EVALUATION OF ANTIOXIDANT PROPERTY AND TOXICOLOGICAL ASSESSMENT OF POLYALTHIA LONGIFOLIA VAR. PENDULA LEAF

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CERTIFICATE

This is to certify that the Ph.D. thesis entitled "**Evaluation of antioxidant property and toxicological assessment of *Polyalthia longifolia* var. pendula leaf**" embodies the original results of bonafide experimental work carried out by **Mr. Rajeshkumar Dave** under my guidance and supervision at the Department of Biosciences, Saurashtra University, Rajkot.

It is further certified that he has put nine terms for research work and that this work has not been submitted to any other University/Institution for the award of Ph.D. degree. His thesis is recommended for acceptance for the award of the Ph.D. degree by the Saurashtra University.

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PUBLICATIONS AND PRESENTATIONS

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CHAPTER 1: INTRODUCTION

"An apple a day keeps the doctor away"

Traditional American rhyme

The 21st century has marked the beginning of a new era, receptive to eastern healthcare philosophy through positive attitudes (Cooper, 2008). Plants, animals and the humans have intimate biological relationships since remote past and have evolved along parallel lines cooperating and depending upon each other for existence. In the present scenario, greater emphasis is being laid on the traditional knowledge of ethnic people imparting that the use of knowledge in bioprospecting of biological resources as a new source of drugs, medicine, food and other industrial and pharmaceutical raw materials. (Dixit et al., 2010). An estimated 300 million indigenous people are living all over the world; nearly half of them are living in Asia. About 53 million indigenous people (tribes) belonging to some 573 tribal communities of 227 ethnic groups are living in India, and they comprise nearly 22% of the world's ethnic population. About 6 different races have migrated to India from different parts of world and have taken root here (Anonymous, 1995).

1.1 Ayurved – ancient science of life

Ayurveda is one of the oldest extant health systems in the world with fundamental principles and theory-based practices. In Sanskrit the meaning of *Ayu* is life and *Veda* is knowledge or science. Therefore, Ayurveda is also generally translated as the science of life. Ayurveda remains one of the most ancient and yet living traditional system of medicine practiced widely in Indian, Sri Lanka and other countries and has a sound philosophical and experiential basis (Chopra and Doiphode, 2002). *Atharvaveda* (around 1200 BC), *Charak Samhita and Sushrut Samhita* (1000-500 BC) are the main classics that give detailed descriptions of over 700 herbs (Dahanukar and Thatte, 2000). Indian healthcare consists of medicinal pluralism and Ayurveda still remain dominant compared to modern medicine, particularly for treatment of a variety of chronic diseases (Chopra and Doiphode, 2002).

1.2 Global endorsement of herbal medicine

Plants have been the basis of many traditional medicine systems throughout the world for thousands of years and continue to provide mankind with new remedies. Use of plants as a source of medicine has been inherited and is an important component of the health care system in India. In the Indian systems of medicine, most practitioners formulate and dispense their own recipes; hence this requires proper documentation and research. In western world also, the use of herbal medicines is steadily growing with approximately 40 % of population reporting use of herbs to treat medical illnesses (Bent and Ko, 2004). Public, academic and government interest in traditional medicines is growing exponentially due to the increased incidence of the adverse drug reactions and economic burden of the modern system of medicine (Dubey et al., 2004)

1.3 India - treasures of medicinal pants

There are about 45,000 plant species in India, with concentrated hotspots in the region of Eastern Himalayas, Western Ghats and Andaman & Nicobar Island. The officially documented plants with medicinal potential are 3000 but traditional practitioners use more than 6000. India is the largest producer of medicinal herbs and is appropriately called the botanical garden of the world (Ahmedullah and Nayar, 1999). There are currently about 250 000 registered medical practitioners of the Ayurvedic system (total for all traditional systems: approximately 291 000), as compared to about 700,000 of the modern medicine system. In rural India, 70 % of the population is dependent on the traditional system of medicine, the Ayurveda (Bent and Ko, 2004). Three of the ten most widely selling herbal medicines in the developed countries, namely preparations of *Allium sativum*, *Aloe barbedensis* and *Panax* spp. are available in India. There are about 7000 firms manufacturing traditional medicines with or without standardization (Dubey et al., 2004).

1.4 Drug discovery from medicinal plants

Plants form a dominant part of Ayurvedic pharmacopoeia where drugs have been classified on the basis of their physiological action (Bhakuni, 1997). Herbal drug is

estimated that approximately one quarter of prescribed drugs contain plant extracts or active ingredients obtained from plant substances. Aspirin, atropine, artimesinin, colchicine, digoxin, ephedrine, morphine, physostigmine, pilocarpine, quinine, quinidine, reserpine, taxol, tubocurarine, vincristine and vinblastine are a few important examples of what medicinal plants have given us in the past (Sekar et al., 2010). Of many strategies for selection of plants as drug source, the most rewarding has been the criteria of their use in folklore medicine. For example, *Rauwellia serpentina* (L.) Benth ex. Kurz provided the hypotensive alkaloids reserpine, reseinnamine and deserpedine; *Digitalis purpurea* L. and *Digitalis lantana* Ehrh. provided digitoxin and digoxin both powerful diatonic agents; *Papaver somniferum* L. provides opium alkaloids, the analgesics codeine and morphine as well as the antitussive noscapine and smooth muscle relaxant, papaverine; *Atropha belladonna* provided the parasympatholytic atropine, scopolamine and 1-hyos-cyamine, Taxol (bark of *Taxus brevifolia* Nutt.) is the most recent anticancer drug discovered from plant source (Bhakuni, 1997).

1.5 Global challenges

The current lifestyle of humans almost everywhere in the world is in sharp contrast than earlier time, and as a consequence, humans suffer from a large number of chronic diseases. In the past, infectious diseases killed our ancestors early, often younger than age 40, so they did not display the current epidemic of chronic diseases that arise in older age. Now medical status has been improved. Nowadays, people live longer and therefore express symptoms of chronic diseases associated with senescence and lifestyle such as obesity, diabetes, hypertension, coronary heart disease, and cancer (Orzechowski et al., 2002).

1.5.1 Inflammation

Chronic inflammation, induced by biological, chemical, and physical factors, is associated with increased risk of human cancer at various sites. Chronic inflammatory processes induce oxidative/nitrosative stress and lipid peroxidation (LPO), thereby generating excess reactive oxygen species (ROS), reactive nitrogen species (RNS), and DNA-reactive aldehydes (Bartsch and Nair, 2006).

1.5.2 Atherosclerosis

Atherosclerosis is a chronic vascular disease in which inflammation and oxidative stress has important role at every stage. The disease process develops and progresses in response to abnormal cholesterol deposits in the intima of large arteries (Dogne et al., 2005).

1.5.3 Cancer

Most tumors form discrete masses but in the leukemias, the tumor cells are spread through the bone marrow or lymphoid tissues and circulate in the blood. DNA damage plays a very important role in carcinogenesis and any agent, which is capable of chemically modifying DNA could be carcinogenic. Hydroxyl radical attack upon DNA generates a whole series of modified purine and pyrimidine bases many of which are known to be mutagenic (Cerutti, 1994).

1.5.4 Diabetes

It has been postulated that the etiology of the complications of diabetes involves oxidative stress perhaps as a result of hypoglycemia (Hunt et al., 1990). Glucose itself and hyperglycemia-related increased protein glycosylation are important sources of free radicals (Wolff and Dean, 1987). Elevated glucose causes slow but significant non-enzymatic glycosylation of proteins in diabetes (Brownlee et al., 1984).

