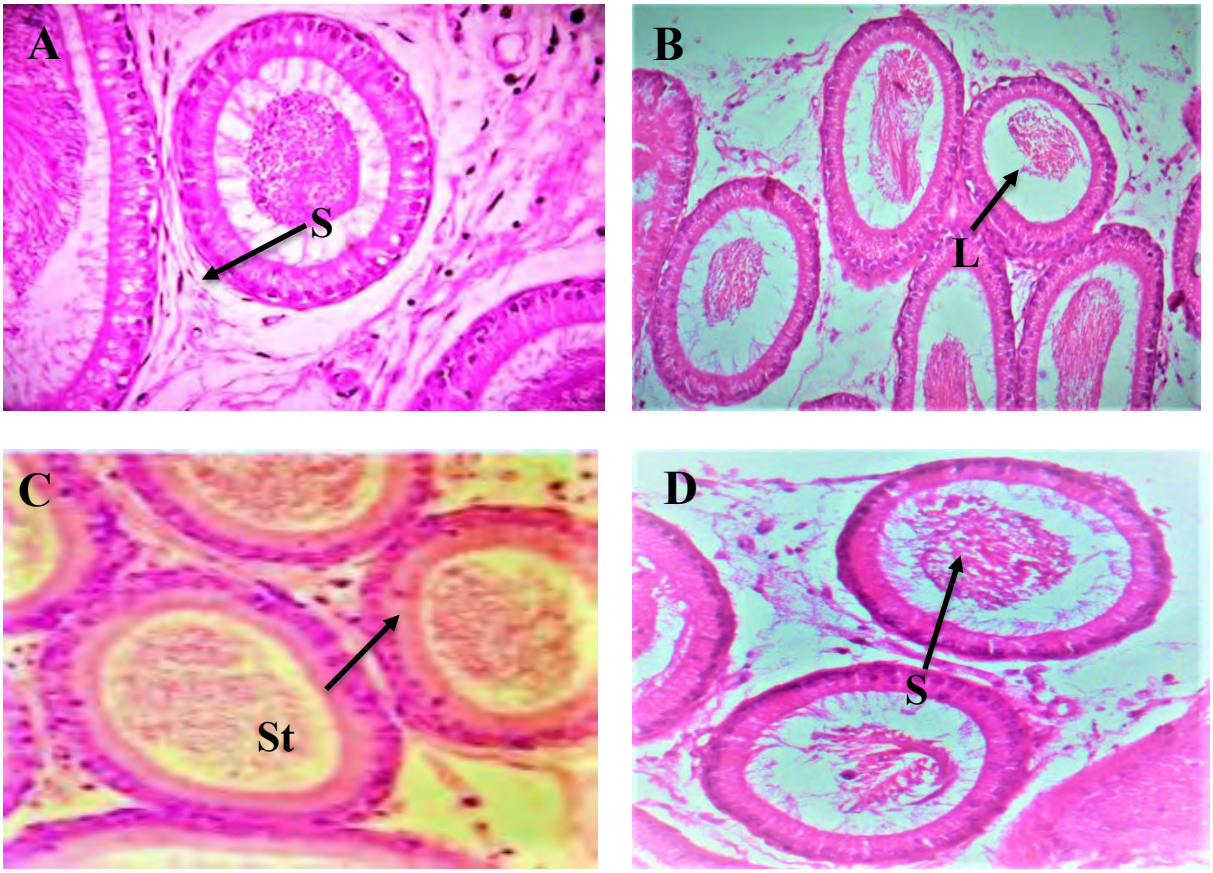


Figure 13. Photomicrograph of Caput epididymis of adult male rats. (A) Control; exhibiting epididymal tubules were closely organized, larger diameter, wider lumen, and thin pseudostratified epithelium and substantial number of spermatozoa present in lumen. (B) Furan; showing epididymal tubules disorganized, reduction in tubular diameter, increase in epithelial height and reduce sperm no in lumen. (C) ACV; showing results contradictory to furan. (D)F+ACV; exhibiting results parallel to control.

Cauda epididymis

Epididymis of cauda in case of control group revealed normal morphology with closely arranged tubules. Stroma surrounded the tubules. Epithelium was thick and pseudostratified, and lumen occupied with spermatozoa. The cells in epididymis are of two kinds: Principal cells (spread from basal lamina short lumen), Basal cells (placed at basal lamina).

