

EFFECT OF AQUEOUS EXTRACT OF FLOWERS OF *Hibiscus rosa-sinensis* Linn ON DMBA INDUCED TUMOURS IN MICE

T H E S I S

Submitted

in partial fulfillment of the requirements for the Degree of

MASTER OF VETERINARY SCIENCE

IN

VETERINARY PATHOLOGY

BY

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Enrollment No. V/11/102

Krantisinh Nana Patil College of Veterinary Science, Shirwal

MAHARASHTRA ANIMAL AND FISHERY SCIENCES

UNIVERSITY, NAGPUR- 440 001.

(INDIA)

2018

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I N

V E T E R I N A R Y P A T H O L O G Y

B Y

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2018

DECLARATION OF STUDENT

I hereby declare that the experimental research work and interpretation of the thesis entitled “**EFFECT OF AQUEOUS EXTRACT OF FLOWERS OF *Hibiscus rosa-sinensis* Linn ON DMBA INDUCED TUMOURS IN MICE**” or part thereof has not been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis/publication of any University or Scientific organization. The sources of materials used and all assistance received during the course of investigation have been duly acknowledged.

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DECLARATION OF ADVISORY COMMITTEE

Dr. HEMANT KUMAR has satisfactorily prosecuted his course of research for a period of not less than one semester and that the thesis entitled “**EFFECT OF AQUEOUS EXTRACT OF FLOWERS OF *Hibiscus rosa-sinensis* Linn ON DMBA INDUCED TUMOURS IN MICE**” submitted by him is the result of research work sufficient to warrant its presentation to the examination in the subject of **VETERINARY PATHOLOGY** for the award of **Master of Veterinary Science** degree by the **Maharashtra Animal and Fishery Sciences University, Nagpur**.

We also certify that the thesis or part thereof has not been previously submitted by him for a degree of any other University.

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CERTIFICATE

This is to certify that the thesis “**EFFECT OF AQUEOUS EXTRACT OF FLOWERS OF *Hibiscus rosa-sinensis* Linn ON DMBA INDUCED TUMOURS IN MICE**” submitted by **HEMANT KUMAR** to the Maharashtra Animal and Fishery Sciences University in partial fulfillment of the requirement for the degree of **Master of Veterinary Science** has been approved by the Student's Advisory Committee after examination in collaboration with the External Examiner.

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Acknowledgements

“Interdependence is certainly more valuable than independence. This page is specifically designed to note my appreciation for those people who stand out most notably in my mind as contributing to the content of what you will find in my research work. As a M. V. Sc. student I am fortunate enough to be a part of such a progressive environment at Krantisinh Nana Patil College of Veterinary Science, Shirwal. I remain grateful to this institute for nurturing me in the field of Veterinary Pathology.

*In the foremost, I would like to express my deepest gratitude to my Guide and Chairman of Advisory committee **Dr. C. S. Mote** Assistant Professor, Department of Veterinary Pathology, KNPCVS, Shirwal for his guidance, advice and supervision from the day I joined this college. I will be indebted to him for life, for giving me extraordinary work experiences and teachings. I am also thankful to him for his timely suggestions, constant encouragement, care and moral support extended towards me.*

*I am grateful to **Dr. V. V. Karande**, Assistant Professor, Department of Veterinary pharmacology & Toxicology, KNPCVS, Shirwal for being a member of my advisory committee, enriching my knowledge in toxicology. His curiosity and keen interest in my research work helped solve many pitfalls without which this work would not have been completed.*

*I am immensely thankful to **Dr. S. N. Jadhav**, Assistant Professor, Department of Veterinary Biochemistry, KNPCVS, Shirwal for accepting to be a member of my advisory committee in midst of all his activities and providing me scientific resources, expert advice and solving all technical problems encountered during my research work.*

*I thank **Dr. P. V. Mehre**, Associate Professor, Department of Physiology, KNPCVS, Shirwal for accepting the proposal for advisory committee, motivation and guidance during the research work.*

*I am thankful to **Dr. S. B. Agwane**, Scientist, Biochemical Division, National Chemical Lab, Pune, for his knowledge, Guidance and positive criticism which helped me a lot in overall personality development which was very essential to proceed with my research work.*

*I express my sincere gratitude towards **Dr. B. P. Kamdi**, **Dr. V. D. Lonkar** and **Dr. K. Kundu** for providing me ever willing help, valuable advice and beneficial suggestions during course of my study.*

*I cannot forget to express my immense sense of thankfulness towards non-teaching staff members viz. **Mr. Sachin Mote, Mr. N. T. Jagtap & Mr. Pisal** for their helping hand during laboratory work.*

*Wholehearted thanks to my friends **Dr. Vipul, Er. Vivek Sharma, Dr. Uday Bagal, Dr. Prerana, Dr. Ajay** and **Dr. Aishwarya** for their constant motivation and support at all times, especially during my research work. I wish, if I could find words which can express my feelings of gratitude, respect, care, regards and love for my dearest and nearest friends **Dr. Amol sir, Dr. Gahininath Yange, Dr. Omkar, Dr. Jyotika, Dr. Bilal sir & Dr. Ankush Dube sir.***

*I take great pleasure to thank my juniors **Nikhil Babar, Akshya Dahatonde, Puneet, Abhijeet Upase, Rajkiran, Nikita Das & Ulrica Cabral** for their helping hand during my research work & stay in this institute.*

*I fall short of words to express my deepest feelings towards my beloved mother **Mrs. Aruna Bharati** & father **Sgt. Hemshankar Vidyarthi (Rtd.)**. They always stood behind me to support in all situations. I highly appreciate their love, care, sacrifice and painstaking efforts which helped me to build my career and to become a good human. I love them a lot and wish to fulfill their every dream. I am cardinally grateful to my deep sense of love and affection towards my brother **Aman Kumar** for being by my side in every stage of life.*

*I acknowledge with greatest regard the facilities provided for conducting the present research by **Dr. A. S. Ranade**, Associate Dean, KNPCVS, Shirwal.*

Last but not the least, I am thankful to all those whom I could not mention but who helped me, directly or indirectly during my stay in this jubilant institute.

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LIST OF ABBREVIATIONS

ALT	Alanine transaminase
ALP	Alkaline phosphatase
AST	Aspartate transaminase
@	At the rate of
bw	Body weight
BUN	Blood UreaNitrogen
mm ³	cubic millimeter
DCIV	Ductal Carcinoma Invasive
DLC	Differential Leukocytic Count
DMBA	7-12-Dimethylbenzanthracene.
<i>et al.</i>	et al/ibi) and elsewhere
etc.	et cetera) and the others
Fig.	Figure
gm	Gram
gm/dl	Grams per deciliter
H & E	Hematoxylin and Eosin
Hb	Hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCH	Mean corpuscular hemoglobin
MCV	Mean corpuscular volume
μl	Micro liter
μg	Micro gram
mcg/L	micro gram per liter
ME	Microscopic examination
mg	Milligram
mg/dl	milligram per deciliter
mg/kg	milligram per kilogram
mg/kg b. wt.	milligram per kilogram body weight
mg/kg /day	Milligram per kilogram per day
ml	Milliliter
viz.	Namely
NBF	Neutral buffered formalin
PCV	Packed Cell Volume
RBC	Red Blood Corpuscles
SEM	Standard error mean
SGOT	Serum Glutamic Oxaloacetic Transaminase
SGPT	Serum Glutamic Pyruvic Transaminase
i.e.	That is
IR	Incidence rate
TEC	Total Erythrocytic Count
TP	Total protein
TLC	Total Leukocyte Count
TSP	Total Serum Protein
WBC	White Blood Corpuscles

CHAPTER I

INTRODUCTION

Cancer is one of the most severe disease and a major public health problem throughout the world. Cancer continues to stand for the major cause of deaths in the world and claims over 6 million lives every year (Hemminkin and Mutanen, 2001). In the year 2012 International Agency for Research on Cancer (IARC) estimates the incidence of mortality and prevalence from major types of cancer for 184 countries of the world revealed that there were 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people were living with cancer worldwide. As per the reports of Siegel *et al.* (2017) It is projected that there will be 26 million new cancer cases and 17 million cancer deaths per year by 2030. In India, 1.1 million new cancer cases were estimated, indicating India as a single country (out of 184 countries) contributing 7.8% of global cancer burden. Mortality figure reached 682830, contributing to 8.3% of cancer deaths. Prevalence affecting 1.8 million individuals with cancer was corresponding to 5.52% of global prevalence (Saranath and Khanna, 2014).

Amongst the domestic and pet animals, canine and bovine are the major species being reported with variety of benign and malignant neoplasm in India. Canine species suffers most commonly with skin tumors and mammary tumors while bovine population is reported with eye and horn cancer, skin papilloma etc.

Due to lack of effective drugs, expensive cost of chemotherapeutic agents and side effects of anticancer drugs, cancer remains to be a cause of death in human medicine. Since last two to three decades, many research studies are reported with use of herbal preparations or plants having the potential to act as anticancer and anti-proliferative activity with beneficial effect in health in different type of diseases in human as well as veterinary medicine. As per World Health Organization (WHO), about 80% of the world's inhabitant's problem could be treated by herbal medicine for their primary health care (Etkin, 1981).

Plants or herbal medical preparations have a long history of being used in the treatment of cancer. Active constituents of *Catharanthus roseus*, *Angelica Gigas*, *Podophyllum peltatum*, *Taxus brevifolia*, *Podophyllum emodii*, *Ocrosia elliptica*, and *Campototheca acuminata* have been used in the treatment of advanced stages of various malignancies (Eva *et al.*, 2006). In last two decades, many plants have been observed to exhibit potent cancer chemo-preventive property. Most of these extracts are known to exert their effects by quenching reactive oxygen, inhibiting lipid peroxidation ornithine decarboxylase activity and DNA synthesis (Kapadia *et al.*, 2003).

Diet containing antioxidant rich fruits and vegetables significantly reduces the risk of many cancer diseases suggesting that antioxidants could be effective agents for the inhibition of cancer spread. These agents are present in the diet as a group of compounds with low toxicity, safe and generally accepted (Fresco *et al.*, 2006). The isolated polyphenols from different plants have been considered as indicator in a number of cancer cell lines at different evolutionary stages of cancer. Anticancer activities of flavonoids were described in various studies (Mavundza *et al.*, 2010).

Hibiscus rosa sinensis (Malvaceae) is widely cultivated in the tropics as an ornamental plant. There is evidence of the anticancer action of *H. rosa sinensis* extract against the tumor promotion stage of cancer development, in mouse skin with ultraviolet radiation (Sharma *et al.*, 2004). Other reports also indicates that *Hibiscus* extract can inhibit the growth of cancer cell types including mammary carcinoma, leukemia and melanoma. On the other hand, the ancient Indian medicinal literature reported that the flowers of *H. rosa-sinensis* have beneficial effects in heart diseases, mainly in myocardial ischemic disease, due to its enhancement of the myocardial endogenous antioxidants by an adaptive response and without producing any cytotoxic effects (Gauthaman *et al.*, 2006).

The recent advances in ayurvedic medicinal therapies and preparation of various extracts with active principles has been reported by many researchers. *H. rosa-sinensis* has been investigated by many researchers for its antipyretic, anti-

inflammatory and antineoplastic potential. However most of these previous studies were performed with organic solvent extracts of leaves from different strains namely *H. sabdariffa*. Hence, the present study is planned to evaluate the anticancer and anti-proliferative potential of extract of flowers of *H. rosa-sinensis* in mice with following objectives:

1. To study the gross and clinico-pathological alterations in DMBA induced tumors of mice treated with aq. extract of flowers of hibiscus (*Hibiscus rosa-sinensis* L.) in mice, if any.
2. To study the anticancer potential of aq. extract of flowers of hibiscus (*Hibiscus rosa-sinensis* L.) in DMBA induced tumors in mice by histopathological examination, if any.
3. To assess the antioxidative status of extract of flower of Hibiscus (*Hibiscus rosa-sinensis* L) .

CHAPTER II

REVIEW OF LITERATURE

2.1 History

Cancer is one of the oldest diseases known to mankind. The earliest known description of cancer appeared from Ancient Egypt in several papyri. In 1600BC Edwin Smith Papyrus was written and contains description of cancer and procedure to remove tumors.

Throughout the history, description of cancer is prevalent with many scientist and doctors trying to solve the mystery. Hippocrates (460BC – 370BC) described several kinds of cancer, referring them by the term *Karkinos*. Celsus (25BC – 50AD) translated *Karkinosto* cancer. Works of a Greek physician Galen helped us in understanding the cancer and derive the modern word “oncology”.

In 16th and 17th century dissection of body to discover the cause of death became more acceptable, thus helped in increasing the knowledge of cancer. The first cause of cancer was identified by a British surgeon Percivall Pott in 1775; he found that cancer of scrotum was common disease among chimney sweeps. The focus on chemical carcinogenesis then sifted to various industrial chemicals.

In 18th century with widespread use of microscope, metastasis was discovered by English surgeon Campbell De Morgan (1871-1874). Rudolph Virchow identified white blood cell in cancerous tissue and for the first time cancer got related with inflammation.

In the year 1902, Theodor Boveri proposed that the cancerous tumor arises from single cell that has gone through chromosomal damage suggesting chromosomal alteration causes the cell to divide uncontrollably. Paul Ehrlich in 1909 proposed a hypothesis that most of the tumor formation is suppressed by immune

system and the concept was called “immune surveillance”. On the principal of this hypothesis in 2010, the first human cancer vaccine was produced and approved by FDA “Sipuleucel-T” in which dendritic cells of patient are used (Boveri, 2008).

Today cancer constitutes an enormous burden on society in more and less economically developed countries equally. The occurrence of cancer is increasing because of the growth and aging of the population. Increasing prevalence of established risk factors such as smoking, overweight, physical inactivity, and changing reproductive patterns associated with urbanization and economic development has also contributed in increase in cancer cases globally. Based on GLOBOCAN estimates, about 14.1 million new cancer cases and 8.2 million deaths occurred in 2012 worldwide. Over the years, the burden has shifted to less developed countries, which currently account for about 57% of cases and 65% of cancer deaths worldwide (Torre *et al.*, 2015).

In India, 1.1 million new cancer cases were estimated, indicating India as a single country (out of 184 countries) contributing 7.8% of global cancer burden. Mortality figure reached 682830, contributing to 8.3% of cancer deaths. Prevalence was 1.8 million individuals with cancer corresponding to 5.52% of global prevalence. (Saranath and Khanna, 2014)

Cancer is not only limited to human population but large population of animal also suffers from different types of cancer with highest incidence in dogs and cats being reported. The annual incidence in dog was reported to be 381 per 100,000 dogs and 155 cats per 100,000 cats compared to 155 per 100,000 in humans in Alameda County.

In a survey by Bastianello, (1983) on neoplasia occurring in pigs and goats in the Republic of South Africa over a period of 40 years, from 1935 to 1974, it was found that, 24 tumors in pigs and 21 tumors in goats were recorded. Of the porcine neoplasm 13 (54%) were lymphosarcomas, 5 (20.8%) were cutaneous squamous cell carcinomas and 2 (8.4%) were embryonal nephromas. In goats, 8 (38%) of the tumors were squamous cell carcinomas, 50% of which occurred in the perineal

region. Malignant melanomas and papillomas each made up 19% of the total, whilst lymphosarcomas accounted for 14% of the total caprine tumors.

The Animal Tumor Registry (ATR) of Genoa, Italy, was established in 1985 with the aim of estimating the occurrence of spontaneous tumors in dogs. It received six thousand seven hundred and forty-three tumor biopsy specimens from local veterinarians in the Municipality of Genoa between 1985 and 2002. Three thousand and three hundred and three (48.9%) biopsy specimen samples were diagnosed as cancer. Mammary cancer was the most frequently diagnosed cancer in female dogs, accounting for 70% of all cancer cases. Incidence of all cancers was 99.3 per 100,000 dog-years in male dogs and 272 in female dogs. The highest incidence rates were detected for mammary cancer and for non-Hodgkin's lymphoma in bitches and for non-Hodgkin's lymphoma and skin cancer in male dogs. All cancer IR increased with age ranging between 23.7 and 763.2 in bitches and between 16.5 and 237.6 in male dogs aged ≤ 3 years and $> 9-11$ years (Merlo *et al.*, 2008).

In a retrospective study by Kimura *et al.* (2012) on cancer in domestic animals in south Brazil, it was found that dogs, cats and horses were most prone to cancer between the age of 6 – 10 years and bovines were more prone between the age range of 1 – 10 years. In dogs the most common neoplasm found was mammary carcinoma whereas, squamous cell carcinoma was the most common in cats bovine and horses.

In another attempt to estimate canine cancer incidence Baioni *et al.* (2017) found that the incidence rate was 804 per 100,000 dogs per year for malignant tumor and 897 per 100,000 dogs per year for benign tumor. The most commonly affected organs were the mammary gland ($n = 585$), skin ($n = 229$), and ovaries ($n = 40$) in the female dogs and the skin ($n = 242$), testicles ($n = 112$), and spleen in the male dogs. Highest rate for all cancer was observed in pure breed dogs.

In a study to find the incidences of neoplasia in canine Arya *et al.* (2018) found that neoplastic conditions were higher in bitches than in male dogs. The highest risk of development of several neoplasm were reported in the age group of

9-12, followed by 6-9 , 12-15, 3-6 and 0-3 years, respectively. Breed wise incidence of neoplasm was highest in Pomeranian (35.48%), German shepherd (25.80%), Labrador retriever (19.35%), Golden retriever (6.45%), Dalmatian (6.45%) and non-descript (3.23%).

2.2 Role of laboratory animals in cancer research

Studies on animals have contributed massively in all the progress that has been made in understanding and treating cancer over the past century. From giving clues to causes of the disease to showing us the best ways to treat it, studies on animals were the vital part. For example, the breast cancer drug Tamoxifen – arguably one of the most important cancer drugs of all time was developed with the aid of animal research. Over the years, it has saved hundreds of thousands of women’s lives. The targeted drug Imatinib can now cure people with chronic myeloid leukemia. The original studies that identified Imatinib’s potential were carried out in mice

The development of antibody treatments for cancer has also relied on animal research. Antibodies are molecules designed to recognize and target cancer cells, and early research in mice helped to find a way to produce large enough quantities of these molecules to be used to treat patients. New immunotherapy drugs called ‘checkpoint inhibitors’ which help the immune system to recognize and attack cancer is one example. These drugs have transformed the outlook for some people with advanced disease, such as melanoma, and wouldn’t have been possible without animal research.

This story is repeated time and time again with other advances in cancer research, and studies in lab animals has proved to be successful as cancer models to study cancer and its related molecular pathology hence continue to be essential in bringing benefits to cancer patients and saving lives around the world.

2.3 7-12-Dimethylbenzanthracene (DMBA)

7-12-Dimethylbenzanthracene or DMBA is a polycyclic aromatic hydrocarbon found in tobacco smoke (Jaiswal *et al.*, 2005) that is a potent carcinogen and acts at several site including skin, mammary gland and oral cavity. It is also very powerful initiating agent that can also cause progression from benign tumor to highly malignant tumors. It is found that carcinogenesis induced by DMBA is due to chronic inflammation and oxidant formation resulting in oxidative stress (Frankel *et al.*, 1995). DMBA is classified as a phenanthrene or a Phenanthrene derivative. Phenanthrenes are polycyclic compounds containing a phenanthrene moiety, which is atricyclic aromatic compound with three non-linearly fused benzene.

2.3.1 Chemical and physical properties of DMBA

- Colour: Yellow to greenish-yellow crystals or a yellow solid
- Odor : Odorless
- Molecular Weight: 256.348 g/mol
- Melting Point: 252 to 253° F
- Solubility in water: 0.061 mg/L water at 25 deg C
- Vapor Pressure: 6.8X10⁻⁷ mm Hg at 25 deg C

(Source: <https://pubchem.ncbi.nlm.nih.gov>)

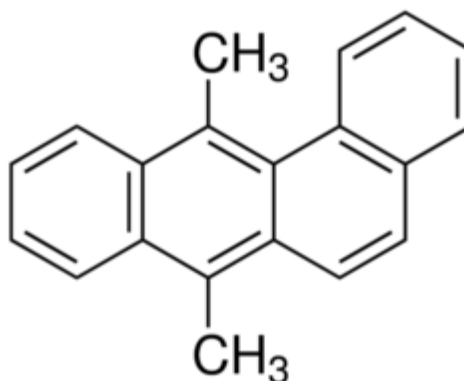


Fig. 2.3.1- 2D Structure of DMBA

2.3.2 Absorption, distribution and excretion of DMBA

Gentil *et al.* (1974) found that 48 hour after the oral administration, 90% DMBA was excreted by mice. Pretreatment of mice with 3-methylcholanthrene slightly increased initial rate of DMBA elimination, but after 48 hour amount of residual DMBA was the same as that in the non-pretreated mice. Ethyl acetate extractable polar metabolites and water soluble metabolites were more abundant in urine and feces of pretreated mice than in nonpretreated ones.

Woodhead (1983) found that two hour after intraperitoneal injection, 7,12-dimethylbenz(a)anthracene (DMBA) was present throughout the body of Amazon mollies, except in brain and ovary. There was an enhanced deposition at sites, in macrophages of atrium of heart and peritoneum, liver, and exocrine pancreas. DMBA was taken up by reticuloendothelial macrophages for 78 hour after injection. Accumulation and disappearance of radioactive label seen in liver and pancreatic cells probably represented pattern of metabolism of the compound. By 400 hour after injection, there was little DMBA remaining. Label accumulated in ameloblasts, which secrete the enamel capping of the teeth. No preferential metabolism of DMBA by spleen was observed.

In a study using oral administration of DMBA in rats at 20mg/kg, it was found that it dissolved in the lipid fraction of the chylomicrons in the lymph (Bingham *et al.*, 2001).

2.3.3 Metabolism and metabolites of DMBA

Tamulski *et al.* (1973) studied and found that metabolism of DMBA to hydroxymethyl derivative differs in hepatic and mammary tissue homogenates. In the hepatic tissue DMBA is transformed into 7-hydroxymethyl-12-methylbenz(a)anthracene (i), 12-hydroxymethyl-7-methylbenz(a)anthracene (ii) and 7,12-dihydroxymethylbenz(a)anthracene (iii) whereas the mammary gland formed only the monohydroxy compounds. Pretreatment of rats with 3-methylcholanthrene induces marked increase in DMBA metabolizing enzymes in the liver causing further conversion of the (i), (ii), and (iii) to more polar components.