1.6 Pharmacognosy

Pharmacognosy basically deals with the standardization, authentication and study of natural drugs. It is closely involved with allied fields, *viz.* phytochemistry and toxicological screening of natural products. Much of the research in pharmacognosy has been done in identifying controversial species of plants, authentication of commonly used traditional medicinal plants through morphological, histological, physico-chemical and toxicological parameters, especially heavy-metal estimation and radiobiological contamination in plants, prescribed by an authoritative source.

The importance of pharmacognosy has been widely felt in recent times (Dinesh Kumar, 2007).

The herbal drug industry is considered to be a high growth industry of the late 90s and seeing the growing demand, it is all set to grow in the next century. The trend for the increasing popularity of medicinal herbs in countries like America, Australia and Germany is well supported by statistical data. Ayurveda strongly believes in polyherbal formulations and scientists of modern era often ask for scientific validation of herbal remedies. The efficacy of some herbal products is beyond doubt, the most recent examples being *Taxus brevifolia* Nutt. (Taxols) and *Silybium marianum* (L.) Garetn. (Silymarin). *Hypericum perforatum* (hypericin & hyperforin), *Allium sativum* L. (allicin or allin), *Ginkgo biloba* L. (Ginkgolic acid) are popularly used herbal remedies among people. All these herbals are standardized for active constituent. Standardization means adjusting the herbal drug preparation to a defined content of the active constituent. Extract refers to a concentrated preparation of active constituent of a medicinal herb. The concept of standardized extracts definitely provides a solid platform for scientific validation of herbals (Soni et al., 2008).

Some drugs of plant origin in conventional medical practice are not pure compounds but direct extracts or plant materials that have been suitably prepared and standardized (Donald, 1986). The World Health Organization has recommended the use of arthemisinin derivatives from *Artemisia annua* (Composite), a Chinese herb with established pharmacognostic data, as a first line drug in the treatment of malaria (WHO, 2002).

Most of the cases of accidental herbal medicine misuse start with wrong identification of a medicinal plant prescribed. Many of the traditional systems have records where one common vernacular name is supplied in place of two or more entirely different species. Ginseng, which is a common Indian drug, is sold under 13 different names in the market. For example Chinese or Asiatic ginseng (*Panax ginseng*), American ginseng (*Panax quinquefolius*), Siberian ginseng (*Eleutherococcus senticosus*), Ayurvedic ginseng (*Withania somnifera* Dunal.) and Russian ginseng (*Acanthopanax senticosus*) (Helena et al., 2002). Such names could create confusion over prescription, which may eventually lead to serious consequences. With this backdrop,

it becomes extremely important to make an effort towards standardization of the plant material to be used as medicine. The process of standardization can be achieved by stepwise pharmacognostic studies (Dinesh Kumar, 2007).

1.7 The need of the hour

A majority of the present day diseases are reported to be due to the shift in the balance of the pro-oxidant and the antioxidant homeostatic phenomenon in the body. Pro-oxidant conditions dominate either due to the increased generation of the free radicals caused by excessive oxidative stress of the present day life, or due to the poor scavenging/quenching in the body caused by depletion of the dietary antioxidants (Halliwell, 1994). In other words, the root cause of all diseases (acute or chronic) is generation of free radicals. Therefore, the dire need of the hour is to discover or identify medicinal plants, rich in antioxidants. Medicinal plants can be economic, natural and easily affordable by all the people.

1.8 Oxidative stress

Reactive oxygen species (ROS) is a term which encompasses all highly reactive, oxygen-containing molecules, including free radicals (Bayr, 2005). Free radicals are electrically charged molecules, i.e., they have an unpaired electron, which causes them to seek out and capture electrons from other substances in order to neutralize themselves. Although the initial attack causes the free radical to become neutralized, another free radical is formed in the process, causing a chain reaction to occur. And until subsequent free radicals are deactivated, thousands of free radical reactions can occur within seconds of the initial reaction (Kaliora et al., 2006). ROS are formed during oxidative processes that normally occur at relatively low levels in all cells and tissues. If ROS are not scavenged; these species may lead to widespread lipid, protein and DNA damage (Jaswal et al., 2003; Mahajan and Tandon, 2004).

Exposure of cellular macromolecules to ROS and reactive nitrogen species (RNS) is strongly controlled. Circulating human erythrocytes possess the ability to scavenge superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD), catalase (CAT) and glutathione reduced (GSH) and glutathione peroxidase

(GPx) dependent mechanisms. It is considered that oxidative damage is attributable to excess active oxygen species generated in the body. Reactive oxygen is also generated by factors such as smoking, strong ultraviolet and radial ray irradiation, and by stress (Osawa et al., 1995). Many studies on stress have been reported (Selye, 1976; Zafir et al., 2009). There seems to be many kinds of 'stressor' which can act as factors that disturb homeostasis at the cell or organ level. Stress may induce or aggravate various types of diseases. The homeostatic regulation of cell numbers in normal tissues reflects a highly regulated balance between cell proliferation and cell death (McDonnell, 1993).

The term "antioxidant" refers to any molecule capable of stabilizing or deactivating free radicals before they attack cells. There are also molecules deserving the "antioxidant" term, because they act as chelating agents binding metal ions (redox activity). Antioxidants are absolutely critical for maintaining optimal cellular and systemic health and well-being (Kaliora et al., 2006). To protect the cells and organ systems of the body against reactive oxygen species, humans have evolved a highly sophisticated and complex antioxidant protection system. It involves a variety of components, both endogenous and exogenous in origin, that function interactively and synergistically to neutralize free radicals. These components include, 1) Nutrient-derived antioxidants like ascorbic acid (vitamin C), tocopherols and tocotrienols (vitamin E), carotenoids, and other low molecular weight compounds such as glutathione and lipoic acid. 2) Antioxidant enzymes, e.g., superoxide dismutase, glutathione peroxidase, and glutathione reductase, which catalyze free radical quenching reactions. 3) Metal binding proteins, such as ferritin, lactoferrin, albumin, and ceruloplasmin that sequester free iron and copper ions that are capable of catalyzing oxidative reactions. 4) Numerous other antioxidant phytonutrients present in a wide variety of plant foods. In nature there are a wide variety of naturally occurring antioxidants which are different in their composition, physical and chemical properties, mechanisms and site of action (Naik, 2003).

1.9 Essential water-soluble antioxidants

1.9.1 Vitamin C

Ascorbic acid (vitamin C) is the major essential water-soluble antioxidant in human serum (Bendich et al., 1986). Vitamin C in humans must be ingested for survival. Vitamin C is an electron donor, and this property accounts for all its known functions. It is present in relatively high concentrations extracellularly in the blood plasma Vitamin C can function as an antioxidant and scavenge the $O_2^{\bullet-}$, $^1O_2$, $^{\bullet}OH$, neutralize hypochlorous acid (HOCl), and prevent lipid peroxidation (Kelly and Tetley, 1997; Anderson, 2001), but cannot scavenge or neutralize H_2O_2 (McCall et al., 1974), rather it may potentiate its toxicity by inhibiting catalase activity (Orr, 1967). Vitamin C can protect DNA from oxidant-mediated damage (Anderson, 1981) and has been reported to neutralize phagocyte-derived oxidants protecting the α1-protease inhibitor (API) from oxidant-mediated functional inactivation (Halliwell et al., 1987; Pryor and Stone, 1993).

1.10 Non-essential water-soluble antioxidants

1.10.1 Glutathione

Glutathione (GSH) in its reduced form is a good scavenger of many free radicals like $O_2^{\bullet-}$, $^{\bullet}OH$ and various lipid hydroperoxides and may help to detoxify many inhaled oxidizing air pollutants like ozone, NO_2 and free radicals in cigarette smoke in respiratory tract (Gupta and Sharma, 2006). GSH is an important water-soluble antioxidant present ubiquitously in cells. Its main function is to detoxify xenobiotic toxins by conjugation giving rise to oxidized glutathione disulfide (GSSG) (Chow and Tappel, 1972). It can also provide reducing equivalents to regenerate reduced vitamin E or vitamin C yielding a thionyl radical ($GS^{\bullet}$) in the process (Kagan et al., 1992).