A negligible decrease in both caudal tubular and lumen diameter while a noteworthy ($p<0.001$) decline in caudal epithelial height was noticed in furan treatment group in comparison with control. A substantial increase in tubular diameter ($p<0.001$), lumen diameter, and epithelial height was perceived in group treated with ACV as

compared to control group. There was a non-significant decrease in both tubular ($p=0.06$) and lumen diameter ($p=0.154$) and highly remarkable ($p<0.001$) decrease in epithelial height in F+ACV group as compared to control. A prominent increase ($p<0.001$) in tubular diameter, lumen diameter, and epithelial height was seen in ACV treatment group compared to furan group. There was a non-significant decrease in tubular diameter ($p=0.836$) and lumen diameter ($p=0.740$) whereas epithelial height showed a highly significant ($p<0.001$) increase in F+ACV as compared to furan. A notable increase in tubular diameter s($p<0.001$) while a highly substantial decrease ($p<0.001$)in diameter of lumen and epithelial height was detected in the F+ACV group in comparison with ACV treated group.

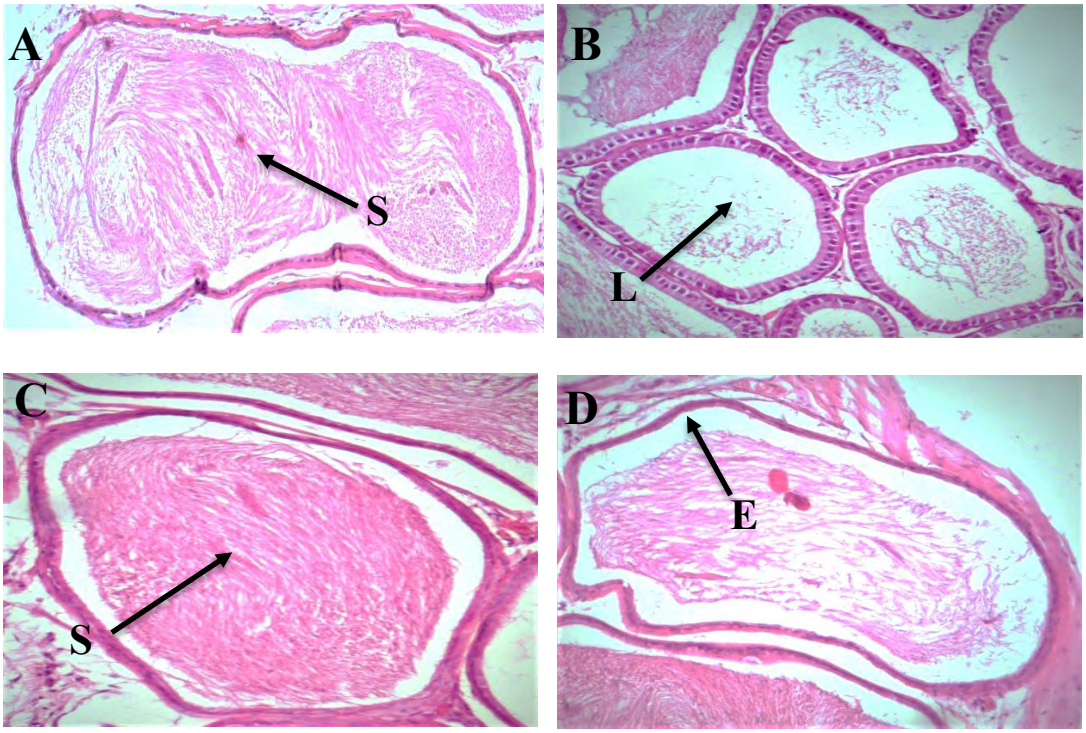


Figure 14. Photomicrograph of Cauda epididymis tubules of adult male rats. (A) Control; exhibiting normal morphology of cauda epididymis closely arranged tubules along with thin epithelium, lumen flushed with spermatozoa. (B) Furan; reduced spermatozoa in lumen and increased epithelial height. (C) ACV; shows results contradictory to furan. (D) F+ACV; Shows results near to control.

Table 5: Mean ± SEM of ductular diameter (µm), diameter of lumen (µm) and height of epididymis cell (µm) of the epididymis after 28 days of exposure among adult male rats.