Wong *et al.* (1980) combined gas chromatography-mass spectrometry subsequent to high-pressure liquid-chromatographic separation of the incubation mixture to examine the metabolism of DMBA in rat liver. In addition to the mono-, di-, and trihydroxylated oxidative metabolites usually associated with the biotransformation of polycyclic aromatic hydrocarbons, formation of a methanolysis product from the K-region epoxide intermediate during the workup procedure was observed. This provides a direct evidence for the presence of the K-region epoxide in the metabolism mixture. Moreover, methanolysis derivative only from the K-region epoxide was observed but none from the non-K-region counterparts. K-region epoxide possesses greater stability and can remain longer in a metabolism mixture reacting with methanol subsequently

Watabe *et al.* (1982) concluded that DMBA gets transformed by liver cytosolic sulfotransferase to reactive 7-hydroxymethyl-12-methylbenz(a)anthracene (7-HMBA) sulfate, which is a major metabolite and was found to be mutagenic toward *Salmonella typhimurium* strain TA98. The mutagenicity of 7-HMBA in the

presence of hepatic sulfotransferase was much higher than that of DMBA or 7-HMBA in the presence of hepatic monooxygenase

2.3.4 Carcinogenesis by DMBA

Joseph *et al.* (1994) conducted experiments to determine if *p53* alterations, which are frequent in human breast cancers, were also common in murine mammary tumors. In 13 mammary tumors from DMBA treated BALB/c mice were immunohistochemically analyzed for over expression of *p53*; *p53* protein was not detectable. The effect of DMBA on mammary tumor development was also tested in mice rendered homozygous for *p53*. These mice and wild type littermate controls had no differences in susceptibility to induction of mammary tumors by oral administration of DMBA. It was found that DMBA induction takes place by a different pathway without altering *p53* expression.

Currier *et al.* (2005) had examined oncogenic signaling pathways that were activated in mammary tumors in mice induced with DMBA. In female mice given 6 doses of 1 mg of DMBA by weekly gavage beginning at 5 weeks of age, all of the mice developed tumors by 34 weeks of age (median 20 weeks after beginning DMBA); 75% of the mice had mammary tumors.

Ranasasmita *et al.* (2010) aimed to explore the influence of dose and time regimens of DMBA on development of mammary carcinogenesis on Sprague Dawley female rats. In the first study 50 rats treated with 20 mg/kg bw of DMBA orally for eleven times at twice a week. First and the latest nodules appeared at the fourth-week and the fourteenth-week after ending DMBA induction, respectively, at the seventh-week the most often nodule appearances was seen. Histological analysis of mammary glands determined that they fell under in Ductal Carcinoma Invasive (DCIV) category. In second study 25 rats were gavaged orally with DMBA at dose 20 mg/kg b. wt. for five times every three days. Histological analysis demonstrated developing of mammary gland carcinogenesis reaching about 60% Ductal Carcinoma *In situ* (DCIS) and 40% Ductal Carcinoma Invasive (DCIV) stages. In

DMBA induction with low frequency, no data showed the incidence and multiplicity of tumor, but histopathologic level carcinogenesis can be distinguished. In DMBA induction with high frequency, incidence and multiplicity of tumor data can be obtained but cannot be distinguished histopathologically.

Olivera *et al.* (2015) used DMBA in rodent experimental models in a study, carcinogen was administered by gavage, diluted in corn oil, to female BALB /mice at doses of 1 mg per animal for 1, 3, 6 or 9 weeks. Remaining animals were euthanized at the age of 53 weeks and representative fragments of neoplasm were collected and routinely processed for histopathological analysis. Of the 70 mice treated with various doses of DMBA, 22 (31.43%) developed mammary tumor adenoacanthoma was the most commonly (18.75%) diagnosed histological type of breast cancer. Lung (15.71%) lymphoid tissue (11.43%), stomach (7.14%) and skin (2.86%) were also primary sites of tumor development. (33.33%) of the mice receiving 1 mg of DMBA developed lung cancer. Therefore, the administration of DMBA was shown to be an efficient model of carcinogenesis in mice, especially for the study of breast cancer.

Yu *et al.* (2015) studied the DMBA-induced mammary tumors, and found that it exhibited elevated expression of the aryl hydrocarbon receptor, *c-myc*, *cyclin D1*, and hyper phosphorylated retinoblastoma protein. Studies have shown that the development of breast cancer is a multi-step process that occurs sequentially from normal to usual hyperplasia, atypical hyperplasia, carcinoma in situ, and finally the invasive stages of carcinoma. In rat model induced by DMBA, estrogen-progestogen (E-P) showed ductal epithelial hyperplasia with forming a second lumen or papillary structure, atypical hyperplasia with atypical proliferative cells, forming a cross-bridge or cribriform structure in breast tissues.

2.4 Herbal medicine

The earliest evidence of human's use of plant for healing dates back to the Neanderthal period (Winslow and Kroll, 1998). Herbal medicine is still the mainstay

of about 75 - 80% of the world population, mainly in the developing countries, for primary health care (Kamboj, 2000). This is primarily because of the general belief that herbal drugs are without any side effects besides being cheap and locally available (Gupta and Raina, 1998). According to the World Health Organization (WHO), the use of herbal remedies throughout the world exceeds that of the conventional drugs by two to three times (Evans,1994). The use of plants for healing purposes predates human history and forms the origin of much modern medicine. Many conventional drugs originated from plant sources a century ago, most of the few effective drugs were plant based. Examples include aspirin (willow bark), digoxin (from foxglove), quinine (from cinchona bark), and morphine (from the opium poppy) (Vickers and Zollman, 1999).

Table 1: Number and percentage of medicinal plant recorded from different country and region (Hamilton, 2004)

Country / region	Total no. of native flora species	No.of medicinal plant species	% of medicinal flora
China	27,100	11,146	41
India	17,000	7,555	44
Mexico	30,000	2,237	7
North America	20,000	2,572	13
World	297,000 - 510,000	52,896	10-18

India is recognized to have a rich system of traditional medicines with over 8,000 plant species of medicinal value, which accounts for nearly 50 % of all the higher flowering plant species. In India, around 25,000 effective plant-based formulations are used in folk medicine, over 1.5 million practitioners of traditional medical system and over 7,800 medical drug manufacturing units consume about 20 tons of herbs annually (Ramakrishnappa, 2002).

2.5 Herbal anticancer

Cancer is one of the leading causes of morbidity and mortality globally. Amongst the non-communicable diseases, after cardiovascular disease cancer is the second leading cause of death (Lopez *et al.*, 2006). Cancer is responsible for one in eight deaths worldwide which is more than AIDS, tuberculosis, and malaria together (Sener and Grey, 2005). Since cancer cells lose many of the regulatory functions present in normal cells, they continue to divide when normal cells do not. This feature makes cancer cells susceptible to chemotherapeutic drugs, which makes chemotherapy a routinely used tool against cancer. However, chemotherapeutic treatments are not devoid of their own intrinsic problems. Various kinds of toxicities may occur as a result of chemotherapeutic treatments. These toxicity creates a significant problem in the treatment of cancer using allopathy or established medicine. Some of the common side effects of routinely used chemotherapeutic drugs are shown in table (2) below.

Table 2: Chemotherapeutic drugs and common side effects associated with them (Desai *et al.*, 2008)

Chemotherapeutic drug	Common side effects
5-fluorouracil	-myelotoxicity - cardiotoxicity - vasospastic effects
Doxorubicin	- cardiac toxicity - renal toxicity - myelotoxicity
Bleomycin	- pulmonary toxicity - cutaneous toxicity
Cyclophosphamide	- bladder toxicity

	- immunosuppression - alopecia - cardiotoxicity
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Anticancer properties of plants have been known for centuries and have been proven safe compared to chemotherapeutic drugs. In recent years many drugs have been prepared using plant, among the 121 medicines for treating cancer, 90 medicines have been extracted from medicinal plants (Wang *et al.*, 2012). Some common medicinal plants with having anticancer activity are depicted in table 3.

Table 3: Anticancer drugs prepared from plant metabolites
(Wilson, 2016)

S.No.	Metabolites	Extracted drugs
1	Vinca	Vincristine
2	Vinca	Vinblastine
3	Vinca	Vindesine
4	Vinca	Vinorelbine
5	Taxan	Paclitaxel
6	Taxan	Docetaxel
7	Podophyllotoxin	Topotecan
8	Podophyllotoxin	Irinotecan
9	Anthracyclines	Doxorubicin
10	Anthracyclines	Daunorubicin
11	Anthracyclines	Epirubicin
12	Anthracyclines	Idarubicin

The National Cancer Institute (NCI) had screened approximately 35,000 plant species for potential anticancer activities. Among them, about 3,000 plant species have demonstrated reproducible anticancer activity (<http://www.ars-grin.gov/duke/>).

2.6 Herbal antioxidant

Ultraviolet (UV) exposure is known to cause oxidative stress and plants protect themselves by producing anti-oxidative phenolic compounds (Larson, 1988). Enhanced UV-B radiation induces greater production of phenolic compounds in plant tissues such as flavonoids (Bassman, 2004). These compounds act as filters by absorbing and effectively reducing the UV flux reaching plant tissues (Caldwell *et al.*, 1989).

Many plants are known for their antioxidant property and were used in traditional medicine in different part of the world throughout. In recent years studies have backed their claims and helped in understanding their potential in reducing the risk of various diseases including cancer.

Phyllanthus emblicais known as '*Embllica officinalisor* (Amla), belongs to family Euphorbiaceae. Its chemical constituents are tannins, other phenolic compounds and flavonoid quercetin. It is a rich source of Vitamin C. It interacts directly with radicals and scavenges them and hence, shows antioxidants activity. It is used for the treatment of common cold, scurvy, cancer and heart disease (Jagetia, 2002).

Citrus lemon belongs to family Rutaceae. The antioxidant activity was estimated by two in vitro assays, DPPH radical scavenging activity and inhibition of Fe-ADP-Ascorbate induced lipid peroxidation (LPO) method. It contains mainly citral and limonene. The antioxidant property is shown due to presence of citral (Kokate *et al.*, 2004).

Aspergillus candidus belongs to family Aspergillaceae. *Aspergillus candidus* broth filtrate, extract was studied using different antioxidant models. It shows antioxidant property due to presence of phenolic compounds, Its ethyl extract scavenge the stable radical diphenylpicrylhydrazyl (DPPH) and radical cation 2,2-azino-bis-(3-ethylbenzothiazoline-6- sulphonate)(Malpure *et al.*, 2006).

Annona squamosa is popular as 'Custard apple or Sitaphal' belongs to family Annonaceae. Chemical constituents known to exert antioxidant property are flavonoids. In a study streptozotocin induced diabetic rats were used and it reduced the lipid peroxidation and increased the activity of antioxidant enzymes and strong super oxide radicals and singlet oxygen quenchers (Kaleen *et al.*, 2006).

Mentha arvensis belongs to family Labiatae. It contains 80% L-menthol and due to this, it shows antioxidant property. It shows antioxidant activity by decreasing lipid peroxidation (Saini *et al.*, 2007).

2.7 *Hibiscus rosa sinensis*

The genus *Hibiscus* (Malvaceae) comprises about 275 species in the tropics and sub-tropics (Dasuki, 2001). The plant *Hibiscus rosa-sinensis* is a perennial shrub with tap root. The leaves are 3.5-12 cm in length and 2-5.5 cm wide. Leaves are simple ovate or ovate-lanceolate. Leaves are entire at the base and coarsely toothed at the apex. Taste is mucilaginous. Flowers are pedicellate, Actinomorphic, pentamerous and complete. Corolla consists of 5 petals, red in colour and about 3 inches in diameter. With attractive and colourful flowers, plants of *Hibiscus* are widely planted as ornamentals and are used in traditional medicine.

Of the species studied, leaves and flowers of *H. mutabilis*, believed to have emollient and cooling effect, are used to relieve swellings and skin infections (Dasuki, 2001). Leaves and flowers of *Hibiscus rosa-sinensis* leaves are used as an antiseptic for boils and ulcers. Leaves and flowers of *Hibiscus rosa* is known for

various pharmacological uses such as abortifacient effect, acid phosphatase stimulation, alkaline phosphatase inhibition, analgesic activity, androgenic effect, anticonvulsive activity, anti-FSH activity, antiestrogenic effect, antifertility effect, antifungal activity, antigonadotropin effect, antihypertensive activity, antiimplantation effect, anti-inflammatory activity, antiovaratory effect, antipyretic activity, antispasmodic activity, antispermatogenic effect, antiviral activity, barbiturate potentiation, beta-glucuronidase inhibition, betaglucuronidase stimulation, CNS depressant activity, contragestative agent, embryo toxic effect., estrogenic effect, estrous cycle disruption effect, gonadotropin synthesis inhibition, hypoglycemic effect, hypotensive activity, hypothermic activity, inotropic effect positive, juvenile hormone activity, plant germination inhibition, lactate-dehydrogenase-X inhibition, luteotropic effect, menstruation induction effect, radical scavenging effect(Rao *et al.*, 2014).

Table 4:Traditional medicinal uses of *Hibiscus rosa-sinensis* in different countries (Jadhav *et al.*,2009)

Sr.No.	Place	Parts	Activity
1.	Bangladesh	Decoction of flowers	Regulation of menstrual cycle
2	China	Hot water extract of flower and bark	Emmenagogue
3	Cook Islands	Hot water extract of flowers & leaves	Ailing infants, Gonorrhea
4	East Indies	I.Hot water extract of flowers. II. Leaf Juice + <i>VernoniaCinerea</i>	Regulate menstruation produce abortion To stimulate expulsion of afterbirth
5	Fiji	Leaf Juice	Digestion , Diarrhea
6	French Guiana	Hot extract of flowers	Grippe
7	Ghana	Peeled Twig	Chewstick
8	Guadeloupe,	Hot extract of flowers Sodorific	Antitussive
9	Guam	Leaves	To Promote draining of abscesses

10	Haiti	Decoction of flowers & leaves	Flu&cough, stomach pain, Eye problems
11	Hawaii	Flowers	Lactation
12	India	Hot water extract of stems & flowers	Abortion, Antifertility, Contraceptive, Diuretic, Menorrhagia, bronchitis, Emmenagogue, Demulcent,Cough, Abortifacient
13	Indonesia	Juice of leaves & flowers	Menstruation,Abortion, Emmenagogue, Women in labor
14	Japan	Decoction of leaves	Antidiarrheal
15	Kuwait	Flowers	Aphrodisiac
16	Malaysia	Hot water extract of roots & flowers	Fever,Expectorant, Emmenagogue
17	Mexico	Infusion of Barks & leaves	Dysentery
18	Nepal	Hot water extract of roots	Cough
19	New Britain	Hot extract of flowers	Menstruation
20	New Caledonia	Decoction of flowers	Abortifacient
21	Northern Ireland	Water extract of flowers	To induce labor
22	Peru	Hot Water extract of flowers	Contraceptive, Emmenagogue
23	Philippines	Hot water extract of flowers	Bronchial Catarrh, Emmolients, Cancerous,Swellings
24	Trinidad	Decoction of flowers	Amenorrhea
25	Vanuata	Decoction of stems & barks	Menorrhagia
26	Vietnam	Infusion of flowers	Dysmenorrhea, Abortive

2.7.1 TAXONOMY (<https://www.itis.gov/>)

Kingdom	Plantae
Subkingdom	Viridiplantae
Infrakingdom	Streptophyta
Superdivision	Embryophyta
Division	Tracheophyta
Subdivision	Spermatophytina
Class	Magnoliopsida
Superorder	Rosanae
Order	Malvales
Family	Malvaceae
Genus	Hibiscus Linn
Species	<i>Hibiscus rosa sinensis</i>

2.7.2 Anticancer properties of Hibiscus rosa-sinensis.

Sharma *et al.* (2004) reported that there is very important evidence of the anticancer action of *H. rosa-sinensis* extract against the tumor promotion stage of cancer development, in mouse skin with ultraviolet radiation. Moreover, they found that the hypoglycemic activity of this extract was not mediated through insulin release and this increase the potential use of this species for human health purposes.

Basha *et al.* (2013) studied aqueous leaf extract of *Hibiscus rosa sinensis* was tested against four human cancer cell lines using MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and found to be active anti-cancerous agents .The results obtained from the Phytochemical analysis and the cytotoxic activity of this plant revealed that further investigations may lead to the development of potent anticancer agents from *H. rosa-sinensis*.

Divya *et al.* (2013) revealed that 90% methanolic leaf extract of *Hibiscus rosa-sinensis* showed significant antioxidant and anticancer activity due to the

increased flavonoids and terpenoids level. The phytochemical analysis of leaf extract indicated the constituents present were responsible for pharmacological effects. The investigation suggested use of *Hibiscus rosa sinensis* as herbal drug for anticancer and antioxidant activity.

Goldberg *et al.* (2016) used extracts from Hibiscus have been shown to inhibit the growth of a variety of cancer cells. In particular, recent studies found that polyphenols extracted from *Hibiscus sabdariffa* by organic solvents can inhibit melanoma cell growth. Aqueous *H. rosa-sinensis* flower extract contains compounds that inhibit melanoma cell growth in a dose dependent manner at concentrations that did not affect the growth of non-transformed cells. In addition, these extracts contain low molecular weight growth inhibitory compounds below 3 kilo dalton in size that combine with larger compounds to more effectively inhibit melanoma cell growth.

2.7.3 Anti-oxidative property of *Hibiscus rosa sinensis*

Bhuvana *et al.* (2011) conducted a study in rats that were fed on 10% D-glucose appreciable decrease in peroxidation product viz. malondialdehyde (MDA) is observed in heart tissues of *H. rosa-sinensis* treated rats. The decreased activities of key antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and non-enzymatic antioxidants such as glutathione, vitamin E and vitamin C in 10% D-glucose-fed rats came back to near normal range upon *H. rosa-sinensis* flower petals treatment. This suggests that the *H. rosa-sinensis* has preventive and curative effects in 10% D-glucose induced oxidative stress in rat heart tissues.

Mak *et al.*, (2013) studied antioxidative and antibacterial properties of aqueous extract hibiscus and ethanolic extract of *Cassia* flowers and found that, both the flower extracts contains high amount of antioxidant compounds and exhibit significant antioxidant activities, which depended on extraction solvents. Ethanolic extracts of *Cassia* had high total phenolic, total flavonoid and total flavonol content, and showed highest activity for inhibition of DPPH, while aqueous extract of hibiscus had high tannin and anthocyanin contents, and showed high ferric reducing

antioxidant power. With regard to antimicrobial activity, aqueous and ethanolic extracts of hibiscus inhibited the growth of food-borne pathogens such as *Salmonella typhimurium* and *Staphylococcus aureus*, while in *Cassia* the growth of *Bacillus cereus* and *Klebsiella pneumoniae* was inhibited.

Khan *et al.* (2014) evaluated antioxidant and antibacterial potential of different solvent extracts of locally grown *Hibiscus rosa sinensis* Linn. The antioxidant activity was assessed by estimation of total flavonoids contents, total phenolic contents, DPPH free radical scavenging activity and percentage inhibition of linoleic acid oxidation capacity. Methanol and ethanol extract of *H. rosa-sinensis* showed total phenolics 61.45 ± 3.23 and 59.31 ± 4.31 mg/100g as gallic acid equivalent, total flavonoids 53.28 ± 1.93 and 32.25 ± 1.21 mg/100g as catechine equivalent, DPPH free radical scavenging activity 75.46 ± 4.67 and $64.98 \pm 2.11\%$ and inhibition of linoleic acid oxidation potential 75.8 ± 3.22 and $61.6 \pm 2.01\%$ respectively, was measured.

Prasanna and Goel (2017) studied the aqueous and Ethanol crude extract of Neem, Hibiscus leaves and the mixture of both leaves' extract for their free radical scavenging properties. Free radical scavenging activity was evaluated using DPPH, NO, FRAP and H₂O₂ free radicals. He concluded that the leaves of Hibiscus, Neem and both leaves mixture have presence of antioxidant and phenol, flavonoids and tannin.

2.7.4 Effect of Hibiscus rosa on hematological parameters

Mishra and Tandon (2012) reported in a study were mice with treated crude extract of *Hibiscus rosa sinensis* flowers (500 mg/kg BW) and *Bougainvillea spectabilis* leaves (800 mg/kg BW) for a period of 30 days. They found a significant increase in the level of hemoglobin and count of RBC but a significant decline in the level of MCH and MCV in the former case. On the other hand, in *B. spectabilis* treated animals, the level of hemoglobin, RBC count & PCV declined significantly. Hence, it was concluded that the use of *H. rosa-sinensis* whereas may not cause

any adverse effect on animals, *B. spectabilis* is to be used with care as its chronic use may cause anemia.

Vishwakarma *et al.* (2014) found no change in body weight and In hematology parameters of *Hibiscus rosa sinensis* treated animals, no reduction were seen in the values of T-RBC, no reduction were seen in the values of Hgb. The decreasing level of RBC and Hemoglobin showed anemic condition in rats.

Nwibo *et al.* (2016) investigated the effects of the leaf products on hematological indices, lipid profile and hepatic parameters using Wistar albino rat bioassay. They found that though the hemoglobin counts, packed cell volume and red blood count were not significantly ($P > 0.05$) affected by the leaf products, lipid profile tests results showed significant decreases ($P < 0.05$) in the TC and LDL levels while the high-density lipoprotein level was further increased. Liver function test (LFT) showed no evidence of hepatotoxicity as assayed liver enzymes and proteins did not vary between HFD administered animals, treated with or without leaf products.

2.7.5 Toxicity of *Hibiscus rosa*

Nath and Yadav (2015) in a study to find the acute and sub-acute toxicity caused by hibiscus found that the extract did not reveal any signs of toxicity or mortality in any animal, during the 14 days observation period. They administered 400 mg/kg and 800 mg/kg doses of extract to mice for two weeks. Animals did not reveal any marked adverse effects on hematological, biochemical parameters and histopathology of liver and kidney in the 400 mg/kg group. In animals that received 800mg/kg dose, hepato-renal toxicity was evident. In addition, in the same group of animals, the histological assessments of liver and kidney also showed various abnormalities such as dilated sinusoids, apoptotic nuclei and inflammatory infiltrate inside sinusoidal capillaries in the liver, and marked the disorganization of tubules and glomeruli, and enlarged interstitial spaces in the kidney.

Administration of *Hibiscus rosa sinensis* flower extract at doses of 100, 200, 400, and 800, mg/Kg did not produce any significant changes in behavior, skin effect, breathing, defecation, postural abnormalities, impairment in food intake and water consumption and yellowing or loss of hair. But at dose 1600 mg/kg showed 20% mortality (Singh *et al.*, 2014)

CHAPTER III

MATERIALS AND METHODS

The present study was designed to study the anti-neoplastic potential and anti-oxidative effects of aqueous extract of *Hibiscus rosa-sinensis* in tumor induced by DMBA in mice. The research work was conducted in Department of Veterinary Pathology, K.N.P. College of Veterinary science, Shirwal, Dist. Satara (India).

3.1 Chemicals

The carcinogen DMBA (7-12-Dimethylbenzanthracene) was used for induction of tumor in experimental animals.

3.2 Reagents, stains, glass wares etc.

All the standard chemical reagents, stains, the glass-wares of neutral glass of Borosil (India) make and the serum vials of Hi-Media Laboratories Pvt. Ltd, Pune were used in the study. Before use of all the glass-ware and plastic-wares were treated and sterilized by following standard bacteriological procedures (Cruickshank *et al.*, 1965).