1.10.2 Lipoic acid

α-Lipoic acid (8-thioctic acid) is another thiol with antioxidant properties (Scott et al., 1994; Sen and Packer, 2000). It has been reported to participate along with vitamin C,

glutathione, vitamin E, and β-carotene in what is defined by Packer (1999) as a biological "redox antioxidant network." Lipoic acid is involved in recycling oxidized vitamin C and/or vitamin E and yields either dehydrolipoic acid (the oxidized form) or another radical. α-Lipoic acid and GSH can scavenge cigarette smoke-contained reactive oxygen species and preferentially react with aldehydes, thus protecting proteins from oxidation (Cross et al., 1993).

1.11 Essential lipid-soluble antioxidants

1.11.1 Vitamin E

Vitamin E (α-tocopherol) is the major intracellular lipophilic, chain breaker, and efficient antioxidant capable of trapping peroxyl radicals intermediates in lipid peroxidation and is responsible for protecting PUFA (Poly unsaturated fatty acid) present in cell membrane and low density lipoprotein (Gupta and Sharma, 2006) and quenching free radicals and reactive oxygen species. It is also essential for structural membrane stability (Patel et al., 1991).

1.12 Non-essential lipid-soluble antioxidants

1.12.1 β-carotene

Carotenoids such as β -carotene are suggested to have antioxidant properties capable of quenching free radicals such as singlet oxygen ($^1O_2$) (Stratton et al., 1993). It was also suggested that a cooperative interaction exists between fat-soluble antioxidants; the relation between β-carotene and vitamin E was reported to be synergistic (Niki et al., 1995).

1.13 Secondary metabolites as antioxidants

1.13.1 Phenolic compounds

Medicinal plant parts (roots, leaves, branches/stems, barks, flowers, and fruits) are commonly rich in phenolic compounds, such as flavonoids, phenolic acids, stilbenes,

tannins, coumarins, lignans and lignins (Cai et al., 2004). Phenolic compounds are ubiquitous bioactive compounds and a diverse group of secondary metabolites. Accordingly, bioactive polyphenols have attracted special attention because they can protect the human body from the oxidative stress which may cause many diseases, including cancer, cardiovascular problems, and aging (Robards et al., 1999). The antioxidant properties of phenolic acids and flavonoids are due to their redox properties, ability to chelate metals and quenching of singlet oxygen (Rice-Evans et al., 1996).

1.13.2 Flavonoids

Flavonoids, which are partly responsible for the pigmentation of flowers, fruits and leaves, are subdivided into flavanols, flavonols, flavones, flavanones and anthocyanins based on the saturation of the flavan ring and also their hydroxylation. They occur mostly as glycosylated derivatives, sometimes conjugated with sulphate or organic acids (Youdim et al., 2002).

1.14 Toxicology

Medicinal plants from time immemorial have been used in virtually all cultures as a source of medicine (Cragg and Newman, 2001). They are considered to be the backbone of traditional medicine and are widely used to treat acute and chronic diseases. The World Health Organization estimated that perhaps eighty percent of the inhabitants of the world rely chiefly on traditional medicines. It, therefore, approved the use of herbal products for national policies and drug regulatory measures in order to strengthen research and evaluation of the safety and efficacy of herbal products. The report has suggested that of the 119 plant derived drug listed by WHO study, 74% were discovered as a result of chemical studies to isolate the active compounds responsible for the use of original plant in traditional medicine (Farnsworth, 1984). The use of plants for healing purpose is getting increasingly popular as they are believed to be beneficial and free of side effects (Leonardo et al., 2000). However, the rationale for the utilization of medicinal plants has rested largely on long-term clinical experience with little or no scientific data on their efficacy and safety (Zhu et al., 2002). Medicinal herbs have their use as medicament based simply on a traditional

folk use that has been perpetuated along several generations. With the upsurge in the use of herbal medicines a thorough scientific investigation of these plants is imperative, based on the need to validate their folkloric usage (Sofowora, 1989).

Toxicological studies help to decide whether a new drug should be adopted for clinical use or not (Anisuzzaman et al., 2001). Depending on the duration of exposure of animals to drug, toxicological studies may be of three types viz. acute, sub-acute and chronic (Baki et al., 2007). Toxicity depends not only on the dose of the substance but also on the toxic properties of the substance. The relationship between these two factors is important in the assessment of therapeutic dosage in pharmacology and herbalism (Hayes, 2001).

1.15 Acute toxicity

Acute toxicity is defined as the toxic effects produced by single exposure of drugs by any route for a short period of time (UWCSDG, 1999). Acute toxicity studies in animals are considered necessary for any pharmaceutical intended for human use. The main objective of acute toxicity studies is to identify a single dose causing major adverse effects or life threatening toxicity, which often involves an estimation of the minimum dose causing lethality. The studies are usually carried out in rodents, and consist of a single dose. In pharmaceutical drug development this is the only study type where lethality or life-threatening toxicity is an endpoint as documented in current regulatory guidelines (Commission Directive, 2003; CDER, 1996). To evaluate toxicity of a compound in animals various routes may be used, but two most commonly used modes of administration for animals studies are via intraperitoneal injection or the oral route (Poole and Leslie, 1989).

Usually acute (single dose) toxicity study is carried out on laboratory animals by using high dose (sufficient to produce death or morbidity) of the substance in question and/or based on previous report on its toxicity or toxicity of structurally related compounds (Demma et al., 2007). Acute toxicity studies are commonly used to determine LD_{50} of drug or chemicals (Baki et al., 2007). The acute study provides a guideline for selecting doses for the sub-acute and chronic low dose study, which may be clinically more relevant (Janbaz et al., 2002; Hasumura et al., 2004).

1.16 Sub-acute toxicity

In sub-acute toxicity studies, repeated doses of drug are given in sub-lethal quantity for a period of 14 to 21 days. Sub-acute toxicity studies are used to determine effect of drug on biochemical and hematological parameters of blood as well as to determine histopathological changes (Baki et al., 2007).

1.17 Chronic toxicity

In chronic toxicity studies, drug is given in different doses for a period of 90 days to over a year to determine carcinogenic and mutagenic potential of drug (Baki et al., 2007). The parameters of chronic toxicity studies are same as that of sub-acute study. Multiple dose studies are necessary to assure the safety of natural products. On the other hand clinical observations of acute assays are valuable tools to define the doses to be tested in multiple dose experiments, along with pharmacological studies in animals and in humans (Alvarez et al., 2004; Hasumura et al., 2004).

1.18 Hematological importance

Hematological status is one of the important ways for the diagnosis of root cause of disease. Hematological disorders include a wide range of abnormal conditions indicating the profile of blood parameters, due to changes in metabolism. Alterations in blood parameters may be due to changes in cellular integrity, membrane permeability of cells or even due to exposure to toxic chemicals (Hoffbrand and Pettit, 1997). Reports regarding toxicological studies of plants on hematological aspects are scanty. However, some reports are available *viz.* Bafor and Igbinuwen (2009); Mbaka et al. (2010).