	Experimental groups				
	Control	**Furan**	**ACV**	**Furan+ACV**	**P value**
Caput epididymis					
Tubular diameter(µm)	435.08±5.41	355.91±10.90^{a***}	470.27±4.07^{a**b***}	309.63±5.50^{abc***}	0.0001
Lumen diameter(µm)	292.89±5.00	293.33±7.35	436.68±5.36	281.20±7.87	0.429
Epithelial height(µm)	76.77±3.45	69.21±3.15	76.77±3.45	68.96±3.16	0.122
Cauda epididymis					
Tubular diameter(µm)	465.78±3.96	457.69±2.08	489.78±4.60^{ab***}	456.69±2.31^{c***}	0.0001
Lumen diameter(µm)	423.84±3.27	419.48±2.93	439.84±1.70^{ab***}	418.16±3.07^{c***}	0.0001
Epithelial height(µm)	50.58±0.56	29.84±0.73^{a***}	66.75±0.90^{ab***}	37.20±0.67^{abc***}	0.0001

Values are specified as Mean ±SEM. *, **, *** showing significance variance at $p< 0.05$, $p<0.01$, $p<0.001$ respectively. **a** represents Value vs control, **b** indicates Value vs furan **c** signifies Value vs ACV.

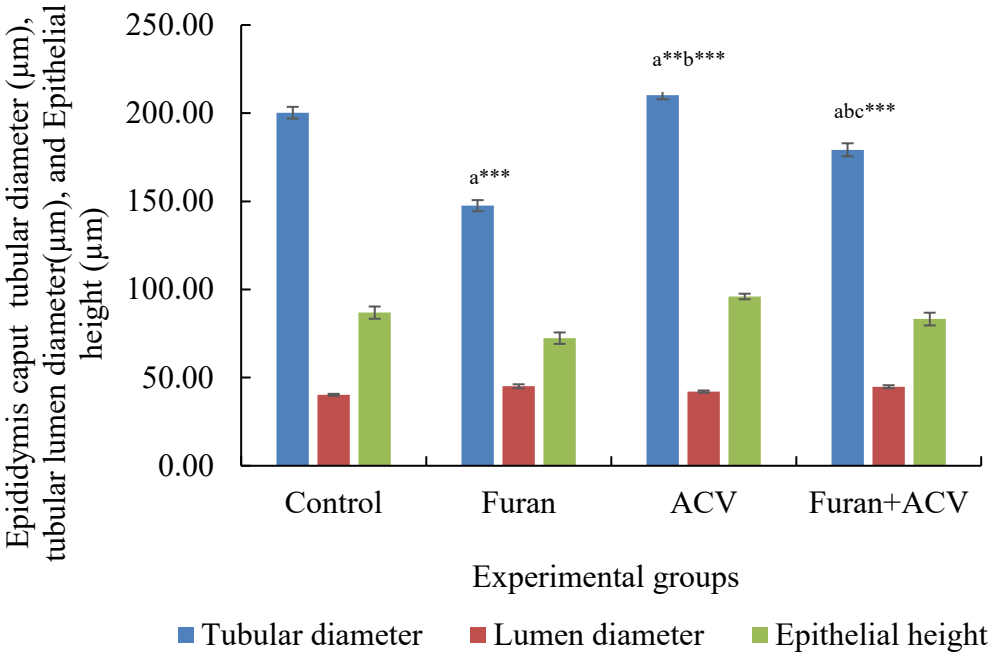


Fig 15: Mean ± SEM caput tubule diameter (µm), lumen diameter (µm) and epithelial height (µm) after 28 days of exposure among adult male rats.