3.3 Experimental Animals

In the present study, adult female mice weighing 35-45g, procured from National Institute of Bioscience; Bhore, Dist-Pune 411051. The animals were kept in the Department of Veterinary Pathology, K.N.P. College of Veterinary science, Shirwal, This lab animal facility is approved by CPCSEA and Institutional Animal Ethics Committee (IAEC) and CPCSEA Registration Number is CPCSEA/309/2000.

3.3.1 Care and management of experimental animals

Adult Swiss female albino mice (35-45g) were used in the present research work. All the animals were maintained in Laboratory Animal House, KNPVC, Shirwal under laboratory conditions of temperature 25 ± 2 °C, and were allowed free access to standard pellet food and water *ad libitum*. Animals were housed in polypropylene cages (Tarsons Make). Rice husk was used for bedding. Photoperiod was maintained at 12 hour light and 12 hour dark cycles. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) in year 2017-2018 (Reg. No 309/CPCSEA).

3.4 Experimental design

In the present study alterations in DMBA induced tumor in mice and study the anti-cancer potential and anti-oxidative status of *Hibiscus rosa sinensis*, was studied using a total of 175 female albino mice (50 normal; 125 DMBA treated mice) were used.

The mice were randomly divided into seven groups comprising of twenty five animals in each group. The groups were designated as group A to E. Group A was kept as control only provided with feed and water, Group B served as positive

control, Group C was given hibiscus extract at the dose rate of 400mg/kg by oral gavage. Group D, Group E and Group F served as treatment groups at low (200mg/kg), mild (400mg/kg), and high (600mg/kg) doses respectively after cancer induction by DMBA. Group G served as treatment standard treated with Doxyrubicine @ 2mg/ kg body weight. Group B, Group D Group E Group F and Group G were induced with cancer using DMBA at the dose of 25mg/kg. DMBA was dissolved in 1ml sunflower oil and injected subcutaneously in the abdomen and flank region one dose per month for three months. Group C, Group D, Group E and Group F were given hibiscus extract after 90 days according to the respected dose rate given in the table 5. Group G was treated with doxyrubicine (referred as anticancer standard drug) @ 2mg/kg after cancer induction (after 90 days).

After completion of 28 days of treatment animals were sacrificed and blood samples for hematology and serum biochemical analysis were collected from retro-orbital plexus. Liver, kidney and ovary tissues were collected for Malondialdehyde estimation and tissue samples from the organs(lung, liver, kidney, heart, spleen, stomach, and intestine) were collected in 10% neutral buffer formalin for histopathological examination.

Table 5: Experimental Design

Sr. No.	Group code & details	No of mice	Sex	Name of drug and dose/kg B. wt. / Day
1	A Normal control	25	F	Nil
2	B Positive control	25	F	DMBA - Chemical induced tumor with 25 mg/kg . S/c. At abdominal region
3	C Treatment Control (drug Control)	25	F	Normal feed + aq. extract of hibiscus flower @ 400mg/kg by gastric

				gavage for 28 days
4	D Low Dose After tumor induction	25	F	Normal Feed + Aq. extract of hibiscus flower @ 200mg/kg by gastric gavage for 28 days, after induction of tumor
5	E Mid Dose after tumor induction	25	F	Normal Feed + Aq. extract of hibiscus flower @ 400mg/kg by gastric gavage for 28 days, after induction of tumor
6	F High dose after tumor induction	25	F	Normal Feed + Aq. extract of hibiscus flower @ 600mg/kg by gastric gavage for 28 days, after induction of tumor
7	G Standard drug, At therapeutic dose after tumor induction	25	F	Normal Feed + Doxyrubicine @2mg/ kg body weight

3.5 Techniques used to administer DMBA and hibiscus extract (*Hibiscus rosa sinensis*) in mice

DMBA was weighed and dissolved in sunflower oil to make the concentration 25mg/kg/ml and injected subcutaneously in the abdomen and flank region. Treatment was divided into three doses, one dose per month for three months i.e. for

90 days. Animals were checked regularly for cancer induction. Hibiscus extract was dissolved in water and administered with predetermined doses as above (table 5). The dose was prepared freshly just before oral feeding throughout the 28 days of treatment. The standard metal oral gavage needle was used for oral feeding. The animals were weighed weekly to adjust the gavage volume to monitor their general health.

3.5.1. Preparation of Hibiscus extract

The flowers of *Hibiscus rosa sinensis* were collected from the local market in and around Shirwal. The flowers were washed with distilled water to remove all impurities, which could have been brought to the flowers by wind, birds, insects, automobiles and so on. The flowers were placed on clean plates and kept in shed for drying. The dried flowers were grounded into a powder with commercial laboratory blender and further milled (mesh size 850 μ m). The aqueous extract was prepared from dried powder, which was soaked in water and left for stirring on a magnetic stirrer for 24 h. The mixture obtained was then filtered and the filtrate was kept. The material left over was subjected to a second and third extraction using each time fresh solvent (Milli-Q water). All filtrates obtained were combined and placed in round bottom flasks in cool temperature. Total yield obtained was 8% after the extraction obtained from the above procedure.

3.5.2 Phytochemical analysis of hibiscus extract

The freshly prepared aqueous extract of flowers of hibiscus were evaluated for presences of various phytochemicals such as alkaloids, sterols, proteins, tannins, amino acid, glycosides, flavonoids and saponins as per the procedure described by Debela (2002).

3.6 Sampling and technique used for collection of blood and tissue materials

3.6.1. Collection of blood

After 28 days of treatment with Hibiscus extract the animals were fasted for 12 hours, and blood was collected from retro-orbital plexus with the help of capillary tube as described by Sorg and Buckner (1964), before sacrificing the animals. The blood was collected in K3-EDTA anticoagulant vials for hematological examination and in plain vials for serum biochemical examination. Both blood and serum sample were processed within 8 hours of post collection. Thin blood smear was prepared for differential leukocyte count.

3.6.2 Collection of tissue

At the end of experiment (118th day) animals were fasted for 12 hours prior to blood collection and sacrifice. After blood collection from retro-orbital plexus animals were sacrificed by using chloroform as per Ethical Committee guidelines. Detailed postmortem examination was carried out as per standard procedure suggested by Feinstin (2011). Gross lesion, if any, in liver, lung, kidney, heart, spleen, stomach and skin were recorded and tissue sample from these organs were collected in 10% neutral buffer formalin in labeled plastic container for further histopathological examination.

3.7 Laboratory techniques and methods to study various parameters

3.7.1 Clinical signs

All the animals were monitored thrice daily (morning, afternoon and evening) for clinical signs, abnormal behavior and ill health.

3.7.2 Body weight

Body weight of all the mice were taken on 0 day, 14 day, 28 day, 56 day, 90 day and at the end of experiment i.e. 118 day.

3.7.3 Hematological examination of blood samples

2.5 – 3ml blood was collected in EDTA vials for complete blood count including hemoglobin, Packed cell volume, Total Leukocyte count, Total Erythrocyte count, Mean Corpuscular Volume (MCV), and Mean Corpuscular Hemoglobin (MCHC), using Hematology Auto-analyzer. The Differential Leukocyte count was conducted by blood smear manually using Leishman's stain as described by Benjamin (2001).

3.7.4 Serum Biochemistry

Biochemical parameters for liver and kidney function impairment were analyzed using automated chemistry analyzer Transasia- Erba (Germany) using biochemical diagnostic kits by Ebra (Germany), according to the manufacturer's guidelines. The parameters measured were serum total protein (TP), alkaline phosphatase (ALP), aspartate amino-transferase (AST), alanine amino-transferase (ALT), urea, creatinine, calcium and phosphorus.

3.8 Pathological Studies

3.8.1 Gross pathology

At the end of experiment, mice from each group were sacrificed and detail post mortem examination was carried out. Skin and visceral organs of the sacrificed mice were examined for gross pathological changes, if any. The gross pathological changes were recorded.

3.8.2 Histopathology

The tissue samples of various organs (liver, lung, kidney, heart, spleen, stomach, and skin with tumor mass) were collected in 10%NBF and processed by routine paraffin embedding method described by Chauhan (1995).at the Central Instrumentation Facility (CIF).The tissue sections of 4 microns were stained with standard Hematoxylin (H) and Eosin (E) protocol suggested by Chauhan (1995).

3.9 Malondialdehyde Estimation

For malondialdehyde estimation, procedure suggested by Buege and Aust (1978) were carried out. The experimental animals after the last dose of hibiscus extract were sacrificed and tissue from liver, kidney and brain were removed and weighed for malondialdehyde estimation.

3.9.1 Reagents for Malondialdehyde estimation:

1. 1.15% KCL (Potassium Chloride):

1.15 gm KCL was mixed thoroughly in 100 ml of distilled water.

2. TCA-TBA-HCL reagent (Trichloroacetic acid- thiobarbutaric acid- hydrochloric acid reagent):

It was prepared by mixing 15 gm of trichloroacetic acid and 0.375 gm of thiobarbutaric acid in 0.25 N HCL.

3. 0.25 N HCL (Hydrochloric acid):

It was prepared by mixing 2.60 ml of HCL with 97.4 ml distilled water.

3.8.2 Procedure for Malondialdehyde eastimation

1. Liver, kidney, ovary and brain tissue were collected and weighed immediately after the sacrifice of the animal. These tissue samples were then homogenized with ice cold 1.15% KCL to make 10 % tissue homogenate.
2. Tissue homogenate was mixed thoroughly with 2ml of TCA-TBA-HCL reagent.
3. The solution was then heated for 15 minutes in boiling water bath. After cooling the flocculent was removed by centrifugation at 1000 rpm for 10 min.
4. The supernatants absorbance was measured at 532 nm against a blank that contained all the reagents except the tissue homogenate. The malondialdehyde concentration of the tissue sample was calculated using extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$

3.10 Statistical analysis

The data generated during experiment were statistically analyzed by using One-way analysis of variance (ANOVA). Values are represented as mean, standard error mean and ($P < 0.05$) considered as significant.

CHAPTER IV

RESULTS AND DISCUSSION

Cancer is a leading cause of death in both more and less economically developed countries. The burden is expected to grow worldwide due to the growth and aging of the population, particularly in less developed countries, in which about 82% of the world's population resides. The change in lifestyle such as smoking, poor diet, physical inactivity, and reproductive changes (including lower parity and later age at first birth) are known to increase the risk of cancer.

The World Health Organization's International Agency for Research on Cancer (IARC) has identified more than 100 chemical, physical, and biological carcinogens. Many of these associations were recognized long before scientists understood much about how cancer develops. Today, research is discovering new carcinogens, explaining how they cause cancer, and providing insight into ways to prevent and treat cancer, still cancer remains to be one of the major cause of deaths in human population.

Plants or herbal medical preparations have long history of being used in the treatment of cancer. Today more than 50% of drugs are derived from plants so it is clear that natural resources, especially plants could be used to find effective drugs for cancer treatment avoiding all the side effects associated with chemotherapeutic.

The present study was conducted at the Department of Veterinary Pathology, Krantisinh Nana Patil College of Veterinary Science, Shirwal (Satara), with an objective to study, the anti-neoplastic potential and anti-oxidative effects of aqueous extract of *Hibiscus rosa-sinensis* in tumor induced by DMBA in mice.

In the present study, a total of 175 female albino mice (50 normal; 125 DMBA treated mice) were used. The mice were randomly divided into seven groups

comprising of twenty five animals in each group. The groups were numbered as group A to G. Group A was kept as normal control only provided with feed and water, Group B served as positive (disease) control for tumour with administration of chemical carcinogen i.e. DMBA, and Group C served as herbal treatment control and was administered with aqueous extract of flowers of hibiscus @ 400mg/kg by oral gavage. Group D, Group E and Group F served as treatment groups at low (200mg/kg), mild (400mg/kg), and high (600mg/kg) doses, respectively after cancer induction by DMBA. Group G served as treatment standard treated with Doxyrubicine @2mg/ kg body weight. Group C, Group D, Group E and Group F were given aqueous extract of flowers of hibiscus after 90 days (post cancer induction). Group G was treated with doxyrubicine @2mg/kg after cancer induction (after 90 days). After completion of 28 days of treatment, the animals were sacrificed and blood samples were collected for hematology and serum biochemical analysis. Tissue pieces of kidney, liver and brain were collected for Malondialdehyde estimation. For histopathological examination, tissue pieces of liver, kidney, spleen, stomach, intestine, lungs, skin, and tumor mass with skin tissue were collected in 10% formalin.

4.1 Phytochemical analysis of aqueous extract of hibiscus

The qualitative analysis of various phytochemicals present in the aqueous extract of hibiscus flower were performed and the results are shown in Table 4.1

Table 6: Phytochemical analysis of aqueous extract of Hibiscus

Sr. No.	Chemical constituents	Aqueous extract
1.	Alkaloids	+
2.	Sterols	-
3.	Proteins	-

4.	Tannins	+
5.	Amino acid	-
6.	Glycosides	+
7.	Mucilage	+
8.	Flavonoids	+
9.	Reducing sugar	+
10.	Saponin	-

Results from the phytochemical analysis in this experiment are in accordance with findings of Gilani *et al.*, (2005) and Sachdava *et al.*, (2001).

4.2 Clinical Signs

Administration of DMBA in 1ml sunflower oil subcutaneously (except group A and group C) at abdominal region of animals did not produce any apparent clinical signs of toxicity in respective groups over a period of 62 days. After 62 days, the animals from Group B, D, E, F, and G were showing the clinical signs of development of tumourous tissue in the abdominal and peripheral skin tissue and on the back of animals.

All the animals in positive control (group B) as well as treatment groups exhibited normal behavioral activities as that of control animals (i.e. group A). The results correlates with the finding of Singh *et al.*(2014) where they found that administration of *Hibiscus rosa sinensis* flower extract at doses of 100, 200, 400, and 800mg/Kg did not produce any significant changes in behavior, skin effect, breathing, defecation, postural abnormalities, impairment in food intake and water consumption of mice as laboratory animals.

Feed intake of the animals in all the groups was non-significant till 56th day of the experiment. Difference in body weight of the animals amongst the control groups and treatment groups was more evident from 90th day onwards. The development of tumor mass was first visible in the animals from group B, D, E, F and G, from 62nd day onwards. The feed intake and body weight in these groups reduced compared to normal and treatment control group animals.

4.2.1 Cancer development

For this experiment 175 albino female mice were used. Out of the total, 125 mice were treated with DMBA at the dose rate of 25mg/kg body weight for induction of tumour by intra-peritoneal route. These animals were given 3 doses at the interval of one month via subcutaneous injection. First signs of tumor development were observed on 62nd day. At the end of the experiment tumor development in each group was analyzed and recorded (as given in Table 7)

Ranasasmita *et al.* (2010) in a study got 74% incidence, they induced cancer by oral administration DMBA at @20mg/kg (11 times, twice a week) and first was observed on 4th week. Sharmila and Manoharam (2012) gave 20µg of DMBA for twice a week for 8 weeks to Swiss albino mice and found 100% tumour formation within 15 weeks. Neagu *et al.* (2016) found the first appearance of tumour on 8 weeks from the 1st DMBA application.

Table 7:Group wise cancer development

Group	Animals treated with DMBA	No. of animals with developed tumour	% of animals with developed tumour	Mean tumour size diameter.(mm)
B	25	16	64	14
D	25	14	56	11
E	25	16	64	13
F	25	12	48	12
G	25	13	52	10

4.3 Mortality

No mortality was observed in control groups (Groups A and C) during entire period of study. Mortality was observed in group B, group C, group D and group E after induction of tumour by DMBA. From group B, 04 animals died between 56th day and 90th day of the experiment, 02 animals from group D and 01 animal each from group E and F died during the same period. Between 90 day to 118 days (i.e. treatment period after 90 days), 02 animals died from group B, 02 animals died from group D, and 01 animal died from group E. No mortality was seen in group F and G.

Our result from group C agree with Nath and Yadav (2014) where they administered @400 mg/kg and @800 mg/kg doses of extract of hibiscus to mice for two weeks and found that the extract did not reveal any signs of toxicity or mortality in the animals during the 14 days observation period. In this experiment, 200 mg/kg, 400 mg/kg and 600 mg/kg doses of hibiscus were administered for 28 days and no sign of toxicity or mortality was observed.

4.4 Body Weights (g)

The average weekly body weights of mice recorded in different groups are summarized and presented table 8 as well as depicted in Figure 1-6 respectively.

The mean body weights (means $\pm$ S.E) of animals at day 0 for groups A, B, C, D, E, F, and G were 29.04 ± 0.633 , 29.59 ± 0.54 , 31.37 ± 1.322 , 29.21 ± 0.765 , 28.54 ± 0.64 , 29.83 ± 0.543 and 29.86 ± 0.456 , respectively. The values did not differ significantly ($P < 0.05$) among the groups. The body weights of all the groups increased from day 0 till the 90th day of experiment.

The mean body weights of the animals on 14th day were 32.52 ± 0.873 in group A, 32.95 ± 0.524 in group B, 33.5 ± 0.95 in group C, 32.32 ± 0.762 in group D, 31.62 ± 0.68 in group E, 32.9 ± 0.748 in group F and 32.94 ± 0.628 in group G. The

values did not differ significantly ($P < 0.05$) among the groups. The average body weights of the animals were comparable suggesting uniformity in the health condition amongst the group.

The mean body weights of the animals at 28th day groups A, B, C, D, E, F and G were 34.55 ± 1.088 , 38.19 ± 0.783 , 35.85 ± 0.999 , 36.05 ± 0.95 , 35.4 ± 0.884 , 37.1 ± 1.062 and 37.89 ± 0.758 , respectively. The difference in the weight on 28th day amongst the group was not significant ($P < 0.05$).

The mean body weights (means $\pm$ S.E) of mice at 56th day of experiment for groups A, B, C, D, E, F and G were 38.72 ± 1.117 , 41.793 ± 0.608 , 41.04 ± 1.194 , 39.55 ± 1.005 , 39.05 ± 0.549 , 41.38 ± 1.05 and 41.025 ± 0.533 , respectively. The growth of the animals were uniform and no significant ($P < 0.05$) difference was recorded amongst the treatment groups and control group.

The mean body weights (means $\pm$ S.E) of mice at 90th day of the experiment for groups A, B, D, E, F and G were 46.47 ± 1.79 , 34.12 ± 1.05 , 48.11 ± 1.128 , 37.98 ± 0.769 , 38.69 ± 1.37 , 40.76 ± 1.328 and 38.98 ± 1.571 , respectively.

The difference in the mean body weight value was significant ($P < 0.05$) on day 90 of the experiment (i.e. last day of tumour induction phase). The mean body weights of Group B were significantly ($P < 0.05$) decreased as compared to other groups. The mean body weights of groups A and C were non-significant from each other. However, the mean body weights of groups D, E, F and G were significantly lower than Groups A and C.

The mean body weights (means $\pm$ S.E) of mice at 118th for groups A, B, C, D, E, F, and G were 49.6 ± 1.472 , 36.17 ± 1.149 , 50.52 ± 1.064 , 40.7 ± 0.562 , 41.71 ± 1.377 , 43.74 ± 1.314 and 41.38 ± 1.708 respectively. The difference in the body weight mean were significant ($P < 0.05$), group A and group C had the maximum mean body weight at the end of the experiment and was significantly higher than rest of the groups. Group B had the least mean body weight value and the treatment groups (D, E, F and G) had significantly comparable mean body weights. Treatment

groups had significantly lower body weight than control group A and C, but were higher than positive control (group B).

The significant reduction in body weight in animals of group B was noticed on 90 and 118 day as compared to control group A and treatment control group C and it could be due to development of tumour leading to para-neoplastic syndrome which leads to disturbance in metabolism and reduced feed intake, ultimately leading to lowered body weight of tumour bearing animals.

Sharmila and Manoharan, (2011) found that, DMBA causes decrease in the body weight. Chwuemeka and Morley (2015) observed anorexia in cancer and reported that it might be due to through inflammatory cytokines, dysphagia/alter gut function and cause of nutrition deficiency such as zinc.

Normal control group A and treatment control group C showed maximum mean body weight on the final day (118 day) supports the reasoning that cancer caused the reduction in body weight. From the treatment groups, group F showed highest mean body weight followed by group E, group D and group G indicating that treatment of aqueous extract of flowers of Hibiscus at high dose i.e. at 600 mg/kg body weight proved to reverse the wasting caused by the development of tumour more effectively after induction of tumour by DMBA .

Vishkarma *et al.*, (2014) reported no change in body weight with treatment of hibiscus .

4.5 Hematological Studies

The mean values of various hematological parameters on 118th day viz. Hemoglobin, PCV, TEC, TLC, DLC, MCV, MCH and MCHC in control and different treatment groups are presented in Table 10 and are depicted in figure 7-9.

4.5.1 Hemoglobin (g/dl)

The mean values of hemoglobin for different treatment groups observed were 14.30 ± 0.308 in group A, 11.65 ± 0.757 in group B, 14.37 ± 0.492 in group C, 13.97 ± 0.758 in group D, 13.57 ± 0.27 in group E, 14.11 ± 0.497 in group F and 14.01 ± 0.407 in group G.

The mean values of hemoglobin were significantly ($P < 0.05$) lowered in group B (tumour positive control) as compared to groups A, C, D, E, F and group G. Treatment groups D, E, F, and treatment control group C had comparable mean hemoglobin values with that of normal control A. In case of positive control group 'B' the hemoglobin mean value decreased significantly; this might be due to oxidative stress caused by DMBA leading to progressive development of tumours in the animals. The herbal treatment groups (i.e. group D, E, and F) as well as standard anticancer drug group (i.e. Group G) showed significantly increased values of hemoglobin as compared to group B which could be attributed to the anti-oxidative activity of the extract administered leading to restoration of metabolic and physiological status with lesser ill-effects of tumour development. When compared to normal control (group A) and treatment control (group C), the herbal treatment groups (group D, E and F) showed significantly comparable mean values this might indicate that extract of hibiscus might have helped in maintaining the hemoglobin levels in the treatment groups.

Mishra and Tondon, (2012) reported no alteration in hemoglobin levels with treatment of hibiscus.

4.5.2 Hematocrit/PCV (%)

The mean hematocrit values in different treatment groups recorded were 47.67 ± 1.647 in group A, 34.74 ± 2.385 in group B, 47.40 ± 1.939 in group C, 42.97 ± 1.26 in group D, 41.99 ± 1.241 in group E, 43.02 ± 1.887 in group F, and 43.83 ± 1.886 in group G.

The mean hematocrit values was significantly ($P<0.05$) lower in cancer positive control group B as compared to in control group A and treatment group C,D, E, F and G. Amongst the treatment and control groups, group D, E, F and group A and C there was no significant difference in their mean hematocrit values.

The decrease in hemoglobin and PCV levels are well documented in animals bearing tumour due to para-neoplastic syndrome with adverse effects on body physiology and metabolism (Arslanagic *et al.*, 2012).