1.19 Serum biochemical importance

The serum biochemical tests are frequently used in diagnosis diseases of hearts, liver, kidney and cardiovascular system etc. they are also widely used in monitoring the response to exogenous toxic exposure (Wang et al., 2006). When an herbal product is ingested, the body interacts with it in an attempt to get rid of any harmful toxins,

especially if the body cannot convert the foreign substance into cellular components. These insults are commonly manifested by changes in enzyme levels and other cell components. The enzymes commonly involved are glutamate oxaloacetate transaminase (GOT) glutamate pyruvate transaminase (GPT), alkaline phosphatase (ALP). Also component like urea and uric acid are vital diagnostic tools for toxicity (Dapar et al., 2007). Generally, liver cell damage is characterized by a rise in serum enzymes like GOT, GPT, ALP, etc. (Brautbar and Williams, 2002). In general, GOT concentrations are consistently higher than GPT levels which are expected since body cells contain more GOT than GPT. Usually, about 80 % of GOT is found in the mitochondria whereas GPT is purely cytosolic enzyme. Therefore, GOT appears in higher concentrations in a number of tissues (Liver, Kidney, heart and pancreas) and is released slowly in comparison to GPT. But since GPT is localized primarily in the cytosol of hepatocytes, this enzyme is considered a more sensitive marker of hepatocellular damage than GOT and within limits can provide a quantitative assessment of the degree of damage sustained by the liver (Al Mammary et al., 2002). The urea and creatinine are good indicatiors for renal function. If kidney function falls, the urea and creatinine levels will rise (Wang et al., 2006).

In preclinical safety studies of new compounds, organ weight changes are often difficult to interpret in relation to primary compound effects when reductions in food consumption are also present. By gaining a better understanding of tissue changes caused solely by feed restriction, it may be possible to differentiate direct compound effects from those of inadequate nutrition. Various studies have yielded information about the effects of inadequate nutrition on body weights, organ weights, histologic tissue changes, and clinical pathology data in rats (Oishi et al., 1979; Levin et al., 1993). On a body weight basis, it is assumed for toxicity data extrapolation that humans are usually about 10 times more sensitive than rodents. On a body surface–area basis, humans usually show about the same sensitivity as test mammals, i.e. the same dose per unit of body surface area will give the same given defined effect, in about the same percentage of the population. Knowing the above relationships, it is possible to estimate the exposure to a chemical that humans should be able to tolerate (Duffus and Worth, 2006). Boby weight and internal organ such as liver, kidney, heart spleen, thymus glands, etc. are simple and sensitive indices of toxicity after exposure

to toxic substance (Teo et al., 2002). Toxicity data are required to predict the safety associated before the use of medical products (McNamara, 1976).

1.20 Histopathological analysis

1.20.1 Liver

The liver has a broad range of functions, such as detoxification, protein synthesis, and production of chemicals which are necessary for digestion Liver is a target organ and primary site of detoxification. Liver is the major site of metabolism and is therefore prone to various disorders as a consequence of exposure to the toxins of extrinsic as well as intrinsic forms. Liver plays an important role in metabolism to maintain energy level and structural stability of body (Paliwal et al., 2009). It is also a site for biotransformation by which a toxic compound gets transformed to less harmful form and reduces toxicity. However, toxic compound damages the liver cells and produce hepatotoxicity.

The liver is surrounded by a thin connective tissue layer, Glisson's capsule which becomes thicker around the inferior cava vein and in hepatic hilum. The duct, vein, and artery divide into left and right lobes. The right lobe is further divided into an anterior and posterior segment by the right hepatic vein. The left lobe is divided into the medial and lateral segments by the left hepatic vein. The connective tissue divides the hepatic parenchyma in lobules and receives the name of periportal connective tissue, since it surrounds the portal triads. Within the lobule, a rigid network of reticular fibers is observed that in the periphery are continued by the interlobular periportal connective tissue (Bustos-Obregon et al., 2008).

1.20.2 Heart

The heart is centrally located in the thoracic cavity. It contains four chambers. The two dark colored chambers at the top are the atria, and the bottom chambers are the ventricles. The heart is covered by a thin membrane called the pericardium. Valves guard the exits of the chambers, preventing backflow of blood. The wall of heart includes the cardiac muscle, a fibrous skeleton for attachment of the valves, and a

specialized internal conducting system involved in the regulation of heart rate. The fibrous skeleton, comprised of dense connective tissue, is situated around the openings of the two arteries leaving the heart and around the openings between the atria and the ventricles. The wall of the heart contains three layers: the epicardium, myocardium and the endocardium. The layers are essentially the same in the atria and the ventricles. The epicardium consists of a lining of mesothelial cells and an underlying layer of connective tissue. Blood vessels and nerves supplying the heart lie within the epicardium, often surrounded by adipose tissue. The myocardium consists of cardiac muscle. The myocardium can be divided into three layers: an inner layer of endothelium and subendothelial connective tissue, a middle layer comprised of connective tissue and smooth muscle cells, and an outer layer, also called the subendocardial layer, which is continuous with the connective tissue of the myocardium. The interventricular septum serves as the wall between the right and left ventricles. The interatrial septum is much thinner than the interventricular septum. Fibrous thread like cords, called the chordae tendinae, extend from the free edge of the atrioventricular valves to muscular projections from the wall of the ventricles called papillary muscles.

1.20.3 Kidney

The kidney is divided into two regions, an outer cortex and an inner medulla. The nephron (the functional unit of the kidney) is organized so that Bowman's capsule and the proximal and distal tubules are located in the cortex and the loop of Henle and the collecting tubules are located primarily in the medulla. In the cortical region Bowman's capsules are relatively abundant and appear as spherical structures with a coiled mass of capillaries, the glomerulus in the center. Surrounding the Bowman's capsule are the elements of proximal and distal convoluted tubules cut in various plains of section. The cells making up the walls of the tubules are cuboid in shape and contain a prominent nucleus. In the medulla there are elements from the loop of Henle and the collecting tubules which cut mostly in longitudinal section.

1.20.4 Spleen

The function of spleen is important in regard to red blood cells and the immune system (Losco, 1992). The spleen is a dark red to blue-black organ located in the left cranial abdomen. It is adjacent to the greater curvature of the stomach and within the omentum. It is an elongated organ, roughly triangular in cross section. The spleen plays a major role in mounting immune responses to antigens in the blood stream. The spleen specializes in filtering blood and trapping blood-borne antigens. The spleen is the site of direct and indirect toxicity and a target for some carcinogens and also a site for metastasis of malignant neoplasms arising in other sites (Goldsby et al., 2002).

The compartments of spleen are of two types, the red pulp and white pulp, which are separated by a diffuse marginal zone. The red pulp consists of a network of sinusoids populated by macrophages and many red blood cells and few lymphocytes; it is the site where old and defective red blood cells are destroyed and removed. Many of the macrophages within the red pulp contain engulfed red blood cells or iron pigments from degraded hemoglobin. The white pulp surrounds the branches of the splenic artery, forming a periarteriolar lymphoid sheath (PALS) populated mainly by T lymphocytes. The marginal zone, located peripheral to the PALS, is populated by lymphocytes and macrophages (Balogh et al., 2004).

1.20.5 Thymus gland

The thymus is the site of T-cell development and maturation. It is a flat, bilobed organ. Each lobe is surrounded by a capsule and is divided into lobules, which are separated from each other by strands of connective tissue called trabeculae. Each lobule is organized into two compartments: the outer compartment, or cortex, is densely packed with immature T cells, called thymocytes, whereas the inner compartment, or medulla, is sparsely populated with thymocytes. The function of the thymus is to generate and select a repertoire of T cells that will protect the body from infection (Goldsby et al., 2002).

1.21 Selection of the plant for the present study

When selecting a plant for pharmacological activities, four basics methods are usually followed (Suffness and Douros, 1979):

a) Random choice of plant species
b) Choice based on ethnomedical use
c) Follow up of existing literature on the use of the species
d) Chemotaxonomic approaches

Comparison of the four methods showed that the choice based on folklore has given about 25% more positive leads than other methods. Based on the second and third approach *Polyalthia longifolia* (Sonn.) Thw. var. pendula (Annonaceae) was selected in our laboratory and was evaluated for antimicrobial, anti-inflammatory, analgesic, antidiabetic and hepatoprotective properties and it showed very promising results (Nair, 2005; Nair and Chanda, 2006; Nair et al. 2007; Tanna et al., 2009; Chanda and Nair, 2010). In continuation of this work, in the present study, *Polyalthia longifolia* (Sonn.) Thw. var. pendula was selected for its antioxidant and toxicological assessment.