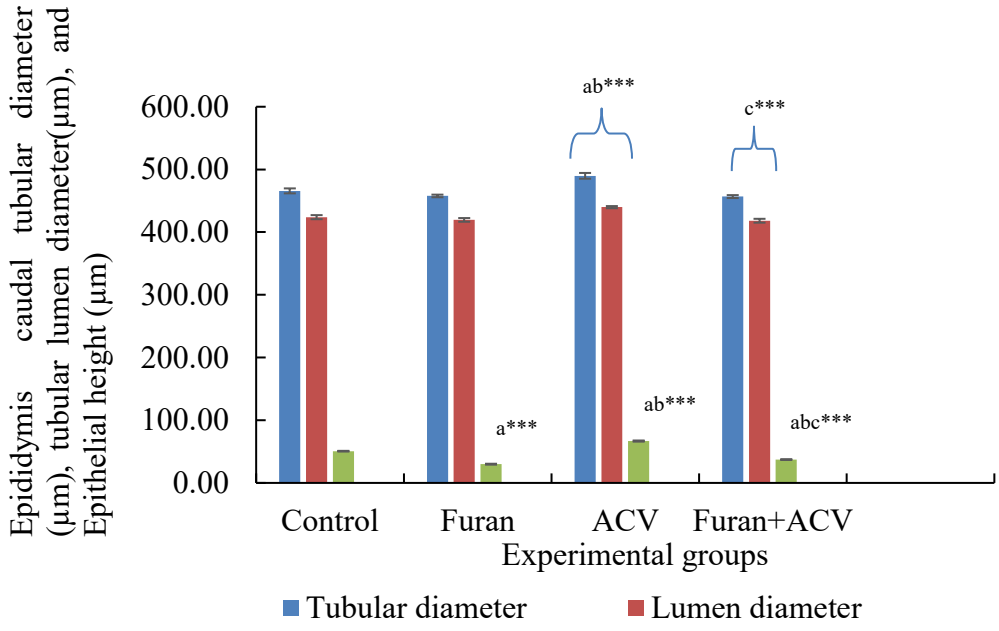


Fig 16: Mean ± SEM cauda tubular diameter (µm), lumen diameter (µm), and epithelial height (µm) after 28 days of exposure among adult male rats.

DISCUSSION

Furan is a compound that is formed as a result of the combination of various precursors molecules that are naturally present in food during processing stages. It has a low molecular weight having a volatile heterocyclic organic "high-production-volume" that may involve Maillard or oxidation reaction (Bolger *et al*.; Limacher *et al*. 2007). International agency for research and cancer has declared furan as a carcinogen for human and is considered to be harmful for rats and other animal models (*IARC,* 2015). Harmful effects of furan includes impairment of the reproductive system altering spermatogenesis resulting in the apoptosis of germ cell lining and Leydig cells (Rehman *et al*. 2020). Common maidenhair fern (*Adiantum capillus-veneris)* 620 inches in height is an evergreen perennial fern belonging to family Pteridaceae having dichotomous leaves. This plant has ornamental properties and is grown in gardens. It is also used for culinary purposes due to its aroma and as a therapeutic plant owing to its healing properties (Ahmed *et al*. 2013; Haider *et al*. 2011; Ibraheim *et al*. 2011). Keeping these facts in mind, current study was designed to investigate the protective effects of methanolic leaf extract of *Adiantum capillus-veneris* L**.** against oxidative stress and histopathological alteration caused by furan exposure in adult male Sprague Dawley rats.

Body mass index (BMI) is a tool used for assessing obesity and to monitor changes in body weight, thus it is useful for assessing the efficiency of weight loss therapy (Knutsen *et al*. 2017). The results of current study revealed that there was a considerable decrease in weight of body and accessory organ in groups treated with furan in comparison with control. These findings are in similar to previous study (Abd El-Hakim, Mohamed, and El-Metwally 2018; Abdel-Rahman *et al*. 2018) where a reduction in the body weight and accessory organs was also noted in the furan administered groups. Furan can easily pass-through biological membranes when administered orally, however its ultimate target is toxicity and carcinogenicity in the liver. In the liver, furan is metabolized to its reactive intermediate known as Z-2-butene-1,4dialdehyde (BDA) through (Cytochrome P450 2E1) cytP2E1 which is known for its cytotoxicity and is notorious for binding irreversibly to nucleotides and proteins (Gill *et al*. 2010). Furan induces hepatocellular alterations and damage the epithelium of bile duct, ultimately causing cholangiofibrosis that leads to

cholangiocarcinoma (Hamberger *et al*. 2010). Furan is eliminated primarily through biliary excretion (Burka, Washburn, and Irwin 1991). Repeated exposure of metabolites of furan in liver are might be associated to reduced appetite and lowered body weight (Bakhiya and Appel 2010). Further our results indicated that in comparison with control group, rise in body weight and accessory organs was observed in ACV and F+ACV treated groups as previously reported by (Gaikwad *et al*., 2013), that higher dose of ACV i.e. 500 mg/kg resulted in increase of body weight of rats. Our findings have been supported by other studies as well, where decrease in the body weight due to the exposure of bisphenol A and cisplatin was regained by the protective effect of *Adiantum capillus-veneris* L. in rats (Gaikwad *et al*., 2013).