Mishra and Tondon, (2012) reported, change in in PCV values with treatment of hibiscus extract. Similar results were found by Nwibo *et al.*, (2016) with aqueous extract of leaves of hibiscus.

4.5.3 Total Erythrocyte Count

The mean $\pm$ S.E. values for total erythrocyte count recorded in different treatment groups were 7.28 ± 0.39 in group A, 5.84 ± 0.295 in group B, 7.10 ± 0.315 in group C, 6.46 ± 0.407 in group D, 6.47 ± 0.365 in group E, 7.41 ± 0.428 in group F and in group G it was 7.30 ± 0.366 .

The mean Total Erythrocyte Count was significantly ($P<0.05$) lowered in DMBA treated group B as compared to control group A, C and treatment groups D, E, F and G.

The mean TEC of treatment groups D and E were non-significant from each other. While in group F, the mean value is significantly ($p<0.05$) higher than groups, D and E. The mean TEC of control group 'A', the treatment control group 'C' and the standard drug treatment group 'G' showed non-significant values from each other and the values were within normal referral range for murine speceis.

The result suggests that, at higher dose (i.e. at 600mg/kg) extract of hibiscus can reverse the decrease in total erythrocyte count caused by DMBA. Whereas in

group D and E the values of TEC are comparable stating at lower doses, the dose difference did not cause a difference in TEC value. This might suggest that there is a positive correlation but is not linear with the dose of extract and erythrocyte count.

Mishra and Tondon, (2012), reported that at 500mg/kg bw hibiscus extract caused a significant increase in TEC value in mice, which correlates with the observations in the present study. However Nwibo *et al.* (2016) in a study on aqueous extract of hibiscus leaves found that there was no significant change in TEC value.

4.5.4 Mean Corpuscular Volume (fl)

The group wise mean MCV values recorded in different treatment groups recorded were 67.25 ± 1.647 in group A, 60.07 ± 2.385 in group B, 62.82 ± 1.939 in group C, 68.17 ± 1.26 in group D, 66.65 ± 1.241 in group E, 58.85 ± 1.887 in group F and 60.81 ± 1.886 in group G.

MCV values did not show any significant difference among various groups when compared to control group. This result suggests that there was no significant effect of administration of aqueous extract of flowers of *Hibiscus* and DMBA on MCV. This suggests that size of RBCs was not altered in any of the groups (i.e. Groups B to G) as compared with control Group A.

The result of the present study is in accordance with the findings of Mishra and Tondon, (2012), and similarly Nwibo *et al.* (2016) also observed no change in MCV.

4.5.5 Mean Corpuscular Hemoglobin Concentration (g/dl)

The mean MCHC values in different treatment groups recorded were 30.6 ± 0.648 in group A, 33.65 ± 0.489 in group B, 32.49 ± 0.46 in group C, 30.31 ± 1.044 in

group D, 32.42 ± 0.556 in group E, 32.92 ± 0.46 in group F and 32.20 ± 0.809 in group G.

All the groups other than normal group showed significant increase ($P < 0.05$) in the MCHC value.

The present finding does not correlates with findings of of Mishra and Tondon, (2012), where they reported no change in the MCHC values.

4.5.6 Total Leukocyte Count

The mean $\pm$ S.E. values for total leukocyte count in control and treatment group are presented in Table 10 and depicted in Figure 8.

The mean $\pm$ S.E. values in different treatment groups recorded were 7.48 ± 0.534 in group A, 10.28 ± 0.348 in group B, 6.72 ± 0.583 in group C, 8 ± 0.466 in group D, 7.53 ± 0.298 in group E, 8.03 ± 0.547 in group F and 7.79 ± 0.564 in group G.

Group B showed significant increase ($P < 0.05$) in the leukocyte count compared to all the other groups. Group A, B, C, D, E, F and G values were significantly comparable with each other. The increase in TLC value in group B might be due to the tumor causing inflammatory response.

Nwibo *et al.* (2016) reported that aqueous extract of leaves of hibiscus inhibited the WBC count. Whereas, the present study did not show any inhibition in WBC count with treatment of aqueous extract of flowers of hibiscus.

4.5.7 Differential Leukocyte Count (%)

The bloodsmears were prepared from the blood collected in EDTA and stained with leishman stain for the differential leukocyte count. The mean differential

leukocyte count values in the mice of control and different groups exposed to DMBA and *Hibiscus rosa sinensis* are shown in Table 10 and depicted in Figure 9.

4.5.7.1 Lymphocyte (%)

The mean lymphocyte count in mice of control and different treatment groups recorded were 79.9 ± 0.957 in group A, 80.4 ± 1.326 in group B, 80 ± 1.065 in group C, 77.2 ± 1.082 in group D, 81.7 ± 0.915 in group E, 79.3 ± 1.04 in group F and 82.1 ± 1.319 in group G. The mean value of lymphocyte values did not show any significant difference among various groups when compared to control group.

4.5.7.2 Neutrophil (%)

The mean neutrophil count in mice of control and different treatment groups recorded were 17.6 ± 0.902 in group A, 17.5 ± 1.166 in group B, 18.2 ± 1.008 in group C, 20.3 ± 1.227 in group D, 16.4 ± 0.933 in group E, 18.3 ± 1.04 in group F and 16.3 ± 1.287 in group G. The mean neutrophil values did not show any significant differences among the various groups when compared to control group. This result suggests animal were not suffering from any disease condition.

4.5.7.3 Eosinophil (%)

The mean eosinophil count in mice of control and different treatment groups recorded were 1.2 ± 0.244 in group A, 0.9 ± 0.278 in group B, 0.9 ± 0.175 in group C, 1.3 ± 0.263 in group D, 1.1 ± 0.278 in group E, 1.1 ± 0.278 in group F and 0.8 ± 0.244 in group G. The mean eosinophil values did not show any significant difference among various groups when compared to control group.

4.5.7.4 Monocyte (%)

The mean monocyte count in mice of control and different treatment groups recorded were 1.3 ± 0.263 in group A, 1.2 ± 0.295 in group B, 0.9 ± 0.278 in group C,

1.2±0.244 in group D, 0.8±0.295 in group E, 1.3±0.263 in group F and 0.8±0.244 in group G. The mean monocyte values did not show any significant difference among various groups when compared to control group.

Overall, it was concluded that differential leukocyte count did not show any significant differences, the tumor bearing animals also did not show any significant difference from the control group.

4.5.8 Platelet Count

The mean ± S.E. values for Platelet count in control and treatment group are presented in Table 10. The mean ± S.E. values of platelet in different treatment groups recorded were 9.09±0.268 in group A, 9.52±0.305 in group B, 8.25±0.441 in group C, 8.65±0.241 in group D, 8.01±0.42 in group E, 8.39±0.537 in group F and 8.48±0.75 in group G.

4.6 Serum Biochemical

The various serum biochemical parameters viz. AST, ALT, ALP, Total Protein, Albumin, Globulin, Albumin:Globulin ratio, Creatinine, BUN, Calcium and Phosphorus were estimated at the end of study on 118th day (i.e. tumor induction for 90 days and followed by treatment in groups for 28 days) to evaluate the effect of DMBA induced tumor and further treatment of aqueous extract of flowers of *Hibiscus rosa-sinensis* if any.

4.6.1 Aspartate aminotransferase (AST) (IU/L)

The mean AST values in mice of different groups have been shown in Table 9.

The mean AST values in mice of control and different treatment groups recorded were 37.16 ± 4.033 , 52.87 ± 10.168 , 27.79 ± 4.386 , 50.24 ± 8.189 , 47.78 ± 8.304 , 42.77 ± 5.97 and 40.98 ± 6.885 in group A, B, C, D, E, F and G respectively. No significant difference was observed for AST values.

Nwibo *et al.* (2016) reported decrease in AST value with treatment of aqueous leaf extract of hibiscus.

4.6.2 Alanine aminotransferase (ALT) (IU/L)

The mean ALT values of different groups are shown in Table. The mean ALT values in mice of control and different treatment groups recorded were 25.01 ± 5.133 in group A, 126.52 ± 14.78 in group B, 30.59 ± 4.008 in group C, 104.78 ± 17.67 in group D, 98.55 ± 10.92 in group E, 80.47 ± 13.009 in group F and 62.41 ± 13.457 in group G.

The ALT values were significantly ($P < 0.05$) increased in group B as compared to other groups. Values of group D and group E were comparable with each other and were higher than group F, G, A and C. Between the group F and G, group F had significantly ($P < 0.05$) higher value. Group A and group C had the lowest ALT values and were significantly comparable with each other.

In the present study high level of ALT are attributed to hepatocellular damage and increased values can be a result of cellular injury due to generation of free radicals during tumor progression leading to metabolic stress.

Nwibo *et al.* (2016) reported decrease in ALT values with treatment of aqueous extract of leaves of hibiscus.

4.6.3 Alkaline phosphatase (ALP) (IU/L)

The mean ALP values in mice of different groups are shown in Table

The mean values of ALP in recorded in groups A,B,C,D,E,F and G were 106.72 ± 14.05 , 174.76 ± 20.85 , 88.9 ± 7.427 , 135.1 ± 15.03 , 144.13 ± 12.5 , 121.01 ± 16.6 and 124.33 ± 15.749 respectively. A significant ($P < 0.05$) difference was observed in the ALP values amongst the groups. Highest and least values for ALP were seen in group B and group C respectively. Group A, F and G showed significantly comparable ALP values and were higher than group C. Between group E and F, group E had significantly higher value followed by group D.

Nwibo *et al.* (2016) found decrease in ALP values with aqueous leaf extract of hibiscus.

4.6.4 Total Serum Protein(g/dl), Albumin (g/dl), and Globulin (g/dl) and A:G ratio.

The mean value of total protein, albumin, and globulin and A:G ratio in animals of different groups are shown in Table 9.

The total protein levels in mice of control and different treatment group be s recorded were 6.97 ± 0.292 in group A, 5.827 ± 0.257 in group B, 6.22 ± 0.278 in group C, 6.04 ± 0.259 in group D, 6.59 ± 0.204 in group E, 6.61 ± 0.402 in group F and 6.41 ± 0.303 in group G. The mean of value of protein level didn't differ significantly from each other and where found to be in referral range for murine species. However, numerically the mean total protein levels in group B,C and D where lower than control group and other treatment group.

The albumin values in mice of control and different treatment groups recorded were 3.57 ± 0.242 in group A, 2.25 ± 0.146 in group B, 2.50 ± 0.204 in group C, 2.49 ± 0.181 in group D, 2.69 ± 0.136 in group E, 2.73 ± 0.271 in group F and 2.85 ± 0.245 in group G. The mean albumin values in treatment groups significantly ($P < 0.05$) decreased from the normal control, group A.

The serum globulin values in mice of control and different treatment groups recorded were 3.4 ± 0.286 in group A, 3.577 ± 0.175 in group B, 3.72 ± 0.443 in group C, 3.54 ± 0.297 in group D, 3.904 ± 0.454 in group E, 3.834 ± 0.311 in group F and 3.551 ± 0.372 in group G. From the table, it was evident that the values of serum globulin did not differ significantly.

The mean values of A:G ratio in animal of control and different treatment groups recorded were 1.17 ± 0.206 in group A, 0.64 ± 0.053 in group B, 0.75 ± 0.249 in group C, 0.79 ± 0.131 in group D, 0.74 ± 0.109 in group E, 0.79 ± 0.12 in group F and 0.929 ± 0.148 in group G. The mean values of A:G did not show any significant differences among treatment groups.

4.6.5 Total phosphorus (mg/dl)

The mean total phosphorus values in mice of different groups are been shown in Table 9.

The mean phosphorus level in animal of control and different treatment groups recorded were 3.2 ± 0.345 in group A, 2.98 ± 0.316 in group B, 2.30 ± 0.249 in group C, 2.24 ± 0.267 in group D, 2.38 ± 0.248 in group E, 2.12 ± 0.25 in group F and 2.35 ± 0.245 in group G. There was no significant difference in mean phosphorus value amongst the groups.

4.6.6 Blood Urea Nitrogen (BUN) (mg/dl)

The mean blood urea nitrogen values in mices of different groups are shown in Table 9.

The BUN values in mice of control and different treatment groups recorded were 12.53 ± 0.778 in group A, 24.1 ± 2.488 in group B, 13.8 ± 1.372 in group

C, 17.33 ± 1.19 in group D, 19.8 ± 1.957 in group E, 15.97 ± 1.623 in group F and 15.61 ± 1.616 in group G.

The mean BUN values were found to be significantly increased ($P < 0.05$) in cancer positive control group B compared to other groups. The normal control group A and treatment control group C did not reveal any significant difference in their mean BUN values. Values in treatment groups D, F and G were significantly comparable. Group E showed significantly increased value amongst the treatment group.

4.6.7 Creatinine(mg/dl)

The mean Creatinine values in different treatment groups recorded were 0.52 ± 0.105 in group A, 1.148 ± 0.19 in group B, 0.325 ± 0.059 in group C, 0.778 ± 0.174 in group D, 0.82 ± 0.095 in group E, 0.30 ± 0.056 in group F and 0.47 ± 0.07 in group G. The result shows significant increased mean value in group B. group A and group G are significantly comparable. Group D had the significantly highest mean amongst the treatment group. Group C and group A had the significantly comparable mean values.

The increased creatinine level in group B might be due to due to kidney damage, due to oxidative stress and para-neoplastic syndrome. Treatment control group, (group C) and high dose treatment group (group F) showed lowered creatinine level compared to all the cancer positive groups suggesting better anti-oxidative activity at higher dose.

4.6.8 Calcium(mg/dl)

The mean calcium level of control and different treatment groups recorded were 6.60 ± 0.244 in group A, 7.30 ± 0.383 in group B, 6.94 ± 0.721 in group C, 6.62 ± 0.444 in group D, 6.48 ± 0.29 in group E, 6.07 ± 0.368 in group F and 6.89 ± 0.211 in group G. The difference in the calcium means values of the groups

were not significant. Although group B showed numerically higher levels as compared to all other groups.

4.7 Malondialdehyde Estimation

Malondialdehyde (MDA) is a naturally occurring byproduct of lipid peroxidation and is mutagenic and carcinogenic.

The MDA was estimated in kidney, liver and brain tissues in mice of control and different treatment groups i.e. group A (Normal Control), group B (Positive control for tumor), group C (Control for Aq. extract of Flower of Hibiscus), group D (Treatment at low dose), group E (Treatment at middle dose), in group F (Treatment at high dose), and in group G (Treatment with standard anticancer drug i.e. Doxorubicin). The mean values of all the group are presented in table 11

4.7.1 MDA Level in Kidney (nmol/gm of tissue)

The MDA values in mice of control and different treatment groups recorded were 30.675 ± 2.353 in group A, 50 ± 2.857 in group B, 26.875 ± 2.069 in group C, 56.175 ± 3.095 in group D, 44.93 ± 2.405 in group E, 41.95 ± 1.719 in group F and 60 ± 2.857 in group G.

Amongst the control groups normal control and treatment control showed the least level of mean value suggesting low lipid peroxidation in the kidney compared to other groups. Between group A and C, group C had lower MDA mean value and this can be attributed to anti-oxidative properties of hibiscus extract. Group B and G had significantly high levels of MDA suggesting increased oxidative stress in these groups. Amongst group B and G, group G had high MDA value suggesting higher oxidative stress in the kidney caused by doxyrubicine. Between the treatment group the MDA level showed inverse relation to dose rate. Group F with highest dose showed least MDA mean value flowed by E and D.

4.7.2 MDA Level in Liver (nmol/gm of tissue)

The mean values in of control and different treatment groups recorded were 20.875 ± 1.082 in group A, 40.5 ± 3.124 in group B, 19.625 ± 1.8 in group C, 41.5 ± 1.192 in group D, 22.975 ± 1.561 in group E, 25.925 ± 1.594 in group F and in group 49.5 ± 5.17 in group G. Similar to the results obtained for MDA in kidney mean MDA values were highest in group G and B as both these organs are related with xenobiotic metabolism thus are more prone to oxidative stress. Group G had higher mean then group B suggesting stress caused by the chemotherapeutic drug. The control groups had the least mean MDA value with treatment control group C having the lower value than group A accounting for the antioxidative activity of hibiscus extract.

In the treatment group dose dependent antioxidative activity was observed with maximum MDA value in group D.then E and at the last F. The result correlates with the findings in kidney and suggest that there is better antioxidative activity at higher dose i.e. at 600 mg/kg then 400mg/kg and 200 mg/kg.

4.7.3 MDA Level in Brain (nmol/gm of tissue)

The mean values of MDA in control and different treatment groups recorded were 10.275 ± 2.39 in group A, 11.8 ± 1.748 in group B, 8.95 ± 0.971 in group C, 11.1 ± 1.832 in group D, 10.275 ± 0.922 in group E, 10.725 ± 1.66 in group F and 12.575 ± 1.724 in group G. The result showed insignificant difference amongst the groups. Group C showed lowest level of the mean MDA value followed by group A. The treatment groups showed comparable values with group G.

Bhuvana *et al.* (2011) reported that, *H. rosa-sinensis* exhibits preventive and curative effects in oxidative stress in rat heart tissues.

4.8 Pathomorphology

The histopathological examination of the tissues viz. liver, lung, kidney, heart, spleen, stomach, and skin tissue with tumor mass was evaluated for cellular details using H&E stain. The descriptive report is narrated groupwise and organwise with lesion score grading with ascending pattern as 1) NAD (No Any Abnormality Detected), 2) Minimal, 3) Mild, 4) Moderate and 4) Severe as per toxicopathological guidelines.

4.8.1 Liver

The histopathological examination of liver tissue sections of groups A (normal control) and group C (herbal treatment control) showed hepatic parenchyma with intact cellular details of hepatocytes. There was absence of any abnormal or metabolic changes in all the sections of liver of these groups. However, the animals from all treatment groups (i.e. D, E, F, and G) showed minimal and focal degenerative changes in hepatocytes in few of the sections of liver. The changes were minimal in group F (High Dose) and G (Standard anticancer drug) as compared to group D (low dose) and E (medium dose of extract of flowers of Hibiscus). The liver sections from group B (positive tumour control) showed mild to moderate pathological changes in hepatocytes with cellular abnormalities like granular cytoplasm, distorted hepatic cords and congested vascular tissue.

The normal cellular details of liver from animals of group C indicated that there is no any hepatic damage due to oral gavage of extract of flowers of Hibiscus for a period of 28 days. As compared to histopathological changes noted in group B, the minimal changes observed in herbal treatment groups (groups D, E, and F) could be due to altered physiological status due to progression of tumour in these animals. Also these cellular changes were of minimal in nature suggestive of metabolic distress at reduced level which could be attributed to the anti-oxidant potential of extract of flowers of Hibiscus administered. The minimal changes observed in group G (treated with Doxorubicin as anticancer drug) could be due to metabolic distress caused by presence of tumour mass as well as administration of drug in the animals.

The anti-oxidant profile using assessment of MDA levels (as mentioned vide point no. 4.7.2 and in Table 11) supports the above histopathological observations. The dose dependent hepato-protective changes (in high dose group F) indicated decreased cellular damage due to administration of

Similar observations are reported by Sharma *et al.*(2004) and Basha *et al.*(2013). However, available literature is scanty on histological observations of administration of extract of flowers of Hibiscus in laboratory animals.

4.8.2. Kidney

The microscopic examination of kidney sections of groups A (normal control) and group C (herbal treatment control) showed renal tissue parenchyma with intact cellular details of glomeruli and renal tubules. There was absence of any abnormal or metabolic changes in kidney.

The animals from all treatment groups (i.e. D, E, F, and G) showed minimal and focal degenerative changes in renal tubules only. Glomeruli did not show any abnormal histological findings. The changes were minimal in group F (High Dose) and G (Doxorubicin as anticancer drug) as compared to group D (low dose) and E (medium dose of extract of flowers of Hibiscus). The kidney sections from group B (positive tumour control) showed mild to moderate pathological changes in renal tubules like cellular swelling and granular cytoplasmic features of tubular epithelium only.

The normal cellular details of kidney tissue from animals of group C indicated that there is no any renal damage due to oral gavage of extract of flowers of Hibiscus for a period of 28 days. As compared to histopathological changes noted in group B, the minimal changes observed in herbal treatment groups (groups D, E and F) could be due to altered physiological status due to progression of tumour in these animals. Also these cellular changes were of minimal in nature suggestive of metabolic distress at reduced level which could be attributed to the anti-oxidant potential of extract of flowers of Hibiscus administered.

Similar observations are reported by Sharma *et al.* (2004) and Basha *et al.* (2013). However, available literature is scanty on histological observations of administration of extract of flowers of Hibiscus in laboratory animals

4.8.3. Heart

The microscopic examination of heart tissue sections of groups A (normal control) and group C (herbal treatment control) showed normal cardiac muscle fibers in myocardium and endocardium. There was absence of any abnormal or metabolic changes in cardiac myofibers in these groups.

The animals from all treatment groups (i.e. D, E, F, and G) also showed normal cardiac muscle fibers in myocardium and endocardium. There was absence of any abnormal or metabolic changes in cardiac myofibers in these groups. However, the heart tissue from group B (positive tumour control) showed mild degenerative changes in cardiac muscles like dilation of fibers, focal cellular swelling and congested vascular tissue in epicardium.

The normal cellular details of heart tissue from animals of group C indicated that there is no any cardiac damage due to oral gavage of extract of flowers of Hibiscus for a period of 28 days. The minimal changes observed in group G (treated with Doxorubicin as anticancer drug) could be due to metabolic distress caused by presence of tumour mass as well as administration of drug in the animals. Doxorubicin is reported to cause cardiac myotoxicity in laboratory animals (Desai *et al.*, 2008).

4.8.4. Spleen

The microscopic examination of splenic tissue sections of groups A (normal control) and group C (herbal treatment control) showed normal cellular population of

lymphocytes in white pulp. There was absence of pathological changes in both red and white pulp of spleen tissue in these groups.

The animals from all treatment groups (i.e. D, E, F, and G) showed minimal focal degenerative changes in splenic tissue with congestion. The changes were minimal in group F (High Dose) and G (Doxorubicin as anticancer drug) as compared to group D (low dose) and E (medium dose of extract of flowers of Hibiscus). The spleen tissue sections from group B (positive tumour control) showed mild to moderate pathological changes in spleen with decreased cellular population of lymphocytes in white pulp and congested splenic parenchyma.

The normal cellular details of spleen tissue from animals of group C indicated that there is no any immunosuppressive or immunostimulant activity in lymphocytes due to oral gavage of extract of flowers of Hibiscus for a period of 28 days.

The available literature is scanty on histological observations of administration of extract of flowers of Hibiscus in laboratory animals.

4.8.5. Stomach

The microscopic examination of stomach tissue sections of groups A (normal control) and group C (herbal treatment control) showed normal glandular and non-glandular regions of stomach. There was absence of any pathological or metabolic or glandular reactive changes in mucosa of glandular epithelium.