Polyalthia longifolia (Sonn.) Thw. var. pendula is a tall handsome evergreen tree which is cultivated all over India. The plant has been used in traditional system of medicine for the treatment of fever, skin diseases, hypertension (Kirtikar and Basu, 1995). The leaf extract showed cytotoxic effect on wide range of human cancer cell line with apoptotic activity in human leukemia HL-60 cells (Verma et al., 2008). The plant possesses compounds like clerodane diterpenoids (Faizi et al., 2008), lactone (Faizi et al., 2003), kolavenic acid, liriodenine, bisclerodane imide (olefinic isomer), clerodane diterpene (olefinic isomer and lysicamine); reduces blood pressure (Saleem et al., 2005). Recently, three new clerodane-type diterpene and four new protoberberine alkaloids were isolated from methanol extract of the stems of *Polyalthia longifolia* var. pendula (Lee et al., 2009).

Considering the above, the objectives set forth are:

- Review of Literature
    - Review of literature for *in vitro* antioxidants activity
    - Review of literature for *in vivo* antioxidants activity
    - Review of literature for toxicity study

- Pharmacognostic investigation of *Polyalthia longifolia* var. pendula leaf
- Physicochemical investigation of *Polyalthia longifolia* var. pendula leaf
- Phytochemical investigation of *Polyalthia longifolia* var. pendula leaf
- To evaluate i*n vitro* antioxidants activity of *Polyalthia longifolia* var. pendula leaf
- To evaluate i*n vivo* antioxidants activity of *Polyalthia longifolia* var. pendula leaf
- To evaluate acute toxicity study of *Polyalthia longifolia* var. pendula leaf
- To evaluate sub-acute toxicity study of *Polyalthia longifolia* var. pendula leaf

CHAPTER 2: REVIEW OF LITERATURE

2.1 Review of literature for *in vitro* antioxidant activity

2.1.1 What is oxidative stress?

Oxidative stress depicts the existence of products called free radicals and reactive oxygen species (ROS), which are formed under normal physiological conditions but become deleterious when not being eliminated by the endogenous systems. In fact, oxidative stress results from an imbalance between the generation of reactive oxygen species and endogenous antioxidant systems. ROS are major sources of primary catalysts that initiate oxidation *in vivo* and *in vitro* and create oxidative stress which results in numerous diseases and disorders (Rackova et al., 2007) such as cancer (Kinnula and Crapo, 2004), cardiovascular disease (Singh and Jialal, 2006), neural disorders (Sas et al., 2007), Alzheimer's disease (Smith et al., 2000) mild congnitive impairment (Guidi et al., 2006), Parkinsons disease (Bolton et al., 2000), alcohol induced liver disease (Arteel, 2003), ulcerative colitis (Ramakrishna et al., 1997), ageing (Hyun et al., 2006), atherosclerosis (Upston et al., 2003). Oxygen derived free radicals such as superoxide anions, hydroxyl radicals and hydrogen peroxide are cytotoxic and give rise to tissue injuries (Jainu and Shyamala Devi, 2005). Excessive amount of ROS is harmful because they initiate bimolecular oxidation which leads to cell death and creates oxidative stress. In addition, oxidative stress causes inadvertent enzyme activation and oxidative damage to cellular system (Wiseman and Halliwell, 1996)

2.1.2 What are ROS?

Free radical is a chemical compound which contains an unpaired electron spinning on the peripheral layer around the nucleus. The family of free radicals generated from the oxygen is called ROS which cause damage to other molecules by extracting electrons from them in order to attain stability. ROS are ions, atoms or molecules that have the ability to oxidize reduced molecules. ROS are various forms of activated oxygen, which include free radicals such as superoxide anion radicals ($^{\cdot}O_2^-$) and hydroxyl radicals ($OH^{\cdot}$), as well as non-free radicals (H_2O_2) and singlet oxygen (Halliwell, 1995). In the body, free radicals are derived from two sources: endogenous sources, e.g. nutrient metabolism, ageing process, etc and exogenous sources e.g.

tobacco smoke, ionizing radiation, air pollution, organic solvents, pesticides, etc (Buyukokuroglu et al., 2001).

2.1.3 How do ROS damage cells?

When oxygen traps single electron, it becomes unstable and thus very reactive, since it generates harmful chain reactions against many biological molecules. The extreme toxicity of oxygen is related to its high capability of generating free radicals and in turn destroying many major biological molecules. They can attack on lipids and proteins and destroy membranes. ROS can damage DNA and lead to mutation and chromosomal damage. Oxidized cellular thiols abstract hydrogen atoms from unsaturated fatty acids to initiate the peroxidation of membrane lipids (Valko et al., 2006). ROS can attack various substrates in body and contribute to development of chronic diseases. For example, oxidatively modified LDL has been hypothesized to be a causative agent in the development of cardiovascular diseases (Touyz, 2004).

Exogenous chemicals and endogenous metabolic processes in human body produce free radicals, especially oxygen derived radicals, which are capable of oxidizing biomolecules, resulting in cell death. Superoxide anion radicals increase under stress conditions such as heavy exercise, certain drugs, infection and various disease states. During normal metabolic processes, human body generates more than 2 Kg of $\cdot O_2^-$ per year (Evans and Halliwell, 1999).

2.1.4 How are cells protected from ROS damage?

Cells are equipped with different kinds of mechanisms to fight against ROS and to maintain the redox homeostasis of cell. For example, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) play important roles in scavenging the free radicals and preventing cell injury (Bergendi et al., 1999). Molecules such as vitamin C and E inhibit lipid peroxidation in cell. When the mechanism of antioxidant protection becomes unbalanced in human body, antioxidant supplement may be used to help reduce oxidative damage.

2.1.5 Natural sources of antioxidants

Medicinal plants are an important source of antioxidants (Rice-Evans, 2004). Natural antioxidants increase the antioxidant capacity of the plasma and reduce the risk of certain diseases such as cancer, heart diseases and stroke (Prior and Cao, 2000). The secondary metabolites like phenolics and flavonoids from plants have been reported to be potent free radical scavengers. They are found in all parts of plants such as leaves, fruits, seeds, roots and bark (Mathew and Abraham, 2006). There are many synthetic antioxidants in use. It is reported, however, they have several side effects (Ito et al., 1983), such as risk of liver damage and carcinogenesis in laboratory animals (Gao et al., 1999; Williams et al., 1999). There is therefore a need for more effective, less toxic, and cost effective antioxidants. Medicinal plants appear to have these desired comparative advantages, hence the growing interest in natural antioxidants from plants.

2.1.6 Evaluation of antioxidant activity

A great number of *in vitro* methods have been developed to measure the efficiency of natural antioxidants either as pure compounds or as plant extracts. *In vitro* methods can be divided in to two major groups. 1) Hydrogen atom transfer reactions like Oxygen Radical Absorbance Capacity (ORAC), Total radical trapping antioxidant potential (TRAP) and β carotene bleaching. 2) Electron transfer reactions like trolox equivalent antioxidant capacity (TEAC), Ferric reducing antioxidant power (FRAP), α, α-diphenyl-β-picryl-hydrazyl radical scavenging assay (DPPH), Superoxide anion radical scavenging assay, Hydroxyl radical scavenging assay, Nitric oxide radical scavenging assay and Total phenol assay (Huang et al., 2005a). These methods are popular due to their high speed and sensitivity. However, it is essential to use more than one method to evaluate antioxidant capacity of plant materials because of the complex nature of phytochemicals (Salazar et al., 2008). The most commonly and uncommonly used antioxidant assays are described below:

2.1.7 Total phenolic content (TPC)

Plant polyphenols possess an ideal structural chemistry for free radical scavenging activity. Antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors from the ability of the polyphenol derived radical to stabilize and delocalize the unpaired electron (chain-breaking function), and from their potential to chelate metal ions (termination of the Fenton reaction) (Rice-Evans et al., 1997). The amount of total phenol content can be determined by Folin-Ciocateu reagent method (McDonald et al., 2001). Gallic acid, tannic acid, quercetin, chlorogenic acid, pyrocatechol and guaiacol can be used as positive controls (Chanda and Dave, 2009). The total phenolic content is expressed in terms of standard equivalent (mg g^{-1} of extracted compound).