Disturbance in antioxidant system results in oxidative stress (Hajhosseini *et al*. 2013). As the indicator of a healthy body is a balanced reactive oxygen species to antioxidants ratio, the antioxidant enzyme content was measured in the present study to assess oxidative stress induced by furan exposure where a significant increase in the levels of reactive oxygen species was noted in furan treated groups compared to the control group. Similar results were also obtained in previous report by Rehman *et al*. (2019), where furan exposure induced elevation in the levels of reactive oxygen species in rats. Other studies have also showed an increase in LPO and reactive oxygen species as a result of exposure to furan and acrylamide (Baş *et al*., 2016). Our findings also observed that decrease in ROS levels in ACV and F+ACV group was reduced. Restoration of increased ROS level due to BPA exposure through protective role of ACV has also been reported previously (Kanwal *et al*., 2018). Exposure to high doses of furan (40 mg/kg) resulted in oxidative damage and increased ROS production in the testicular tissues as it caused reduction in diameter of seminiferous tubular lumen, height of epithelia and reduced sperm number in epididymis lumen and all these changes could be attributed to increase production of ROS (Rehman *et al*. 2019). ROS production occurs as a result of oxidative phosphorylation which takes place in mitochondria (Devasagayam *et al.,* 2004). proteins, lipids and DNA also faces damages due to high ROS production (Radák *et al*. 1999). ROS levels are controlled through the self-defense mechanism which are initiated in response to formation of ROS in cells that limits ROS production (Kaul and Forman, 2000). Lower antioxidant levels alter the sensitivity of cell to cope with oxidative stress which further compromises the ability of cell to detoxify the effects of ROS.

However, ACV helped in restoration of antioxidant enzyme due to its antioxidant properties.

In the current study, a negligible increase in TBARs level in furan group (p=0.139) when a comparison was made with control. Our results are in accordance with previous literature by (Rehman *et al*., 2019) as a slight elevation in TBARs activity was noticed in group treated with high-dose (40mg/kg). Further, decrease in TBARs levels was seen among ACV and F+ACV groups in comparison with control these results are in line with preceding literature that thiobarbituric acid reactants levels diminished in groups treated with (ACAE) and (ACHE) compared to the control group (Khoramian *et al*.,2020).

Two important antioxidant enzymes, catalase and sodium oxide dismutase have the potency to eliminate free radicals in male reproductive organs. In present findings decrease in CAT and SOD levels were perceived in the furan treatment group in comparison with control. As reported earlier that a significant decline in the SOD and CAT were seen in furan exposed (40mg/kg) groups (Rehman *et al*., 2019). On the contrary, a substantial increase in SOD and CAT levels were noticed in ACV treated group when a comparison was made with control (Singh *et al*., 2020). These findings are contrary to previous literature by Ahmadpoor *et al*. (2019) where decrease in SOD levels following cisplatin exposure were restored by protective effects of ACV. These results showed that although ACV restored the effects of furan on SOD and CAT levels, but this restoration was not prominent because furan might be more toxic than cisplatin. This suggests that if rats are exposed to ACV for longer time, then there is a chance that CAT and SOD levels might be restored.

Another important antioxidant enzyme that plays a pivotal role in cell defense is POD. Through oxidation reduction reaction this antioxidant helps to covert hydrogen peroxide into water (Chen and Schopfer 1999). In the absence of POD, this reaction cannot be performed which results in the injury and subsequent cell death. In current study, diminished POD levels were noticed in group treated with furan in comparison with control. Comparable results were obtained in previous studies where significant decrease in POD level was observed as a result of exposure to furan (40mg/kg) (Pandır 2015; Rehman *et al*. 2019). Reduction in POD levels may be a reason behind the reduced sperm mobility and viability in the current study. A significantly

increased levels of POD was observed in ACV treated group. Negligible change in POD levels in F+ACV group when a comparison was made with control. No previous literature was available to explain the possible effects of ACV on POD levels in rats.

Seminiferous tubules were closely placed to each other and held tightly due to the presence of intact cellular organization in the control group. Sertoli cells were also bound closely to each other while the luminal compartment had a high presence of mature spermatozoa. Large number of circular and oval shaped Leydig cells were present in the interstitium and large blood vessels were observed surrounding seminiferous tubules (Rehman *et al*. 2020). Testicular organization was adversely affected in the furan exposed group (40mg/kg) as normal organization of seminiferous tubules was missing and they were deranged, due to the lack of Leydig cells and restricted/small blood vessels, Sertoli cells were also apart from each other, and the basement membrane was distorted due to disruption of blood testis barrier, lumen had lower mature spermatozoa or it was completely lacking (Karacaoğlu and Selmanoğlu 2010; Rehman *et al*. 2019). All these observations related to the histopathology confirms the disrupting nature of furan. The CYP2E1 family member of CYP (cytochrome P450) play an important role in bioactivation of many pro-carcinogens, environmental pollutants, such as furan acrylamide and xenobiotics. CYP2E1 not only expressed in liver but also in various other tissues, including brain, kidney, and the testis. It is well-known to produce the reactive oxygen species (ROS). Therefore, the presence of CYP2E1 in the testis might reveal the metabolic activation of environmental contaminants like furan, and ensuing production of oxidative stress and all these changes are due to this oxidative stress (El-Akabawy and El-Sherif 2016). In the ACV treated group, normal organization of rat testis were observed which was comparable to the control group. Seminiferous tubules were visibly normal and sheathed with germinal epithelium. All kinds of spermatogenic cells were available including spermatogonia, primary spermatocytes, secondary spermatocytes spermatids, and mature sperm were present. Interlobular spaces contained sufficient number of Leydig cells. The ACV group recovered the disorganization of testicles and epididymis caused by the furan exposure. Similar results were observed by Yousaf *et al*. (2016) where ACV restored deteriorations caused by BPA in testicular tissue .Testis histology of the ACV group in the current study showed results closer to