The animals from all treatment groups (i.e. D, E, F, and G) also revealed normal glandular and non-glandular regions of stomach. There was absence of any pathological or metabolic or glandular reactive changes in mucosa of glandular epithelium. The animals from tumour control group (Group B) also showed normal glandular and non-glandular regions of stomach.

The normal cellular details of stomach tissue from animals of group C indicated that absence of any pathological changes in mucosal tissue of stomach due to oral gavage of extract of flowers of Hibiscus for a period of 28 days.

4.8.6. Lungs

The microscopic examination of lung tissue sections of groups A (normal control) and group C (herbal treatment control) showed lung parenchyma with normal cellular details of bronchi and alveolar tissue as well as blood vascular tissue. There was absence of any pathological or inflammatory changes in lung parenchymatous tissue in all animals.

The animals from all treatment groups (i.e. D, E, F, and G) also showed lung parenchyma with normal cellular details of bronchi and alveolar tissue as well as blood vascular tissue. The lung tissue sections from group B (positive tumour control) showed focal and mild pathological changes of congested lung vascular tissue and focal pneumonic consolidative changes of alveoli.

The normal histological features of lung tissue from animals of group C indicated that absence of any pathological changes in respiratory organs due to oral gavage of extract of flowers of Hibiscus for a period of 28 days. The mild pathological changes observed in group B could be due to para-neoplastic syndrome with altered physiological status of animals due to progression of tumour.

4.8.7. Skin tissue / skin with tumour tissue

The histopathological examination of skin tissue sections of groups A (normal control) and group C (herbal treatment control) showed normal dermis and epidermis skin tissue. There was absence of any pathological changes in skin tissue layers as the animals of these groups were not administered the chemical carcinogen (i.e. DMBA).

However, the animals from all treatment groups (i.e. D, E, F, and G) as well as group B (positive tumour control) showed progressive development of tumorous tissue mass in abdominal region as well as on the lateral aspect of abdomen and back of animals.

The animals from all treated groups (i.e. D, E, F, and G) as well as group B (positive tumour control) showed presence of tumours which were classified as fibrosarcoma as well as tumour of mammary gland (adenoma and adenocarcinoma).

The skin tissue sections with tumours mass revealed neoplastic cells of fibrous connective tissue with solid and compact arrangement in all the groups. The neoplastic tissue showed proliferative changes with large elongated nucleus with mitotic figures suggestive of Fibrosarcoma. The tumours were classified based on histological features as fibrosarcoma, mammary gland adenoma and adenocarcinoma.

In group B, out of 16 tumours, 10 were fibrosarcoma and 6 were mixed tumour growth with fibrosarcoma and adenocarcinomas. In group D, out of 14 tumours, 9 were fibrosarcoma and 5 were adenoma and adenocarcinomas. In group E, out of 16 tumours, 11 were fibrosarcoma and 5 were adenoma. In group F, out of 12 tumours, 10 were fibrosarcoma and 2 were adenoma. In group G, out of 13 tumours, 9 were fibrosarcoma and 4 were adenocarcinomas.

With respect to cellular changes in tumour tissue of fibrosarcoma, it was observed that neoplastic cells in group B (positive tumour control), group D (low dose@200mg/kg) and E (medium dose@400mg/kg) as well as of group F (High Dose @600mg/kg of aqueous extract of flowers of Hibiscus) showed proliferative tumour cells and there was absence of any degenerative or necrotic changes in the tumour mass.

However, the cellular changes in tumour tissue from animals of group G (Doxorubicin as anticancer drug) showed mild to moderate degenerative changes in neoplastic tissue suggestive of anticancer therapeutic potential. The tissue sections showed areas of cellular debris of eosinophilic nature with degenerated cells and foci of necrosis.

It was noted that administration of aqueous extract of flowers of Hibiscus @ 200, 400 and 600 mg/kg of body weight of mice for a period of 28 days did not show any anticancer activity as evident by presence of proliferative tumour cells as well as absence of significant degenerative changes in tumour cells. Also, the biochemical

differences in chemical properties between aqueous and ethanolic extract of flowers of hibiscus are not documented in the scientific literature.

Although some researchers (Sharma *et al.*, 2004 and Basha *et al.*, 2013) have reported anticancer property of aqueous extract of leaves of Hibiscus, in the present study at the administered dosage, the anticancer potential of aqueous extract of flowers of Hibiscus was not evident. However, available literature is very scanty on histological observations of administration of extract of flowers of Hibiscus in laboratory animals with respect to anticancer potential.

CHAPTER V

SUMMARY AND CONCLUSIONS

Cancer is one of the most severe diseases and a major public health problem throughout the world, it is reported to claim over 6 million lives every year and estimated to reach around 17 million cancer deaths per year by the year 2030. Cancer is not only limited to human population but a large population of animals also suffer from different types of cancer, with highest incidence being reported in canine and feline followed by bovines. Due to lack of effective anti-cancer drugs and expensive cost of treatment and severe side effects associated with the chemotherapeutics, cancer remains to cause huge loss of life in human and animal medicine.

Ayurveda and herbal medicine are known to be safe and has been used by mankind since ages and shows a promising future in treatment of cancer. In the last two decades many plants have been observed to exhibit potent anticancer properties. *Hibiscus rosa-sinensis* Linn is a widely cultivated in the tropics and has been mentioned in many ancient texts for its medicinal properties. In recent years many researchers have studied hibiscus for anticancer properties and found it to have a good potential against cancer.

The present study is undertaken to evaluate the anticancer and anti proliferative potential of aqueous extract of flower of *Hibiscus rosa sinensis* Linn in mice induced with cancer using DMBA. A total of 175 female albino mice (50 normal; 125 DMBA treated mice) were used.

The mice were randomly divided into seven groups comprising of twenty five animals in each group. The groups were numbered as group A to G. Group A was kept as control, Group B served as positive control, Group C was treatment control. Group D, Group E and Group F served as treatment. Group G served as treatment standard treated with Doxorubicin.

After cancer induction for 90 days animals were treated with aqueous extract for 28 days at different doses. Animals were then fasted for 12 hours and then sacrificed and samples for various parameters (hematology, biochemistry, MDA and histopathology) were collected.

The significant reduction in body weight gain in animals of group B was noticed on 90 and 118 day as compared to control group A and treatment control group C. It could be due to development of tumor leading to para-neoplastic syndrome which leads to disturbance in metabolism and reduced feed intake, ultimately leading to lowered body weight of tumour bearing animals. Whereas, in extract treated group the mean body weight of the animals on 118th day didn't reduced as much as group B. This might be due to reversal of wasting seen in cancer.

The hematological parameters viz. hemoglobin, packed cell volume and total erythrocyte count decreased significantly in group B as compared to control groups. Treatment groups showed significant increase hemoglobin, packed cell volume and total erythrocyte count with respect to the dose given, with highest values shown by groups with high dose treatment (@ 600 mg/kg). In Group B, the total leukocyte count increased significantly whereas, control and treatment groups showed lowered total leukocyte counts. There was no significant difference seen in differential leukocyte count amongst the control, positive and treatment groups. The MCV values didn't show any significant difference but MCHC values were significantly increased in all the groups except group A.

The serum biochemistry values of various parameters showed that the extract had a good protective effect on kidney and liver as the values of ALT, AST, ALP, Urea and Creatinine in treatment groups were significantly reduced as compared to positive control group.

The histopathological examination of vital organs revealed the metabolic and systemic stress caused by progression of tumour by DMBA in group B animals. The histopathological examination of liver tissue sections of groups A and group C

showed hepatic parenchyma with intact cellular details of hepatocytes. The group B animals showed hepatocytes with cellular abnormalities like granular cytoplasm, distorted hepatic cords and congested vascular tissue. These lesions were less marked in treatment group with high dose treatment suggesting, the cellular damage caused by free radicals could be reduced by administration of aqueous extract of flowers of hibiscus at higher dosage.

The kidney sections from group B showed mild to moderate pathological changes in renal tubules like cellular swelling and granular cytoplasmic features of tubular epithelium. These changes were minimal in group F and G where as in low treatment groups D and E these degenerative lesions were present in renal tubule but were not seen in glomeruli. Group C showed no sign of degenerative changes suggestive of the extract to be safe.

The microscopic examination of organs like heart, lung, spleen and stomach showed no alteration in their histology in groups A, C, D, E, F and G where as in group B mild to moderate degenerative changes were observed in all these organs.

The skin tissue sections with tumours mass revealed neoplastic cells of fibrous connective tissue with solid and compact arrangement in all the groups. The neoplastic tissue showed proliferative changes with large elongated nucleus with mitotic figures suggestive of fibrosarcoma. Some animal presented tumors which were classified as adenoma and adenocarcinoma

With respect to cellular changes in tumour tissue of fibrosarcoma, it was observed that neoplastic cells in group B, group D and E as well as of group F showed proliferative tumour cells and there was absence of any degenerative or necrotic changes in the tumour mass. However, the cellular changes in tumour tissue from animals of group G (Doxorubicin as anticancer drug) showed mild to moderate degenerative changes in neoplastic tissue suggestive of anticancer therapeutic potential. The tissue sections showed areas of cellular debris of eosinophilic nature with degenerated cells and foci of necrosis. The histopatholgy concludes that the administration of aqueous extract of flowers of hibiscus at lower and higher doses was not found to be useful to control the proliferative changes in the tumor.

Conclusions

On the basis of present study following conclusions were drawn:

1. Extract of flowers of *Hibiscus rosa sinensis* Linn showed preventive effect against oxidative stress and holds a good antioxidative properties.
2. Extract of flower of *Hibiscus rosa sinensis* Linn had no toxic effect at 200, 400 and 600mg/kg dose in mice and can be said to be safe at these doses.
3. DMBA induction in mice proved to be an effective model to study cancer.
4. Aqueous extract of flowers of *Hibiscus rosa sinensis* Linn showed no therapeutic effect in induced cancer, in this study.

Table 8: Group-wise mean body weights (g)

Days	Weight of Groups (g)							SEM	P Value
	A	B	C	D	E	F	G		
Day 0	29.04	29.59	31.37	29.21	28.54	29.83	29.86	0.289	0.221
Day14	32.52	32.95	33.5	32.32	31.62	32.9	32.94	0.279	0.705
Day28	34.55	38.19	35.85	36.05	35.4	37.1	37.89	0.365	0.151
Day56	38.72	41.79	41.04	39.55	39.05	41.38	41.02	0.356	0.097
Day90	46.47 ^c	34.12 ^a	48.11 ^c	37.98 ^b	38.69 ^b	40.76 ^b	38.98 ^b	0.729	0
Day118	49.6 ^c	36.17 ^a	50.52 ^c	40.7 ^b	41.71 ^b	43.74 ^b	41.38 ^b	0.732	0

Note: Superscripts are to be read row wise for mean comparison.

Mean bearing similar superscripts in a row do not differ significantly.

Means with different superscripts in a row differ significantly at $P < 0.05$.

Table 9: Group-wise mean of biochemical parameters with standard error mean and P-value

Tests	Groups							SEM	P Value
	A	B	C	D	E	F	G		
ALT	25.01 ^a	126.52 ^e	30.59 ^a	104.78 ^{de}	98.55 ^d	80.47 ^{cd}	62.76 ^{bc}	6.06	0
AST	37.16	52.87	27.79	50.24	47.78	42.77	40.98	2.757	0.201
UREA	12.53 ^a	24.1 ^c	13.8 ^a	17.33 ^{ab}	19.8b ^c	15.97 ^{ab}	15.61 ^{ab}	0.739	0
CRT	0.52 ^{ab}	1.14 ^c	0.32 ^a	0.77 ^b	0.82b ^c	0.39 ^a	0.47 ^{ab}	0.054	0
PHO	3.2	2.98	2.3	2.24	2.38	2.12	2.35	0.11	0.056
CA	6.59	7.29	6.95	6.61	6.48	6.06	6.88	0.131	0.28
TP	6.97	5.82	6.22	6.04	6.59	6.61	6.4	0.119	0.06
ALB	3.57 ^b	2.25 ^a	2.50 ^a	2.49 ^a	2.69 ^a	2.73 ^a	2.85 ^a	0.091	0
GLB	3.4	3.57	3.72	3.54	3.9	3.83	3.55	0.119	0.939
A;G	1.17	0.64	0.84	0.79	0.74	0.79	0.92	0.059	0.32
ALP	106.72 ^{ab}	174.76 ^c	88.9 ^a	135.1 ^{abc}	144.13 ^{bc}	121.01 ^{ab}	124.33 ^{ab}	6.5	0.012

Note: Superscripts are to be read row wise for mean comparison.

Mean bearing similar superscripts in a row do not differ significantly.

Means with different superscripts in a row differ significantly at $P < 0.05$.

Table 10: Group-wise mean of hematological parameters with standard error mean and P-value

Parameters	Groups							SEM	P Value
	A	B	C	D	E	F	G		
HB	14.3 ^b	11.65 ^a	14.37 ^b	13.97 ^b	13.57 ^b	14.11 ^b	14.01 ^b	0.218	0.009
PCV	47.67 ^b	34.74 ^a	47.4 ^b	42.97 ^b	41.99 ^b	43.02 ^b	43.83 ^b	0.78	0
TLC	7.48 ^a	10.28 ^b	6.72 ^a	8 ^a	7.53 ^a	8.03 ^a	7.79 ^a	0.214	0
MCV	67.25	60.07	62.82	68.17	66.65	58.85	60.81	1.303	0.296
MCHC	30.6 ^a	33.65 ^b	32.49 ^b	30.3 ^b	32.42 ^b	32.92 ^b	32.2 ^b	0.269	0.028
TEC	7.28 ^b	5.84 ^a	7.1 ^b	6.46 ^{ab}	6.47 ^{ab}	7.41 ^b	7.3	0.148	0.029
PLT	9.09	9.52	8.25	8.65	8.01	8.39	8.48	0.152	0.125
LYMP	79.9	80.4	80	77.2	81.7	79.3	82.1	0.44	0.063
NEU	17.6	17.5	18.2	20.3	16.4	18.3	16.3	0.421	0.179
MONO	1.3	1.2	0.9	1.2	0.8	1.3	0.8	0.1	0.63
EOS	1.2	0.9	0.9	1.3	1.1	1.1	0.8	0.094	0.801
BASO	0	0	0	0	0	0	0	0	0

Note: Superscripts are to be read row wise for mean comparison.

Mean bearing similar superscripts in a row do not differ significantly.

Means with different superscripts in a row differ significantly at $P < 0.05$.

Table 11: Group-wise mean values of MDA in different tissues

Tissues	Groups							SEM	P Value
	A	B	C	D	E	F	G		
LIVER	20.87 ^a	40.5 ^b	19.62 ^a	41.5 ^b	22.97 ^a	25.92 ^a	48.5 ^c	2.3	0
KIDNEY	30.67 ^a	50 ^{cd}	27.87 ^a	56.17 ^{de}	47.93 ^b	41.95 ^b	60 ^e	2.35	0
BRAIN	10.27	11.8	8.95	11.1	10.27	10.72	12.57	0.59	0.81