2.1.8 Total flavonoid (TF)

The antioxidative properties of flavonoids are due to several different mechanisms, such as scavenging of free radicals, chelation of metal ions, such as iron and copper, and inhibition of enzymes responsible for free-radical generation (Benavente-Garcia et al., 1997). Depending on their structure, flavonoids are able to scavenge practically all known ROS. The amount of total flavonoid content can be determined by Aluminum chloride method (Chang et al., 2002). Quercetin and catechin can be used as positive controls (Chanda and Dave, 2009). The flavonoid content is expressed in terms of standard equivalent (mg g^{-1} of extracted compound).

2.1.9 Reducing power (RP)

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity (Oktay et al., 2003). Compounds with reducing power indicate that they are electron donors, and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Yen and Chen, 1995). The reducing power can be determined by the method of Athukorala et al., (2006). Ascorbic acid, butylated hydroxyanisole (BHA), α-tocopherol, trolox and butylated hydroxytoluene (BHT) can be used as positive controls (Chanda and Dave, 2009).

2.1.10 Free radical scavenging assays

2. 1.10.1 α, α-Diphenyl- β-picryl-hydrazyl radical scavenging (DPPH) assay

The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant compounds. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen–donating antioxidant due to the formation of the nonradical form DPPH-H (Blois, 1958). This transformation results in a color change from purple to yellow, which is measured spectrophotometrically. The disappearance of the purple color is monitored at 517 nm. The free radical scavenging activity can be measured by using 2, 2-diphenyl-1-picryl-hydrazyl or 1, 1-diphenyl-2-picryl-hydrazyl by the method of McCune and Johns (2002). Ascorbic acid, gallic acid, BHA, α-tocopherol, quercetin, BHT, rutin, catechin and glutathione can be used as positive controls (Chanda and Dave, 2009).

2. 1.10.2 Superoxide anion radical scavenging (SO) assay

Although superoxide anion is a weak oxidant, it gives rise to generation of powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress (Meyer and Isaksen, 1995). Numerous biological reactions generate superoxide anions which are highly toxic species. In the PMS/NADH-NBT system, the superoxide anion derived from dissolved oxygen from PMS/NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. The superoxide anion scavenging activity is measured as described by Robak and Gryglewski (1988). Gallic acid, BHA, ascorbic acid, α-tocopherol, curcumin, quercetin or trolox can be used as controls.

2. 1.10.3 Xanthine oxidase method

To determine superoxide anion-scavenging activity, two different assays were used: the enzymatic method with cytochrome C (McCord and Fridovich, 1969) and nonenzymatic method with nitroblue tetrazolium (NBT) (Zhang and Lu, 1990). BHT and catechin can be used as positive controls (Chanda and Dave, 2009).

2. 1.10.4 Hydrogen peroxide radical scavenging (H_2O_2) assay

Hydrogen peroxide occurs naturally at low concentration levels in the air, water, human body, plants, microorganisms, food and beverages. It is widely used as a bleaching agent in the textile, paper, and pulp industries. Human beings exposed to H_2O_2 indirectly via the environment are estimated as 0.28 mg/kg/day with intake from leaf crops contributing most to this exposure. Hydrogen peroxide enters the human body through inhalation of vapor or mist and through eye or skin contact. In the body, H_2O_2 is rapidly decomposed into oxygen and water, and this may produce hydroxyl radicals ($OH^{\cdot}$) that can initiate lipid peroxidation and cause DNA damage.The ability of plant extracts to scavenge hydrogen peroxide is determined according to the method of Ruch et al. (1989). Ascorbic acid, rutin, BHA, α-tocopherol and quercetin can be used as positive controls (Chanda and Dave, 2009).

2.1.10.5 Nitric oxide radical scavenging (NO) assay

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacted with oxygen to produce nitrite ions, which were measured using the Griess reaction (Green et al., 1982). Curcumin, caffeic acid, sodium nitrite, BHA, ascorbic acid, rutin, BHT and α-tocopherol can be used as a positive control (Chanda and Dave, 2009).

2. 1.10.6 Hydroxyl radical scavenging (HO) assay

Hydroxyl radical is one of the potent reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell (Halliwell and Gutteridge, 1981). Gallic acid, mannitol, catechin, vitamin E, quercetin, BHA , α-tocopherol, rutin and ascorbic acid can be used as positive controls (Chanda and Dave, 2009).

2. 1.10.7 Metal chelating activity

Ferrozine can quantitatively chelate with Fe^{2+} and form a complex with a red color. This reaction is limited in the presence of other chelating agents and results in a decrease of the red color of the ferrozine-Fe^{2+} complexes. Measurement of the color reduction estimates the chelating activity to compete with ferrozine for the ferrous ions (Soler-Rivas et al., 2000). EDTA or citric acid can be used as positive control (Chanda and Dave, 2009).

2. 1.10.8 Total antioxidant activity

The oxidation of linoleic acid generates peroxyl free radicals due to the abstraction of hydrogen atoms from diallylic methylene groups of linoleic acid (Kumaran and Karunakaran, 2006). The free radicals then will oxidize the highly unsaturated beta carotene. Consequently, the orange coloured chromophore of beta carotene would be degraded and the results can be monitored spectrophotometrically. The antioxidant activity is determined by the conjugated diene method (Lingnert et al., 1979). Ascorbic acid, BHA, α-tocopherol and trolox can be used as positive controls.

2. 1.10.9 Oxygen radical absorbance capacity (ORAC) assay

The capacity of a compound to scavenge peroxyl radicals, generated by spontaneous decomposition of 2, 2'-azo-bis, 2- amidinopropane dihydrochloride (AAPH), was estimated in terms of standard equivalents, using the ORAC assay (Prior et al., 2005).The method of Ou et al. (2002a; 2002b) is used for the estimation.

2. 1.10.10 Ferric reducing antioxidant power (FRAP) assay

FRAP assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), forming an intense blue Fe^{2+}-TPTZ complex with an absorption maximum at 593 nm. This reaction is pH-dependent (optimum pH 3.6). The absorbance decrease is proportional to the antioxidant content (Benzie and Strain, 1996). $FeSO_4$ is used for calibration. BHT, BHA, ascorbic acid,

quercetin, catechin and trolox (Benzie and Strain, 1996) can be used as positive control.

2. 1.10.11 Trolox equivalent antioxidant capacity (TEAC) assay

The $ABTS^{\bullet+}$ formed from the reaction ABTS-e^{-} $\longrightarrow$ $ABTS^{\bullet+}$ reacts quickly with ethanol/hydrogen donors to form colourless 2, 2'-azinobis (3-ethyl-benzothiazoline 6-sulfonate (ABTS). The reaction is pH-independent. A decrease of the $ABTS^{\bullet+}$ concentration is linearly dependent on the antioxidant concentration. The ABTS free radical-scavenging activity of plants samples is determined by the method of Stratil et al. (2006). The $ABTS^{+}$ scavenging rate is calculated. Trolox, BHT, rutin, ascorbic acid and gallic acid can be used as positive controls (Chanda and Dave, 2009).