the control group, showing no side effects of *A. capillus-veneris* plant extract (APE) on testes. Damages caused by the furan exposure were healed by APE. These findings demonstrated that extract of *A. capillus-veneris* plants may have antioxidant and androgenic properties.

In current study there was a reduction in tubular and lumen diameter of testis in furan treated group while epithelial height showed an increase as compared to control. Our results are in line with previous literature by Rehman *et al*. (2019), where tubular and lumen diameter showed a highly significant decrease while epithelial height showed an increase in furan treated group as compared with control. Testicular tubular diameter showed an increase while testicular lumen diameter and epithelial height were elevated in group treated with ACV as compared to control. There was a highly significant decrease in tubular diameter, a highly significant increase in lumen diameter, and a non-significant decrease in epithelial height in the F+ACV group when a comparison was made with control. Previous literature have not shown the effect of ACV on testicular tubules diameter, lumen diameter and height of the epithelia in any laboratory tested animal. This study is considered the first to show the effect of this plant on testicular parameters of male albino rats.

In present study tubular diameter of caput presented a considerable decline while there was no obvious change in epithelial heights and lumen diameter of caput in the furan treated group in comparison with control. These results are in accordance with previous literature by Rehman *et al*. (2019), where no clear changes were seen in caput epithelial heights and diameter of lumen. However, furan treated groups with high dose (40mg/kg) demonstrated remarkable ($p < 0.001$) difference in term of tubular diameter of caput in comparison with control while a significantly increased caput tubular diameter was observed in ACV treated group. Additionally, non-significantly increased lumen diameter and epithelial height was noticed in ACV group as compared to furan exposed group. Significantly decreased tubular diameter and a non-significant decrease in lumen diameter and height of epithelia in F+ACV group was observed compared to control. Previous literature have not shown the effect of ACV on epididymal tubules diameter, lumen diameter and height of the epithelia in any laboratory tested animal. This study is considered the first to show the effect of this plant on epididymal parameters of male albino rats.

Current study observed a decrease in lumen and tubular diameter. Additionally, a decrease in height of caudal epithelia was seen in furan treatment group when a comparison was made with control. Previous reports suggested that diameter of cauda (tubular and lumen) exhibited a reduction while epithelial height of cauda was remarkably decreased in furan group treated with high-dose (40mg/kg) as compared to control (Rehman *et al*. 2019). ACV treated group showed a significantly increased ($p<0.001$) tubular and lumen diameter and an increased epithelial height compared to control group. However, a decrease in the tubular, lumen diameter, and significantly higher decrease in epithelial height was detected in F+ACV group in comparison with control.

Conclusions.

The present study concludes that furan induces oxidative stress and severe testicular toxicity in male albino rats. Furan administration resulted in significant reductions in body weight, testes weight as well as accessory organ weights. Antioxidant levels show a significant decline (catalase ($p<0.01$), peroxidases ($p<0.05$) and sodium dismutase ($p<0.01$)) due to the overproduction of oxidative stress markers or oxidants. Histopathological changes in testicular tissues were induced by furan, while ACV restored these effects. Additionally, ACV treated group showed contradictory results, further we found that ACV restored toxicological effects caused by furan in F+ACV group. Therefore, it is suggested that reproductive toxicity of furan in rats can be restored by ACV due to its antioxidant, antiobesity and protective effects on reproductive system and thus, can be used to treat reproductive health problems. For better understanding of the role of furan in inducing oxidative stress and testicular damage, more investigations on other animal models are needed. Further comprehensive and multidimensional research should be performed to fully understand the protective effect of ACV against furan.

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