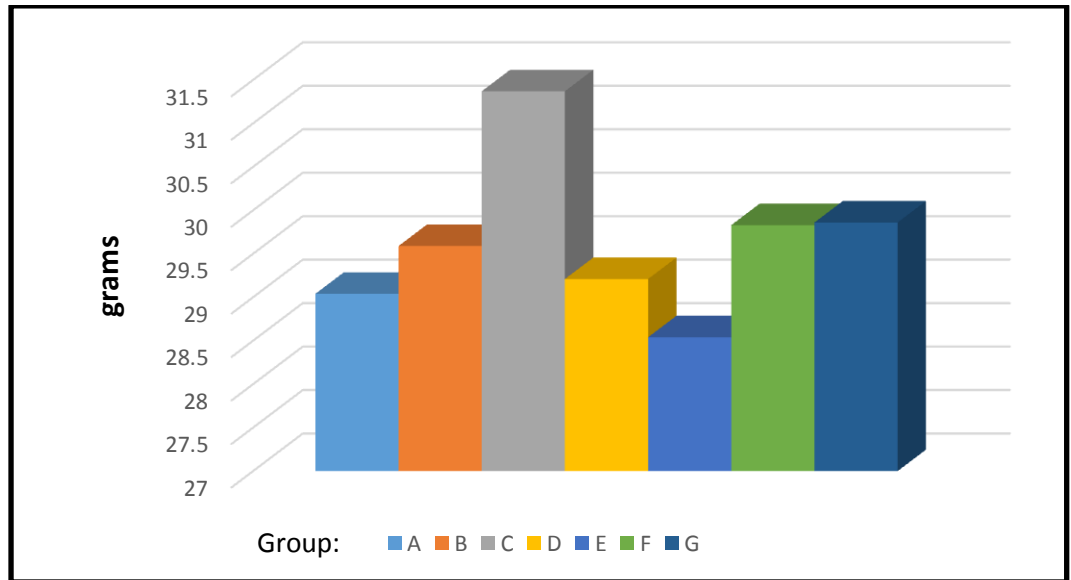


Fig.1: Body Weight (g) at Day 0

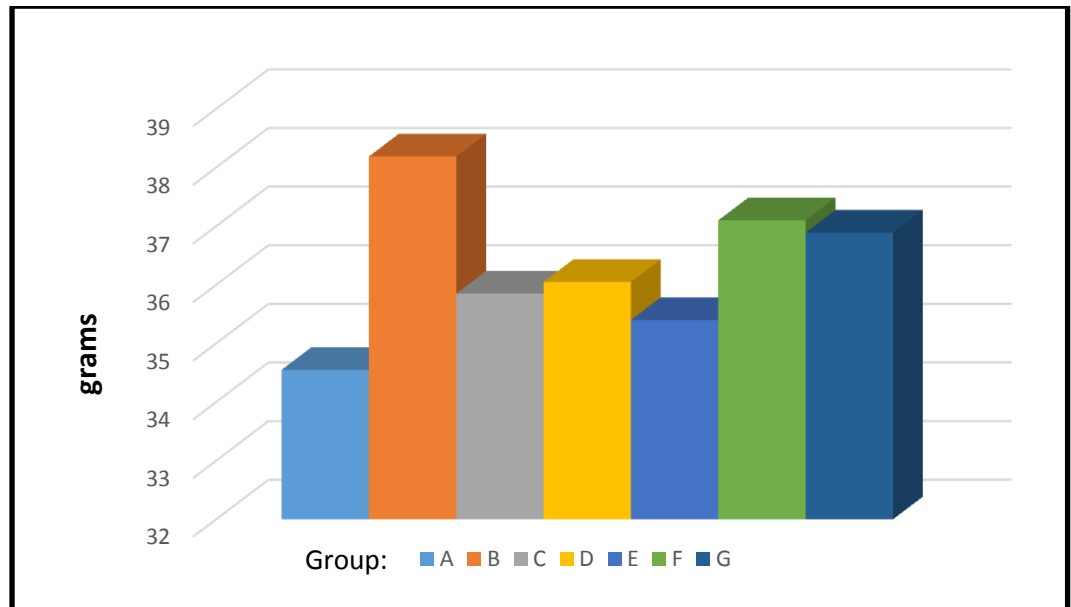


Fig.2: Body Weight (g) at Day 14

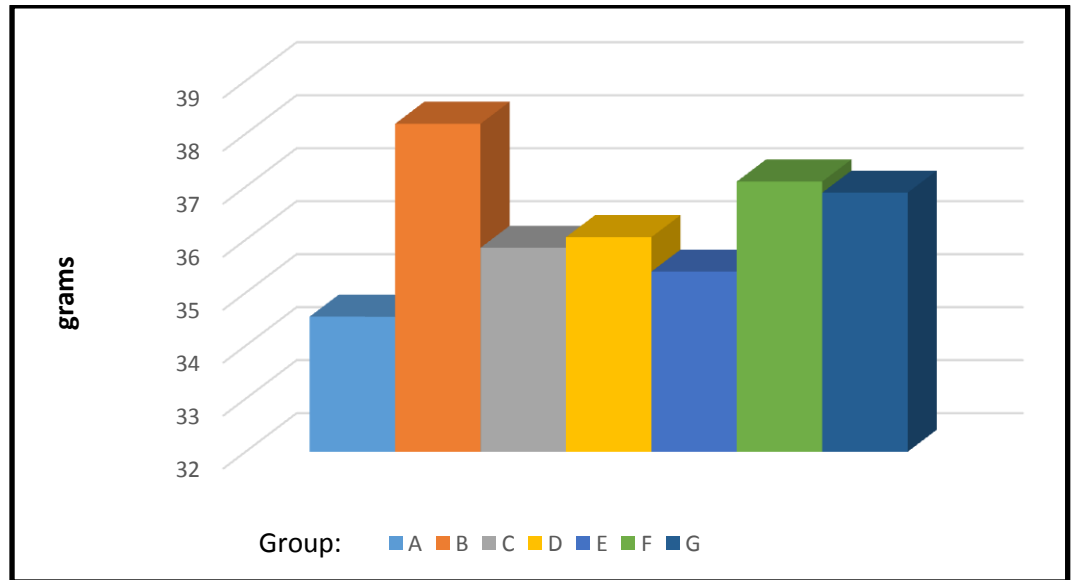


Fig.3: Body Weight (g) 28 days

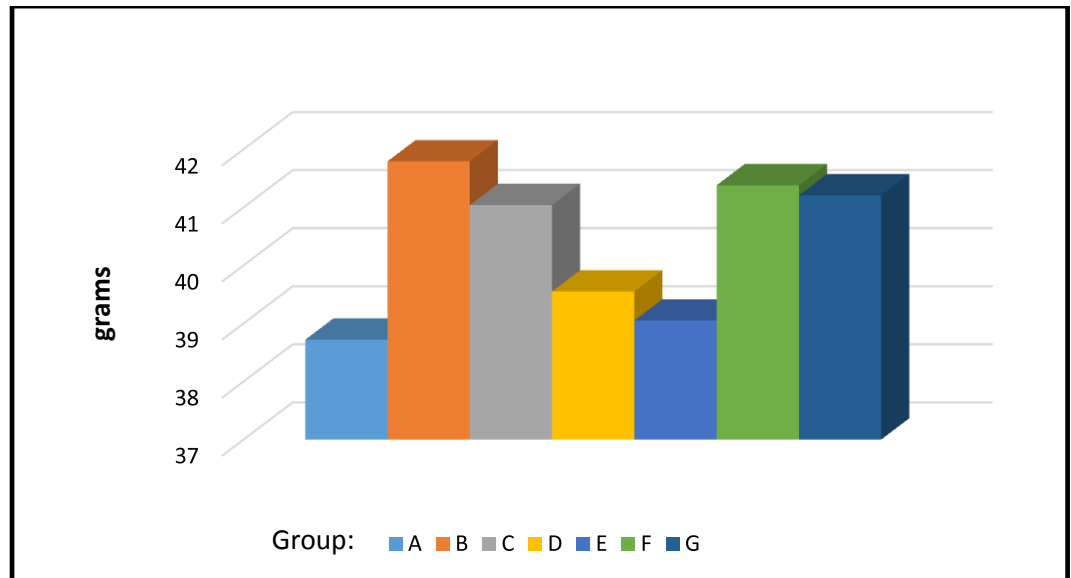


Fig.4: Body Weight (g) 56 days

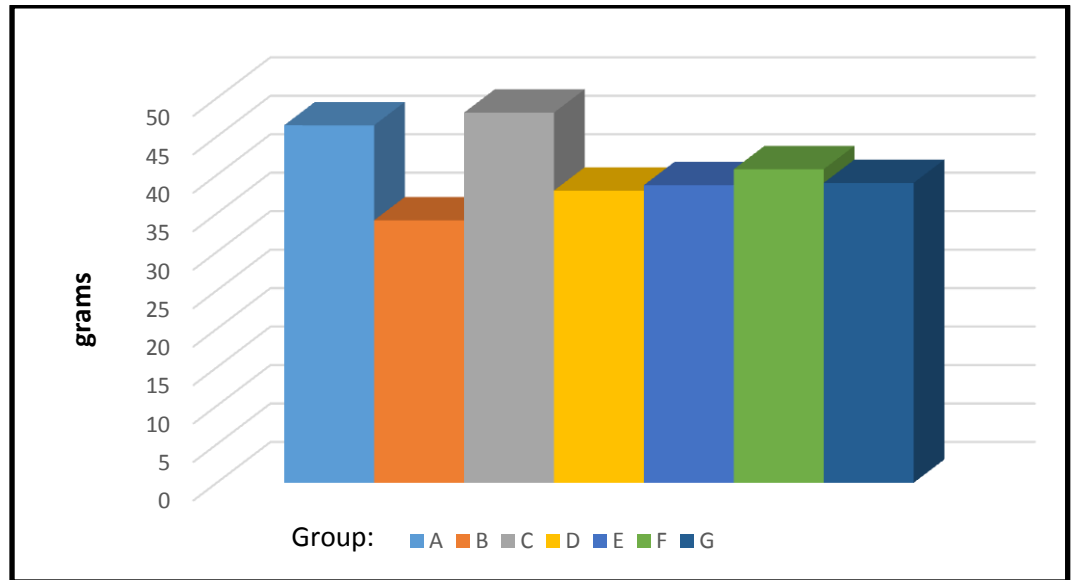


Fig.5: Body Weight (g) 90 days

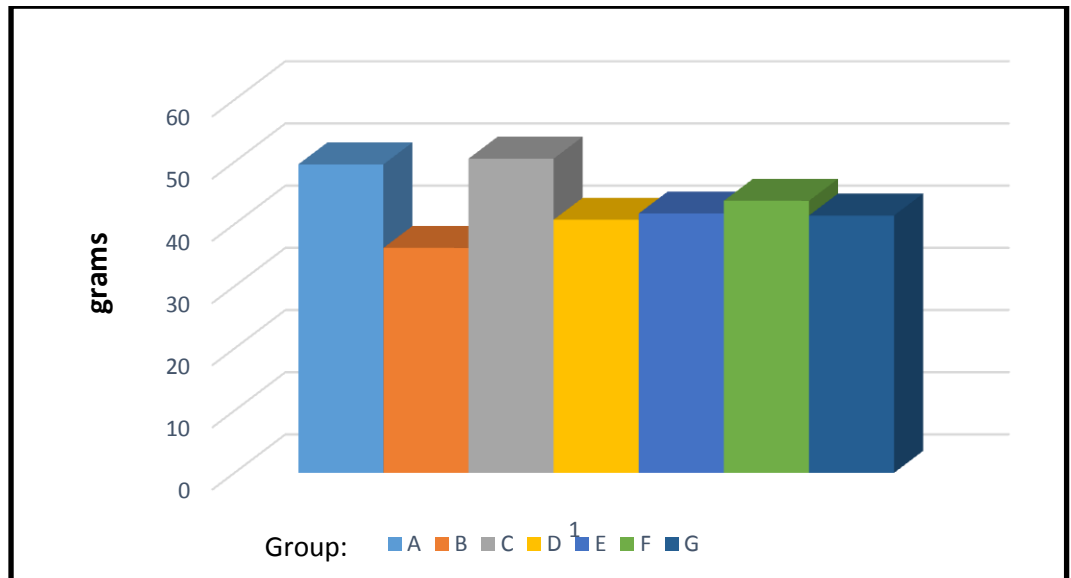


Fig.6: Body Weight (g) 118 days

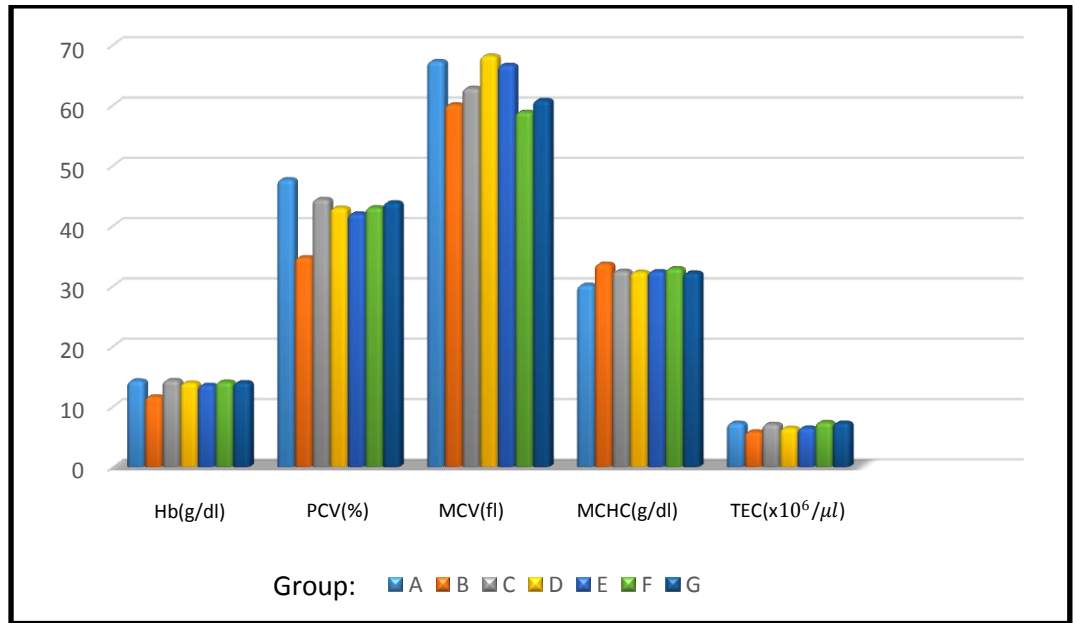


Fig.7:Group-wise hematological values

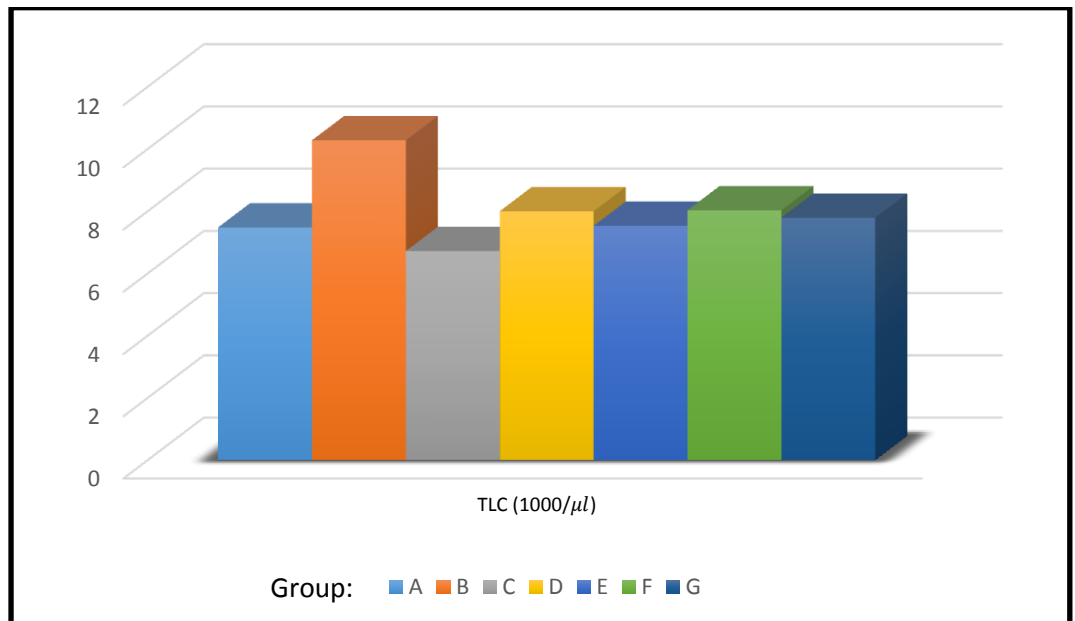


Fig.8:Group-wise TLC values

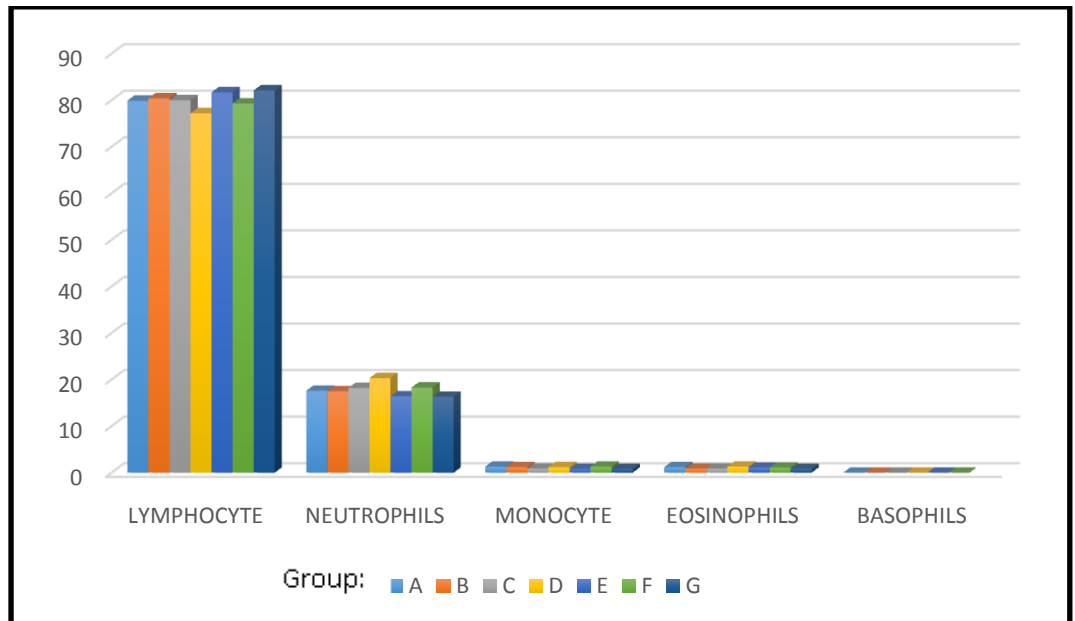


Fig.9:Group-wise DLC values (%)



Plate 1: Handling of mice while induction

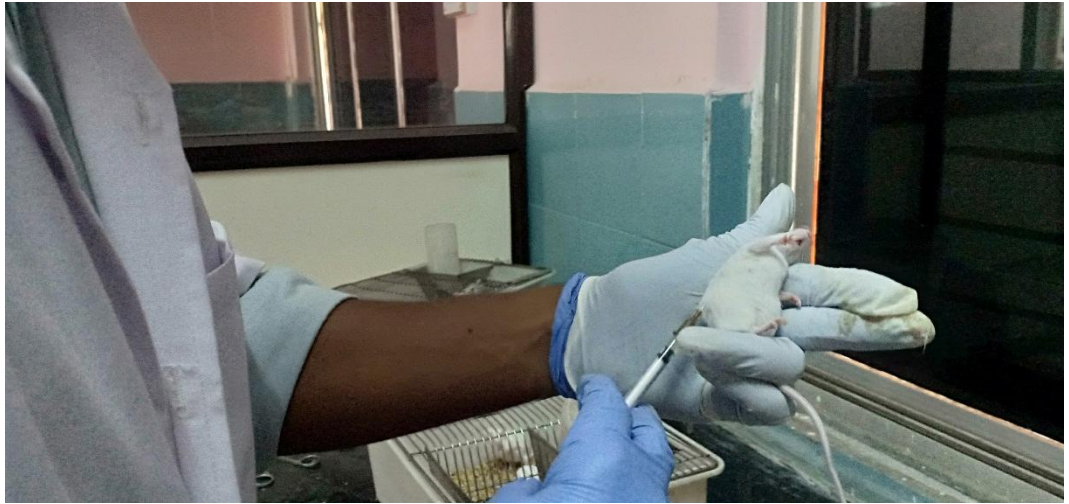


Plate 2: DMBA sub-cut Induction



Plate 3: Weighing of mice



Plate 4: Gavaging of Hibiscus aqueous extract



Plate 5: Tumorous growth observed

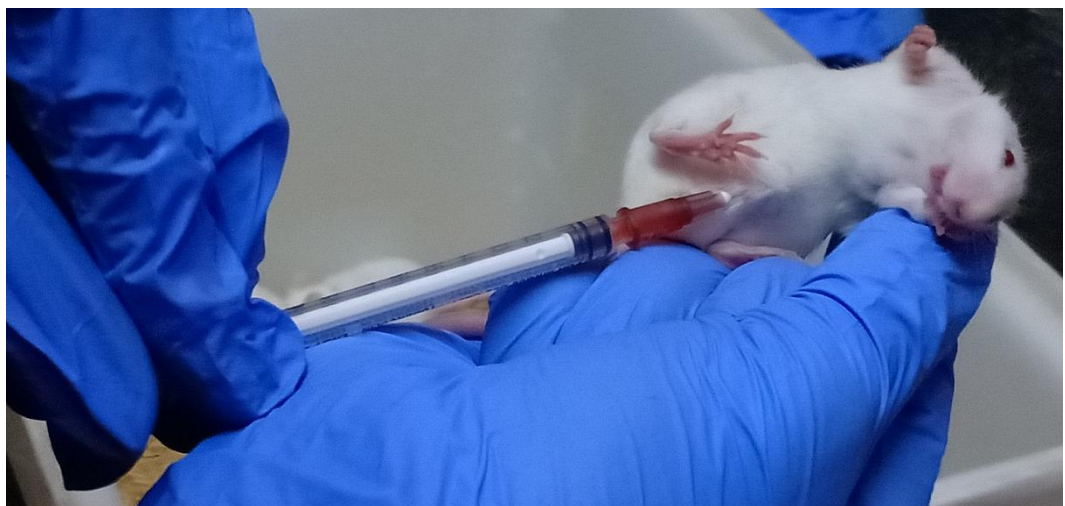


Plate 6: Treatment of group 'G' with Doxorubicin (Standard drug)

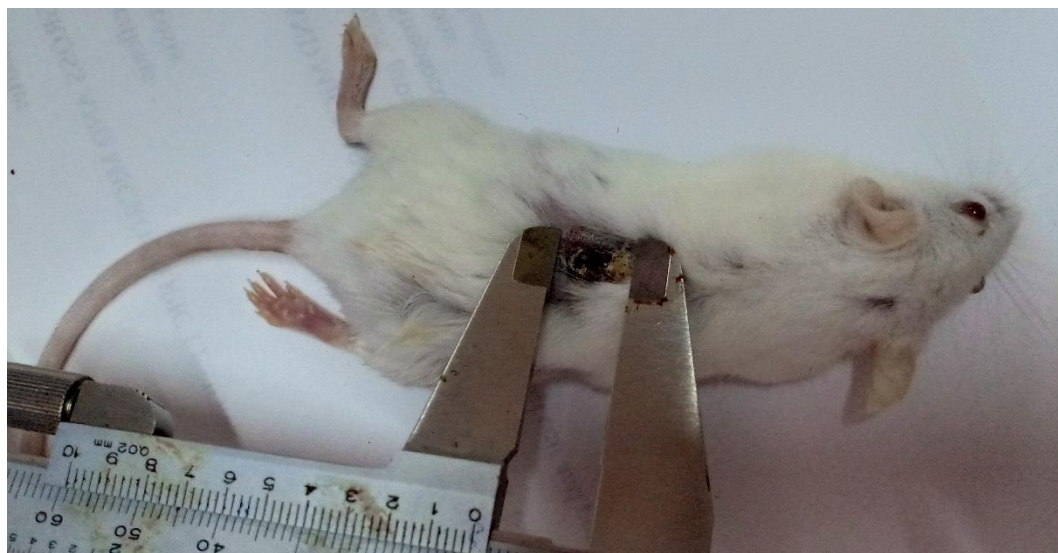


Plate 7: Measurements of tumorous growth

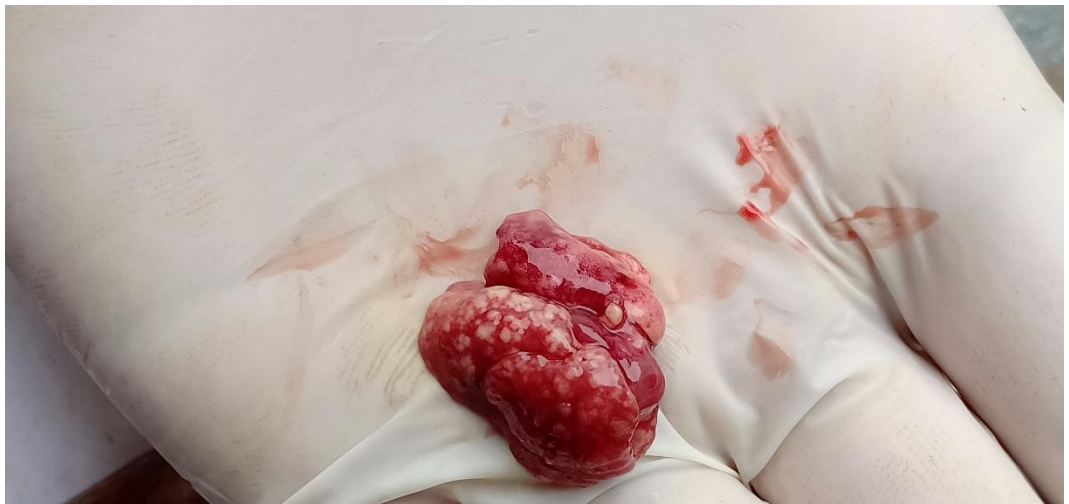
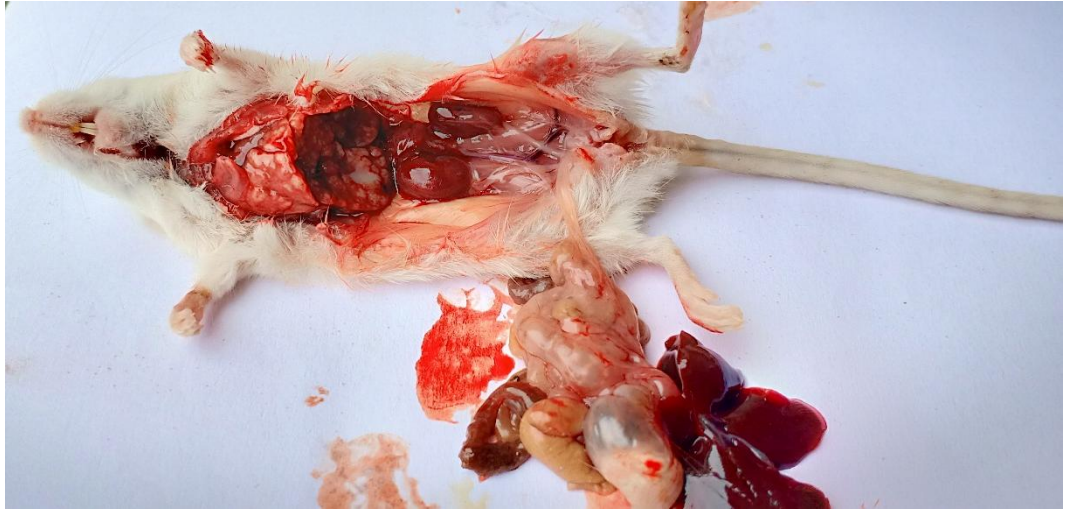