Many solvents are used for extraction of bioactive compounds from the plants. The solvents used vary in their polarity. The most commonly used solvent is methanol as shown in Fig. 1. Traditionally water is used for extraction but it is next to methanol. The use of non polar solvents is comparatively less indicating that the active constituents are soluble in polar solvents only. Generally any part of the plant can be used for antioxidant studies but most commonly used part is leaf followed by fruit as shown in Fig. 2.

Table 1 lists some of the plants which show antioxidant activity. It also shows which part of the plant is used for antioxidant studies, which solvent is used and which assays are employed. It is generally believed that plants which are having more phenolic content show good antioxidant activity i.e. there is a direct correlation between total phenol content and antioxidant activity (Salazar et al., 2008; Saravana Kumar et al., 2008). However there are reports which do not show this correlation (Agbor et al., 2005; Maisuthisakul et al., 2007). It can be stated that phenolic content of the plant may be a good indicator of its antioxidant capacity.

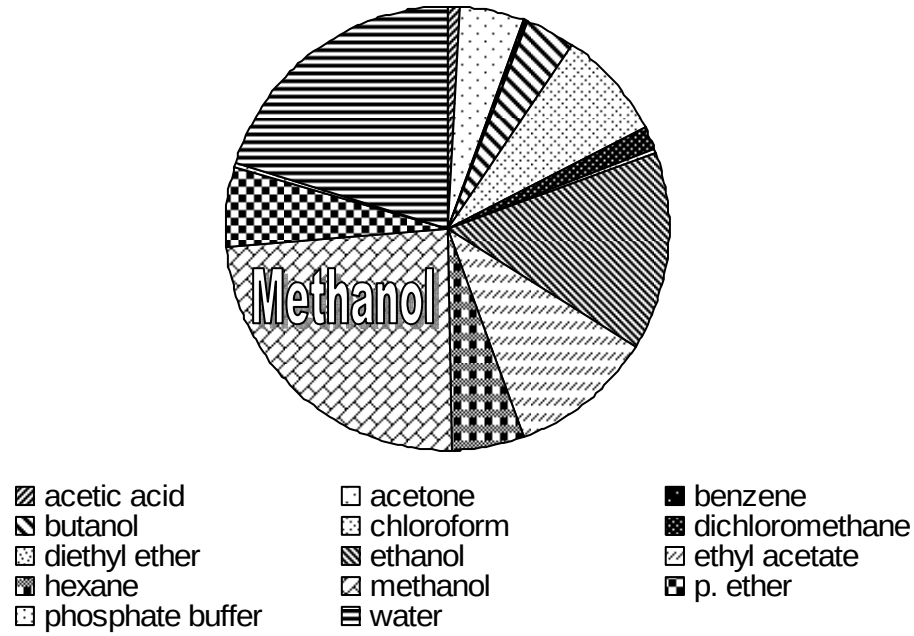


Fig. 1 Various solvents used for antioxidant activity

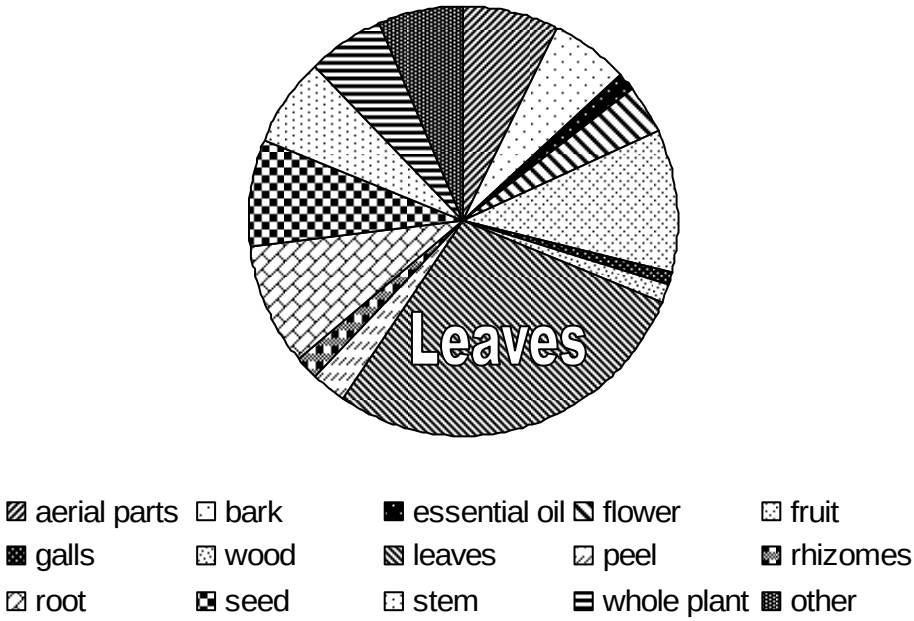


Fig. 2 Different plant parts used for the study of antioxidant activity

Table 1 List of medicinal plants, their family, parts and solvents used, assay employed for *in vitro* antioxidant studies

No	Plants (family)	Parts used	Solvent	Assay	References
1.	*Acacia auriculiformis* A. Cunn. ex Benth. (Mimosaceae)	bark	HE, C, A, ET, ME	TPC, DPPH, HO, RP	Singh et al., 2007
2.	*Achillea millefolium* subsp. Millefolium Afan. (Asteraceae)	essential oil	C, ME, Water	DPPH, SO, HO	Candan et al., 2003
3.	*Aegle marmelos* Correa (Rutaceae)	fruit	ME	DPPH, RP, NO, SO	Dhalwal et al., 2008
4.	*Allanblackia floribunda* Oliv (Guttiferae)	leaves, fruit	ME	DPPH, TPC, TF	Ayoola et al., 2008
5.	*Amaranthus lividus* L. (Amaranthaceae)	stem, leaves, flower	EA, ME Water	TEAC, DPPH , RP, Metal chelating, HO	Ozsoy et al., 2009
6.	*Aporosa lindleyana* Baill. (Euphorbiaceae)	root	PE, C, EA, ME	DPPH, NO	Badami et al., 2005a
7.	*Argyreia cymosa* R. Sweet (Convolvulaceae)	bark	PE, C, EA, ME	DPPH, HO, ABTS, NO, H_2O_2	Badami et al., 2008
8.	*Aristotelia chilensis* Maqui (Elaeocarpaceae)	fruit	EA, ME, Water	ORAC, FRAP , TPC	Céspedes et al., 2008
9.	*Azadirachta indica* A. Juss var. siamensis Valeton (Meliaceae)	leaves, raw fruit, ripe fruit, flower stem bark	HE, ME, Water	DPPH, total antioxidant activity	Sithisarn et al., 2005
10.	*Azadirachta indica* A. Juss var. siamensis Valeton (Meliaceae)	leaves	ET	DPPH	Sithisarn et al., 2006
11.	*Byrsonima crassifolia* H. B. & K.(Malpighiaceae)	leaves, bark, fruit	ME, Water	TPC, TF	Souza et al., 2008
12.	*Bergia suffruticosa* (Delile) (Elatinaceae)	whole plant	ME	TPC, DPPH, SO, RP	Anandjiwala et al., 2007