Plate 8: Post-mortem

Plate 9 : GroupWise histopathological observations of Liver tissue

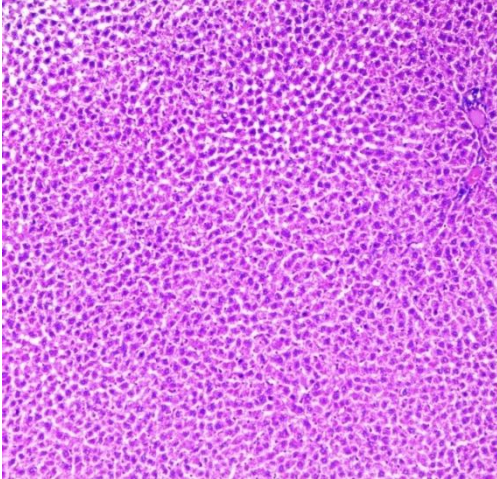
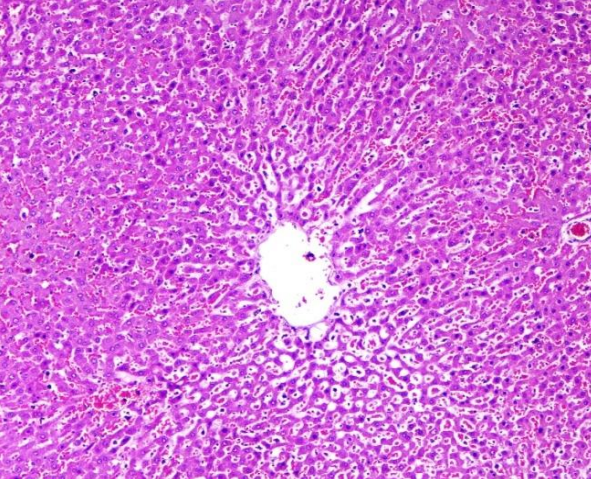
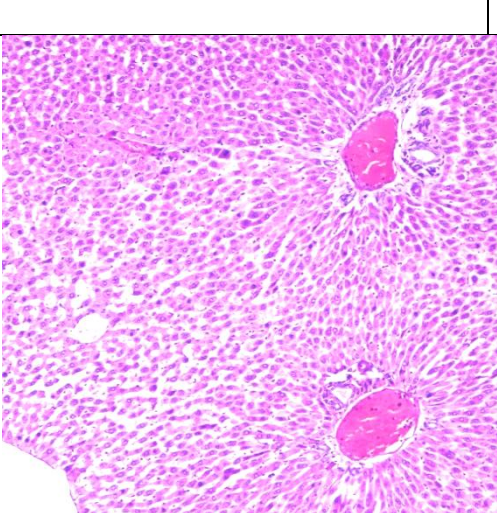
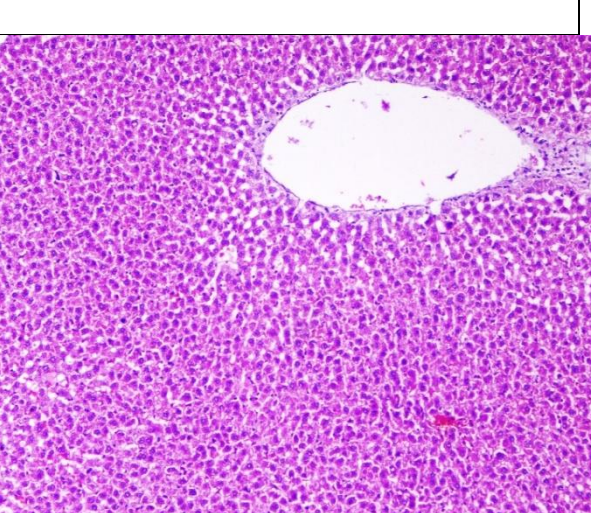
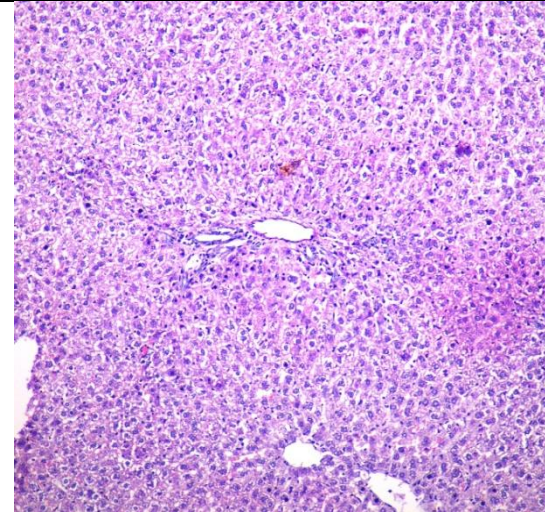
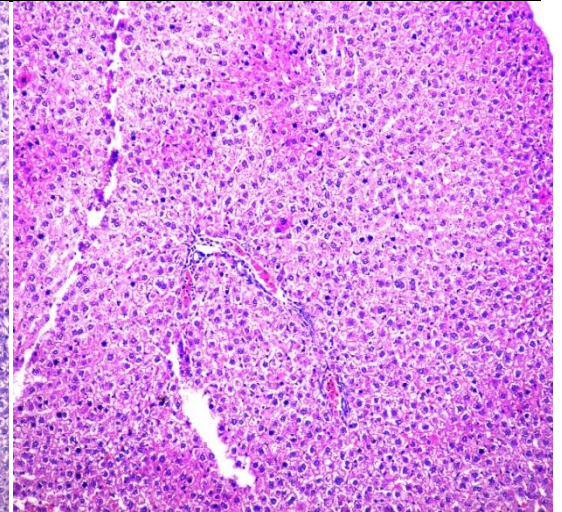
	
Gr. A: Liver section showing normal hepatic parenchyma with normal hepatocytes. H&E, x100	Gr. B: Liver section showing mild pathological changes of hepatic parenchyma with cellular swelling, degeneration of hepatocytes, congested parenchyma and distorted hepatic cords. H&E, x100
	
Gr. B: Liver section showing mild pathological changes of hepatic parenchyma with cellular swelling, degeneration of hepatocytes, congested vascular tissue and distorted hepatic cords. H&E, x100	Gr C: Liver section showing minimal to mild pathological changes of hepatic parenchyma with focal areas cellular swelling congested blood vessels and distorted hepatic cords. H&E, x100

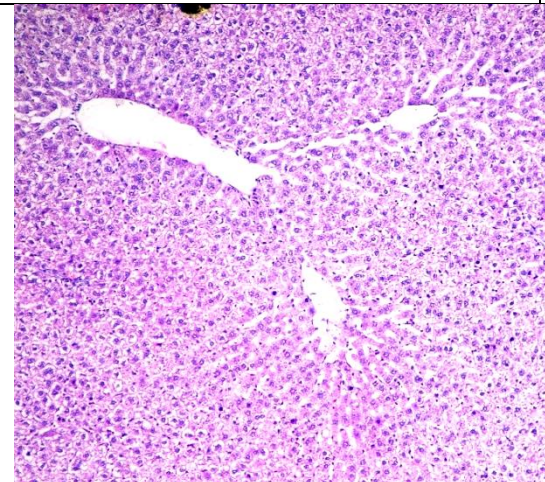
Plate 10 : Groupwise histopathological observations of Liver tissue



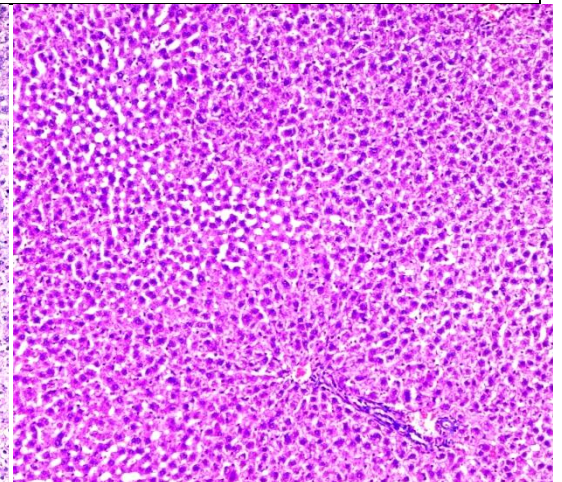
Gr D: Liver section showing mild pathological changes of hepatic parenchyma with degeneration of hepatocytes with granular cytoplasmic features, focal areas cellular swelling, and distorted hepatic cords. H&E, x100



Gr E: Liver section showing mild pathological changes of hepatic parenchyma with degeneration of hepatocytes with granular cytoplasmic features, focal areas cellular swelling, and distorted hepatic cords. H&E, x100

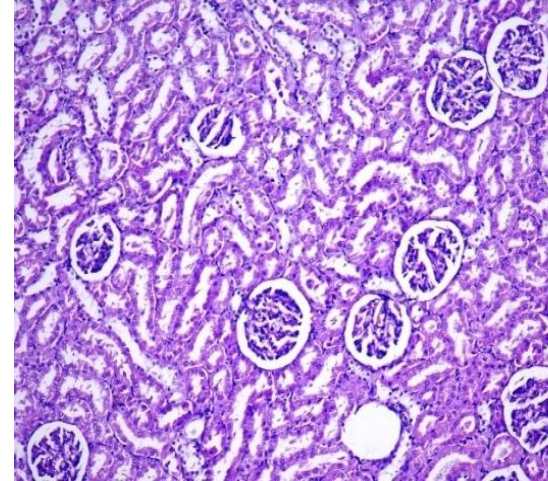


Gr E: Liver section showing mild pathological changes of hepatic parenchyma with degeneration of hepatocytes with granular cytoplasmic features, focal areas cellular swelling, and distorted hepatic cords. H&E, x100

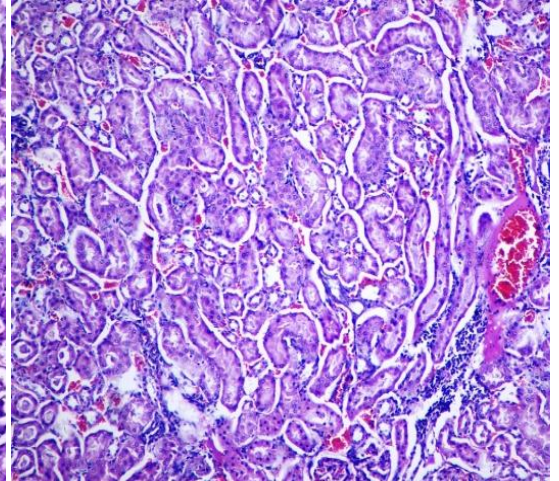


Gr G: Liver section showing minimal and focal pathological changes of hepatic parenchyma with degeneration of hepatocytes with granular cytoplasmic features, focal areas cellular swelling, and distorted hepatic cords. H&E, x100

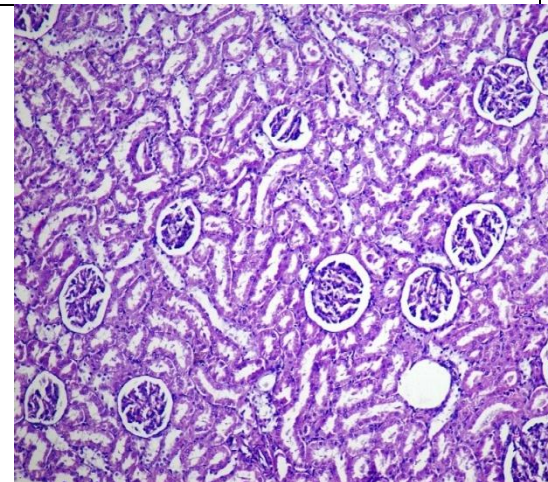
Plate 11 : GroupWise histopathological observations of Kidney tissue



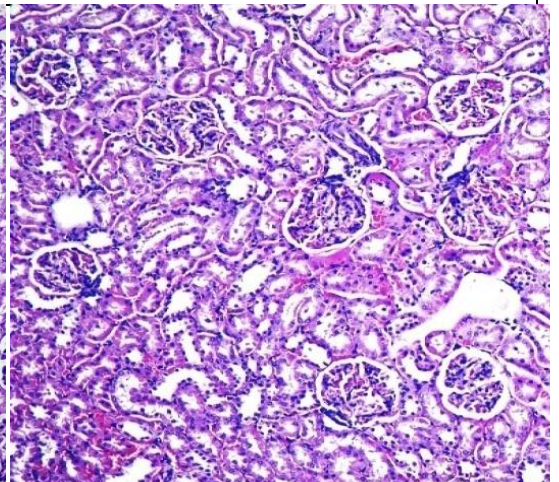
Gr. A: Kidney section showing normal renal parenchyma with intact tubules and normal glomeruli. H&E, x100



Gr. B: Kidney section showing mild pathological changes of renal tubules with cellular swelling, degeneration of tubules, interstitial hemorrhages. H&E, x100

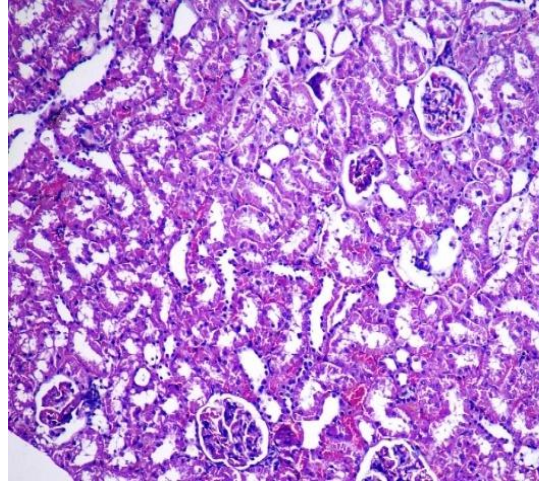


Gr. C: Kidney section showing normal renal parenchyma with intact tubules and normal glomeruli. H&E, x100

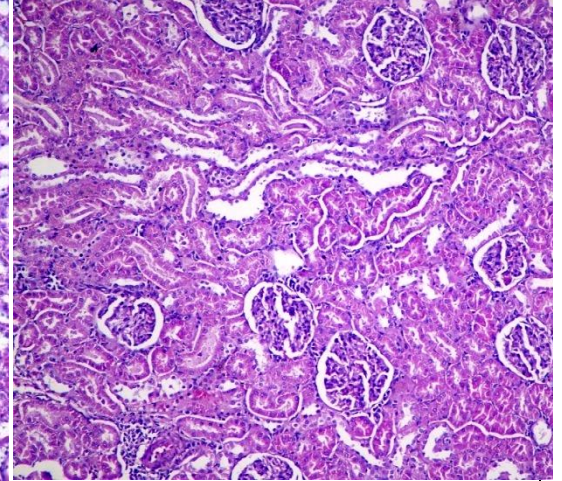


Gr D: Kidney section showing minimal pathological changes of renal tubules and swelling of glomeruli, degeneration of tubules and distorted parenchyma. H&E, x100

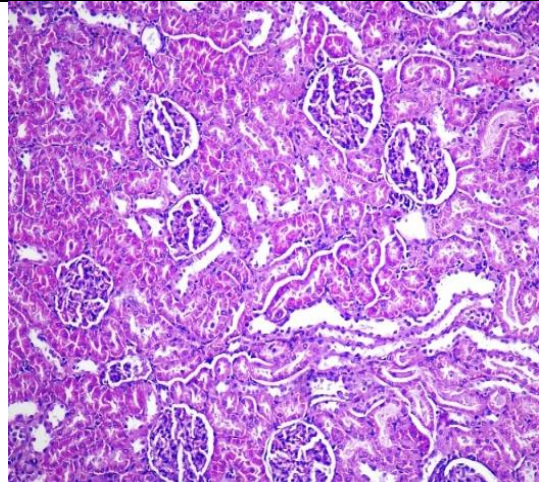
Plate 12 : Groupwise histopathological observations of Kidney tissue



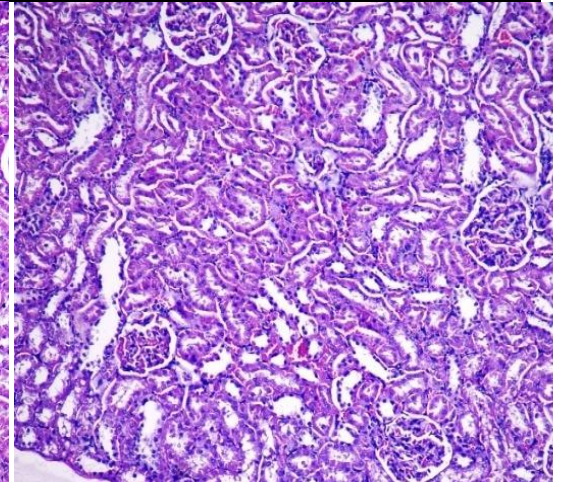
Gr. E: Kidney section showing minimal to mild pathological and degenerative changes of renal tubules, swelling of glomeruli, and interstitial hemorrhages . H&E, x100



Gr. F : Kidney section showing minimal pathological changes of renal tubules, swelling of glomeruli. H&E, x100

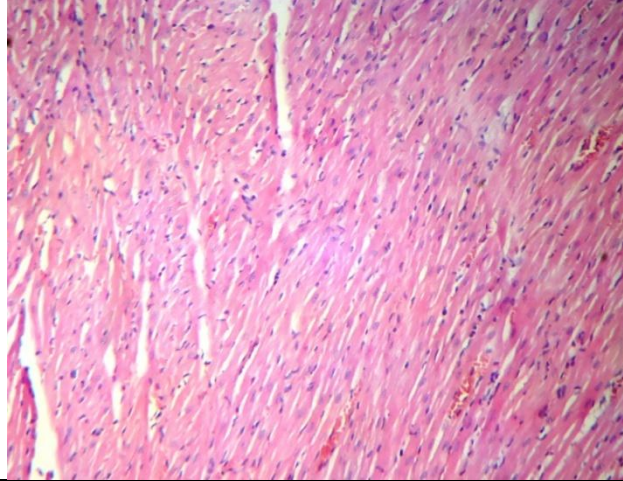


Gr. G: Kidney section showing minimal pathological changes in renal parenchyma with focal cellular swelling of tubules, and swelling of glomeruli. H&E, x100

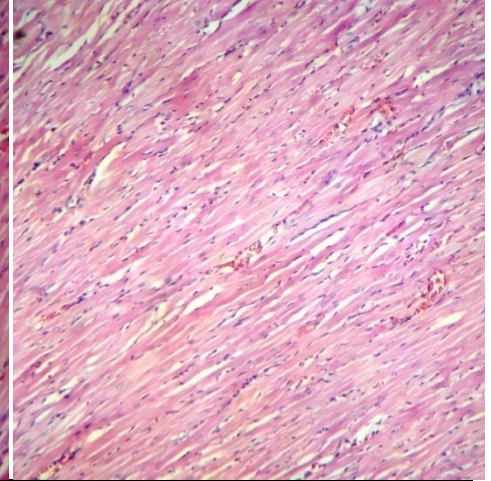


Gr. G: Kidney section showing minimal pathological changes in renal parenchyma with focal cellular swelling of tubules, and swelling of glomeruli. H&E, x100

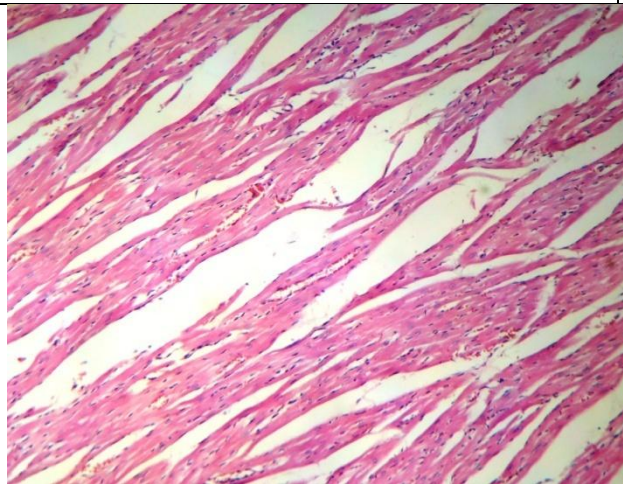
Plate 13 : Groupwise Histopathological observations of heart



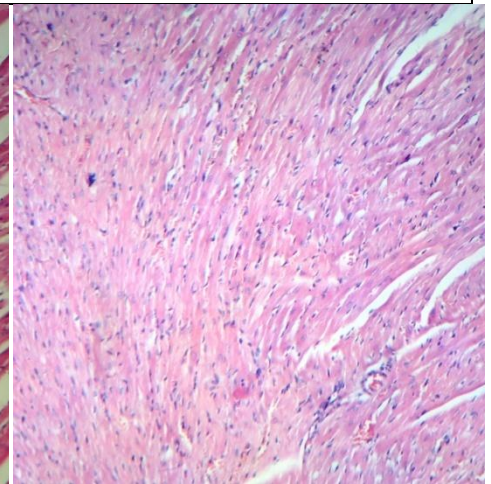
Group A: Normal histomorphological features of heart tissue showing intact cardiac muscle fibers. H&E stain. x100



Group B: Heart tissue showing mild degenerative features of cardiac muscle fibers. H&E stain. x100

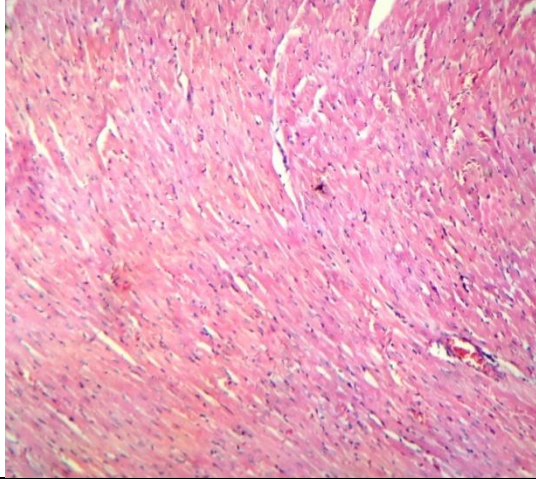


Group B: Heart tissue showing dilation of cardiac fibers and focal mild degenerative changes of cardiac muscle fibers and hemorrhages in myocardium. H&E stain. x100

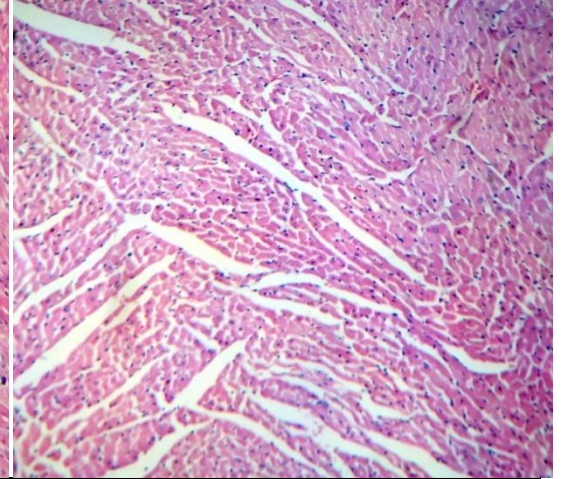


Group C: Normal histomorphological features of heart tissue showing intact cardiac muscle fibers. H&E stain. x100

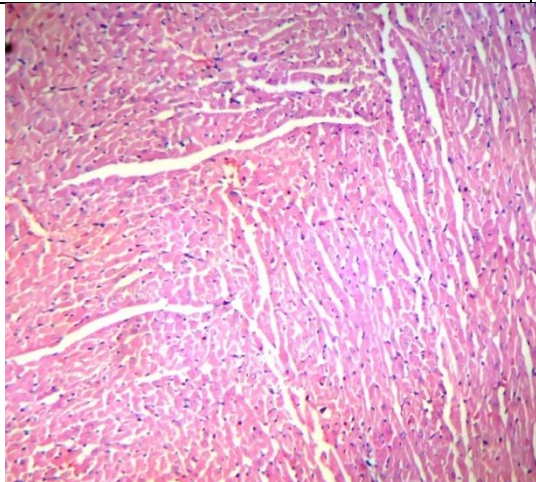
Plate 14 : Groupwise Histopathological observations of heart



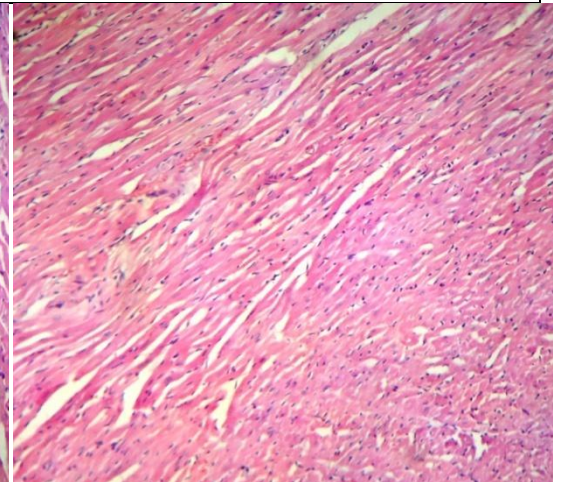
Group D: Normal histomorphological features of heart tissue showing intact cardiac muscle fibers.
H&E stain. x100



Group E: Normal histomorphological features of heart tissue showing intact cardiac muscle fibers.
H&E stain. x100

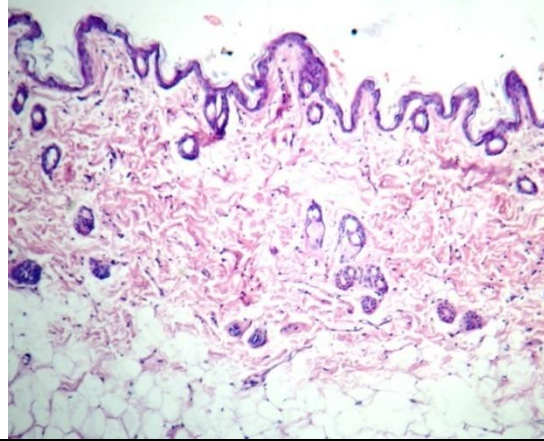


Group F: Heart tissue showing focal and minimal degenerative features of cardiac muscle fibers.
H&E stain. x100

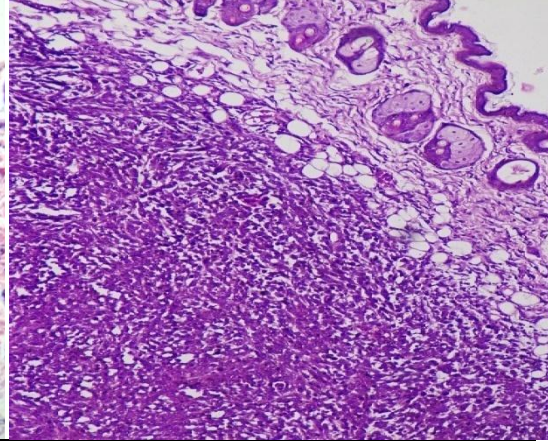


Group G: Heart tissue showing dilation of cardiac fibers and focal mild degenerative changes of cardiac muscle fibers in myocardium.
H&E stain. x100

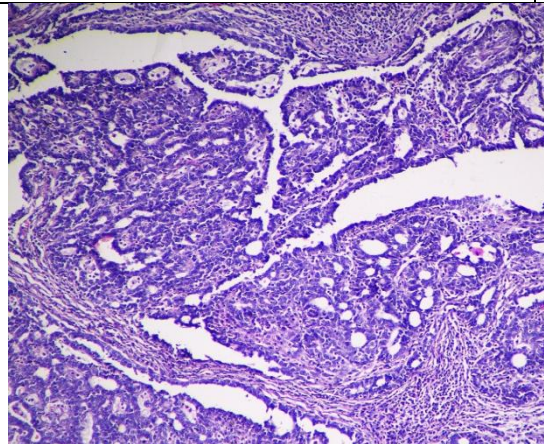
Plate 15 : Groupwise Histopathological observations of Skin and /or Tumour tissue



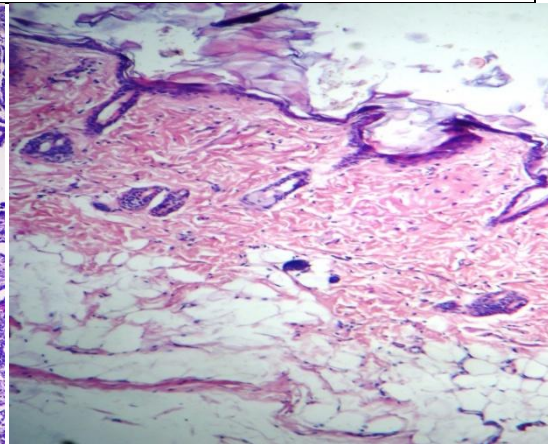
Group A: Normal histomorphological features of skin showing dermis and epidermis and subcutaneous layer. H&E stain. x100



Group B: The skin with tumour tissue showing well developed fibrosarcoma in the subcutis layer. H&E stain. x100

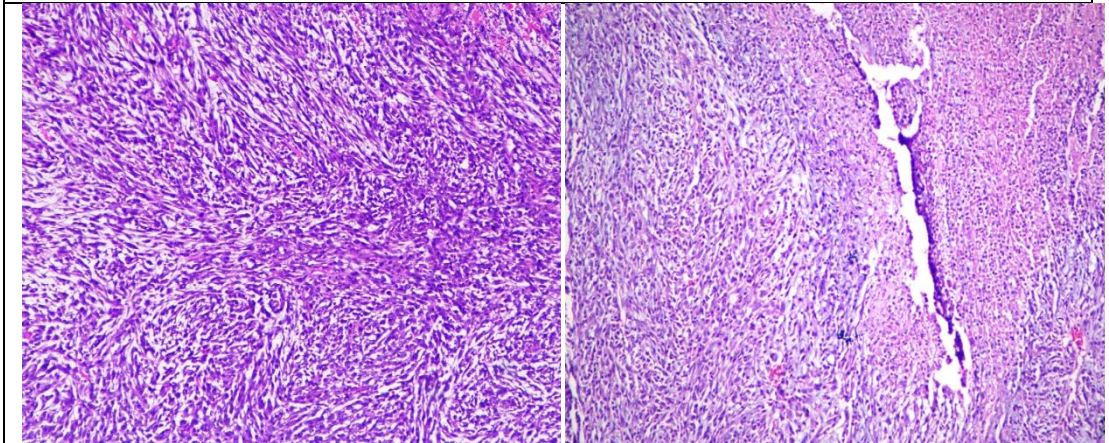


Group B: The skin with tumour tissue showing Adenocarcinoma of mammary gland. H&E stain. x100



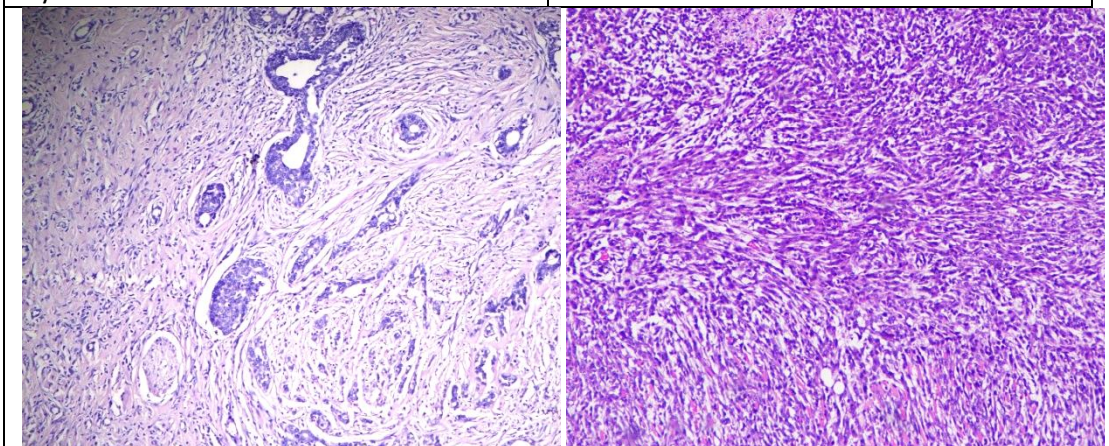
Group C: Normal histomorphological features of skin showing dermis and epidermis and subcutaneous layer. H&E stain. x100

Plate 16 : Groupwise Histopathological observations of Skin and /or Tumour tissue



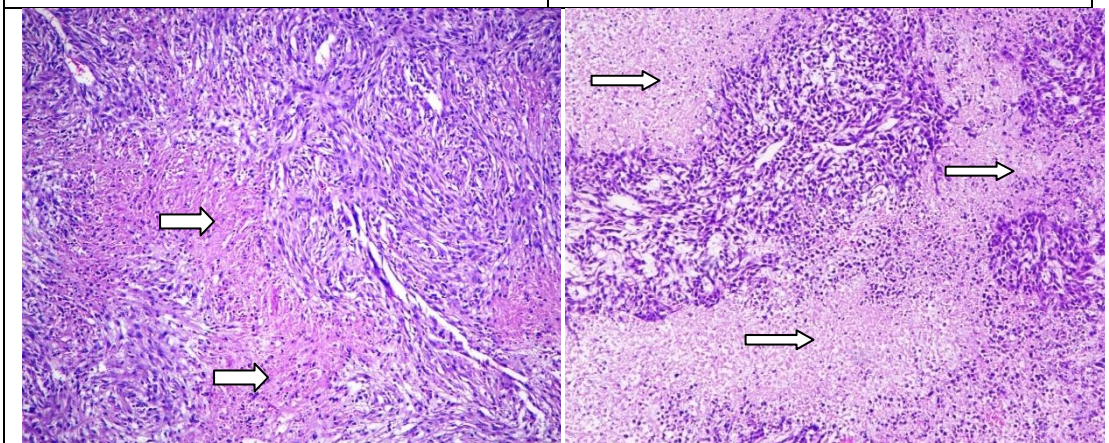
Group D: The skin with tumour tissue showing neoplastic features of well developed fibrosarcoma in the subcutis layer. H&E stain. x100

Group E: The skin with tumour tissue showing fibrosarcoma with proliferative features. H&E stain. x100



Group F: The skin with tumour tissue showing neoplastic features of Adenocarcinoma of mammary glandular tissue. H&E stain. X400

Group F: The skin with tumour tissue showing fibrosarcoma with proliferative features with degenerative changes. H&E stain. x100



Group G: The tumour tissue mass showing degenerative and necrotic changes of neoplastic cells in fibrosarcoma. H&E stain. x100

Group G: The tumour tissue mass showing degenerative and necrotic changes of neoplastic cells in fibrosarcoma. H&E stain. x100

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VITA

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His immense passion for helping animals in need ultimately zeroed down his career choice to becoming a Veterinary Doctor. Author joined Nagpur Veterinary College, Nagpur in year 2011 and successfully completed B.V.Sc. & AH degree in July 2016 with 2nd class. He is national cadet corp and holds "C" certificate from R & V SQN NCC. Author has represented the Maharashtra Animal and Fishery Sciences University at State level Inter university sports competition in 2012 and 2013.

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Appendix - G

THESIS ABSTRACT

- a) Title of thesis : EFFECT OF AQUEOUS EXTRACT OF FLOWERS OF *Hibiscus rosa-sinensis* LINN ON DMBA INDUCED TUMOURS IN MICE
- b) Full name of student : HEMANT KUMAR
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- e) Year of awarded of degree : 2018
- f) Major subject : DEPARTMENT OF VETERINARY PATHOLOGY
- g) Total number of pages in the thesis : 63
- h) Number of words in the Abstract : 661
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Abstract

Cancer is one of the oldest diseases known to mankind and the occurrence is growing enormously with growing population with increase in average life expectancy and changing lifestyle predisposing more lives today. The lack of effective, safe and economical treatment makes treating cancer more difficult. Plants and its preparations are used for medicinal purposes since ancient times and are reported to be effective and safe. WHO stated that 80% of global population's problems can be solved by using herbal medications for their primary healthcare. Around 52,896 plant species are found to have medicinal properties and many of them have shown potent anti cancer property. *Hibiscus rosa sinensis* Linn has been reported to have large no of medicinal uses in different parts of the world and in recent years studies have shown that it possess a good potential to be used as anticancer drug.

This study was carried out to evaluate the anti cancer properties of aqueous extract of *Hibiscus rosa sinensis* Linn in DMBA induced tumours in mice. The mice were randomly divided into seven groups comprising of twenty five animals in each group. Group A was kept as normal control only provided with feed and water, Group B served as positive (disease) control for tumour with administration of chemical carcinogen i.e. DMBA, and Group C served as herbal treatment control and was administered with aqueous extract of flowers of hibiscus @ 400mg/kg by oral gavage. Group D, Group E and Group F served as treatment groups at low (200mg/kg), mild (400mg/kg), and high (600mg/kg) doses, respectively after induction of tumour by DMBA. Group G was treated with anticancer drug Doxyrubicine @2mg/ kg body weight.

The tumours were induced by DMBA in mice by intra-peritoneal route. The tumours were developed after 62 days after DMBA administration. After 90 days of tumour induction dosage, the animals were treated with aqueous extract of flowers of hibiscus for 28 days. The body weights were decreased in tumour induced groups on day 90 and on day 118 as compared to normal control group and extract control group.

The hematological parameters viz. hemoglobin, packed cell volume, and total erythrocyte count were found to be significantly decreased in tumour induced animals as compared to control group while treatment with aqueous extract of flowers of hibiscus showed significant increase in hemoglobin, packed cell volume and total erythrocyte count in dose dependent manner. The groups with higher dose (@ 600 mg/kg) showed higher hemoglobin and PCV levels than other treatment groups. There was no significant difference for differential leucocyte count in all the groups. The total leucocyte count was found to be significantly increased in group B due to tumour development as compared to other treated groups with aqueous extract of flowers of hibiscus.

Serum biochemical evaluation revealed that the administration of aqueous extract of flowers of hibiscus helped in protecting liver and kidney from damage caused by free radicals in tumour bearing animals. This was evident by reduced levels of ALT, AST, ALP, Urea, and Creatinine in group D, E and F as compared with group B.

The MDA results have shown that the extract of flowers of hibiscus possesses good anti-oxidative properties and can effectively reduce the oxidative stress caused by cancer at cellular level as observed in liver and kidney.

The overall histopathological examination of vital organs revealed that metabolic and systemic stress caused by progression of tumour by DMBA could be reduced by administration of aqueous extract of flowers of hibiscus at higher dosage. The cellular pathological changes in organs like liver and kidney caused by oxidative damage in tumour bearing animal could be reduced by aqueous extract of flowers of hibiscus. However, the administration of aqueous extract of flowers of hibiscus at lower and higher doses were not found to be useful to control the proliferative changes in the tumour.

It was concluded that administration of aqueous extract of flowers of hibiscus was useful to reduce the oxidative damage caused by the tumour.

प्रबंध सारांश			
१)	प्रबंध शीर्षक	:	मूषकामधील डीएमबीए व्दारे उद्भवित केलेल्या कर्क रोगावर जास्वंदाच्या फुलांच्या अर्काचा परिणाम .
२)	विद्यार्थ्याचे पुर्ण नाव	:	हेमंत कुमार
३)	मार्गदर्शकाचे नाव व पत्ता	:	डॉ . चं .सं .मोटे सहाय्यक प्राध्यापक,पशुविकृती शास्त्र विभाग, क्रा .ना .पा . पशुवैद्यकीय महाविद्यालय, शिरवळ .जि .सातारा
४)	पदवीचे नाव	:	एम .व्ही .एस .सी .
५)	पदवीचे वर्ष	:	२०१८
६)	विषय	:	पशुविकृती शास्त्र विभाग
७)	प्रबंधातील एकूण पाने	:	६३
८)	प्रबंध सारांशामधील एकूण शब्द	:	६०६
९)	विद्यार्थ्याची स्वाक्षरी	:	
१०)	पुढे पाठवणाऱ्या अधिकाऱ्याची स्वाक्षरी नाव व पत्ता	:	डॉ . चं .सं .मोटे सहाय्यक प्राध्यापक,पशुविकृती शास्त्र विभाग, क्रा .ना .पा . पशुवैद्यकीय महाविद्यालय, शिरवळ .जि .सातारा

गोषवारा

मानव जातीला ज्ञात असलेल्या रोगांपैकी कर्करोग हा एक प्रमुख रोग असून दिवसेंदिवस जागतिक स्तरावर विविध कर्करोगाचे प्रमाण वाढत असल्याचे आढळून आले आहे. प्रामुख्याने मानव जातीची बदललेली जिवनशैली, वाढलेले आयुर्मान आणि वाढलेली लोकसंख्या यामुळे जगभरात कर्करोगाचे प्रमाण वाढलेले आहे. प्रभावी, सुरक्षित आणि आर्थिक दृष्ट्या परवडणाऱ्या उपचारांच्या कमतरतेमुळे तसेच उशिरा होणाऱ्या निदानामुळे कर्करोगावर योग्य प्रमाणात औषधोपचार करता येत नाही.

प्राचीन काळापासून विविध वनस्पतीपासून तसेच भागांपासून बनवलेली औषधे अनेक रोगावर तसेच कर्करोगावर परिणामकारकरित्या सुरक्षितपणे वापरली जात आहेत. जागतिक आरोग्य संघटनेच्या (WHO) संदर्भानुसार जवळपास ८० टक्के लोकसंख्येचे विविध प्राथमिक आरोग्य अपाय वनस्पतीजन्य (हर्बल) औषधांचा वापर करून सोडवता येऊ शकतो. जगभरातील एकदरीत ५२८९६ प्रजातींच्या वनस्पतींमध्ये औषधी गुणधर्म असल्याचे आढळून आले आहे आणि त्यापैकी बऱ्याच वनस्पतींमध्ये कर्करोग प्रतिबंधक तसेच औषधी गुणधर्म दिसून आले आहेत.

जास्वंद (हिबीस्कस रोझा सेनेसिस) हि जगभरात आढळणारी वनस्पती असून विविध संशोधकांनी जास्वंदाच्या पाने तसेच फुलांमध्ये कर्करोग विरोधी औषधी गुणधर्म असल्याचे नमुद केले आहे.

प्रस्तुत संशोधनामध्ये जास्वंदाच्या फुलांचा 'डिएमबीए' या रसायनाद्वारे मुषक वर्गिय प्राण्यामध्ये उद्भवित केलेल्या कर्करोगावर औषधी गुणधर्माचा परिणाम तपासण्यासाठी अभ्यास करण्यात आला. एकूण १७५ मुषकांना प्रत्येक गटात २५ याप्रमाणे एकूण ७ गटांमध्ये (गट ए, बी, सी, डी, ई, एफ आणि जी) समप्रमाणात विभागण्यात आले. गट 'ए' मधील प्राण्यांना सामान्यपणे देण्यात येणारे मुषक खादय आणि पाणी देण्यात आले. गट 'बी' मधील उंदरांना 'डिएमबीए' या रसायनाद्वारे कर्करोग उत्पन्न करण्यात आला, परंतु कोणताही कर्करोग प्रतिबंधक उपचार देण्यात आला नाही. गट 'सी' मधील प्राण्यांना जास्वंदाच्या फुलांचा अर्क देण्यात आला. गट 'डी' 'ई' 'एफ' आणि 'जी' मधील प्राण्यांमध्ये 'डिएमबीए' या कर्करोगजन्य रसायनाद्वारे एकूण ९० दिवसांमध्ये कर्करोगाच्या गाठी उद्भवित करण्यात आल्या.

तदनंतर प्रयोगशाळेत तयार केलेल्या जास्वंदाच्या फुलांचा अर्क गट 'डी' (कमी प्रमाणात ① २०० मिलीग्रम प्रति किलो) गट 'ई' (मध्यम प्रमाण ② ४०० मिलीग्रम प्रति किलो) आणि गट 'एफ'

(उच्च प्रमाण ① ६०० मिलीग्रम प्रति किलो) या प्रमाणात सलग १८ दिवसापर्यंत देण्यात आला. गट 'जी' मधील प्राण्यांना डॉक्सोरेबीसिन या कर्करोगावरील औषधाची मात्रा ② २ मिलीग्रम प्रतिकिलो याप्रमाणे २८ दिवस देण्यात आली. अशा प्रकारे विविध प्रयोगगटामध्ये एकूण ९० दिवसांनंतर 'डिएमबीए' द्वारे कर्करोग उत्पन्न झाल्यानंतर जास्वदांच्या फुलांच्या अर्काची औषधीमात्रा एकूण २८ दिवस देण्यात आली. प्रयोगाच्या ९० व्या दिवशी तसेच ११८ व्या दिवशी असे आढळून आले की गट 'ए' आणि गट 'सी' च्या तुलनेत इतर सर्व गटांमधील प्राण्यांचे सरासरी वजन कमी झाले होते. प्रयोगाच्या शेवटच्या दिवशी (११८ व्या दिवशी) प्रत्येक गटातील १० प्राण्यांच्या विविध रक्तचाचण्या, रक्तजलचाचण्या तसेच विविध अवयवांचा ऊती अभ्यास करण्यात आला. रक्तातील विविध चाचण्या जसे की हिमोग्लोबीन, एकूण लाल पेशींचे प्रमाण आणि हिमॅटोक्रिट यांचे प्रमाण जास्वदांच्या फुलांच्या अर्क दिलेल्या गटामध्ये तुलनात्मकरित्या सुधारल्याचे आढळून आले तसेच उच्च प्रमाण गटामध्ये (उच्च प्रमाण ① ६०० मिलीग्रम प्रति किलो) हे प्रमाण सर्वाधिक प्रमाणात वाढल्याचे आढळले. एकूण पांढऱ्या पेशींच्या संख्येत सर्व उपचार गटांमध्ये कर्करोग उत्पन्न झाल्यामुळे गट 'ए' आणि गट 'सी' च्या तुलनेत लक्षणीय वाढ आढळून आली.

रक्तजलातील विविध जैवरासायनिक चाचण्याद्वारे असे आढळून आले की गट 'बी' च्या तुलनेत जास्वदांच्या फुलांचा अर्क दिलेल्या गटांमध्ये एएलटी, एएलपी, युरीया तसेच क्रियॅटिनिनचे प्रमाण काही प्रमाणात कमी झालेले आहे. प्राण्यांमध्ये उद्भवित केलेल्या कर्करोगामुळे वाढलेल्या फ्री - रॅडिकल मुळे होणारी यकृत आणि मूत्रपिंडाची उतीस्तरावरील हानी जास्वदांच्या फुलांच्या अर्क सेवनाने कमी होण्यास मदत झाल्याचे आढळून आले. यकृत आणि मूत्रपिंडामधील मॅनोल्डीहाईड (एमडीए) चे प्रमाण गट 'बी' च्या तुलनेत इतर सर्व गटांमध्ये लक्षणीयरित्या कमी झाल्याचे आढळून आले. यावरून असे सिद्ध झाले की यकृत आणि मूत्रपिंडाची पेशीस्तरावरील होणारी ऑक्सीडेटिव्ह हानी जास्वदांच्या फुलांच्या अर्क सेवनानंतर कमी होणे शक्य आहे. कर्करोग बाधित प्राण्यांच्या विविध पेशींच्या उतीजन्य अभ्यासातून असे आढळून आले की जास्वदांच्या फुलांचा अर्क (उच्च प्रमाण ① ६०० मिलीग्रम प्रति किलो) दिल्यास यकृत तसेच मूत्रपिंडाची पेशीस्तरावरील होणारी हानी कमी करता येऊ शकते. मात्र केवळ जास्वदांच्या फुलांच्या अर्क सेवनाने कर्करोगाच्या गाठींमधील कर्कपेशींमध्ये कुठलाही लक्षणीय बदल आढळून आला नाही.

सदर प्रयोगांती असा निष्कर्ष काढण्यात आला कि जास्वदांच्या फुलांच्या अर्क सेवनाने मुषक वर्गीय प्राण्यांमध्ये कर्करोग पेशींवर कुठलाही सकारात्मक परिणाम दिसून आला नाही, मात्र कर्करोगामुळे होणारी ऑक्सीडेटिव्ह हानी पेशी स्तरावर काही प्रमाणात कमी करता येणे शक्य आहे.