Table 1 Continued

13.	*Burkea africana* Hook (Leguminocaea)	bark	PE, BT, EA, Water	DPPH	Mathisen et al., 2002
14.	*Camellia crassicolumna* Var. multiplex (Theaceae)	leaves	C, EA, Water	DPPH	Liu Q et al., 2009
15.	*Camellia sinensis* var. sinesis (L.) Kuntz (Theaceae)	leaves	ET, Water	HO, SO	Chen et al., 2008
16.	*Campanula alliariifolia* Willd.(Campanulaceae)	whole plant	C, ME	DPPH, RP	Dumlu et al., 2008
17.	*Campis grandiflora* (Thunb.) K. Schum	flower	50% ET	DPPH, SO	Cui et al., 2006
18.	*Careya arborea* Roxb (Barringtoniaceae)	bark	PE, C, EA, ME	ABTS, DPPH, H_2O_2, NO, TPC, Total antioxidant capacity	Senthilkumar et al., 2007
19.	*Cassia siamea* Lam. (Caesalpiniaceae)	flower	95% ET	TPC, RP, DPPH, H_2O_2, NO, Protein oxidation, Metal chelating	Kaur et al., 2006
20.	*Cassia tora* L. (Caesalpiniaceae)	seeds	ME	RP, DPPH, Metal Chelating	Zhenbao et al., 2007
21.	*Celtis africana* Burm.f. (Ulmaceae)	stem, leaves	ME	TPC, TF, ABTS, DPPH, FRAP	Adedapo et al., 2009
22.	*Chaerophyllum hirsutum* L. (Apiaceae)	root, aerial parts	C, ME	DPPH	Acqua and Innocenti, 2004
23.	*Chamaecyparis lawsoniana* (A. Murr.) Parl. (Cupressaceae)	wood, bark	ME	ABTS, DPPH, TPC	Gao et al., 2007
24.	*Chlorophytum tuberosum* baker (Liliaceae)	whole plant	PE, 80% ET	DPPH, NO	Narasimhan et al., 2006
25.	*Cissus quadrangularis* L. (Vitaceae)	stem	ME	SO, DPPH	Jainu and Shyamala Devi, 2005
26.	*Citrullus colocynthis* L. (Cucurbitaceae)	fruit	ME	TPC, TF, DPPH, HO, NO, SO	Kumar et al., 2008
27.	*Cocos nucifera* L. (Arecaceae)	mesocarp	ME	DPPH, FRAP	Chakraborty and Mitra, 2008

Table 1 Continued

28.	*Cordia gilletii* De Wild (Boraginaceae)	root bark	HE, DM,EA, ME, Water	DPPH	Okusa et al., 2007
29.	*Cydonia vulgaris* Pers. (Rosaceae)	leaves	ET	RP, total antioxidant activity	Yildirim et al., 2001a
30.	*Cynara cardunculus* L. (Compositae)	involucral bracts	C, BT , EA, ET	FRAP, DPPH	Kukic et al., 2008
31.	*Cytisus scoparius* Linn. (Leguminosae)	aerial parts	70% ET	DPPH, NO, SO, HO, RP, TPC	Sundararajan et al., 2006
32.	*Dimocarpus Longan* Lour (Sapindaceae)	peels	ET	DPPH, TPC, HO, RP, total antioxidant activity	Pan et al., 2008
33.	*Dimocarpus Longan* Lour (Sapindaceae)	seeds	PE, C, BT, ME	DPPH, SO	Zheng et al., 2009
34.	*Diospyros ebenum* Roxb. (Ebenaceae)	leaves	PE, EA, ME, Water	DPPH, TPC, TF	Baravalia et al., 2009
35.	*Dipsacus asper* Wall (Dipsacaceae)	root	C, BT, EA, ME	DPPH	Hung et al., 2006
36.	*Ecklonia cava* (Alariaceae)	prothallus	HE, C, EA, 70% ME	DPPH, SO, HO, H_2O_2, RP, NO	Senevirathne et al., 2006
37.	*Elephantopus tomentosus* L. (Asteraceae)	whole plant	ET	DPPH, total antioxidant activity, RP, HO, TPC	Yam et al., 2008
38.	*Emblica officinalis* Gaertn. (Euphorbiaceae)	fruit	ME	TPC, DPPH, ABTS	Scartezzini et al., 2006
39.	*Garcinia kola* Heckel (Guttiferae)	seeds	PE, EA, A	RP, DPPH, SO, H_2O_2, HO	Farombi et al., 2002
40.	*Gracilaria changii* (Gracilariaceae)	prothallus	80% ME	TPC, DPPH (TLC)	Sreenivasan et al., 2007
41.	*Vitis vinifera* L. (Vitaceae)	seeds, bagasse, berries	PE, A, AA, Water	total antioxidant activity, TPC, H_2O_2	Baydar et al., 2007
42.	*Gynura procumbens* (Merr.) (Compositae)	leaves	PE, C, BT, EA, ME, Water	ABTS, total, RP, TPC antioxidant activity, DPPH, Xanthin oxidase,	Rosidah et al., 2008

Table 1 Continued

43.	*Haplopappus baylahuen* Remy (Asteraceae)	leaves	ME	DPPH (TLC)	Vogel et al., 2005
44.	*Heracleum nepalense* D Don (Apiaceae)	root	70 % ME	DPPH, HO, SO	Dash et al., 2005
45.	*Hordeum vulgare* L. (Poaceae)	seeds	HE, ME	TPC, DPPH, metal chelating	Madhujith and Shahidi, 2008
46.	*Hymenocardia acida* Tul. (Hymenocardiaceae)	leaves	ME , Water	DPPH, RP, ABTS, TF	Sofidiya et al., 2009
47.	*Hypericum venustum* Fenzl (Hypericaceae)	flower	ET, Water	RP, SO, Metal chelating, H_2O_2	Spiteller et al., 2008
48.	*Hyphaene thebaica* (L.) Mart. (Arecaceae)	fruit	Water	TPC, RP, DPPH, Metal chelating, HO, SO	Hsu et al., 2005
49.	*Hypsizigus marmoreus* (Peck) Bigelow (Tricholomataceae)	prothallus	ET, Water	Total antioxidant activity, RP, DPPH, Metal chelating	Lee et al., 2008
50.	*Ilex kudingcha* C.J. Tseng (Aquifoliaceae)	leaves	C , BT, EA, Water	TPC, DPPH, TEAC, FRAP	Liu L et al., 2009
51.	*Inonotus obliquus* (Hymenochaetaceae)	prothallus	EA, 80% ET, Water	DPPH, SO	Cui et al., 2005
52.	*Ipomoea aquatica* Forsk (Convolvulaceae)	leaves, veins	95% ET	DPPH, TPC, TF, RP, Ferric thiocynate	Huang et al., 2005b
53.	*Jasminum sambac* Linn. (Oleaceae)	leaves	BT, EA, Water	DPPH, NO, HO, β-carotene, RP	Tenpe et al., 2008
54.	*Kadsura coccinea* (Lem.) A.C. smith (Schisandraceae)	peels, pulp	EA, A, ME, Water	TPC, DPPH, FRAP, metal chelating	Sun et al., 2009
55.	*Kappaphycus alvarezii* (Doty) Doty (Solieriaceae)	prothallus	HE, EA, ET, ME, Water	TPC, DPPH, Metal chelating, RP, Total antioxidant activity	Suresh Kumar et al., 2008
56.	*Lannea velutina* A. Rich (Anacardiaceae)	root bark	ET, ME, Water	DPPH	Maiga et al., 2007

Table 1 Continued

57.	*Laurus nobilis* L. (Lauraceae)	leaves	ET	Total antioxidant activity, Metal chelation, SO, DPPH, RP, TPC	Elmastas et al., 2006
58.	*Lawsonia inermis* L. (Lythraceae)	leaves	HE, C, A, ET	TPC	Khodaparast et al., 2007
59.	*Lecaniodiscus cupanioides* Planch. (Sapindaceae)	leaves	ME	DPPH, ABTS, TPC, TF	Sofidiya et al., 2008
60.	*Lithospermum erythrorhizon* Sieb. & Zucc. (Boraginaceae)	root	PE, C	DPPH, RP, ABTS	Han et al., 2008
61.	*Mahonia aquifolium* (Pursh) Nutt. (Berberidaceae)	stem bark	ET	DPPH	Rackova et al., 2007
62.	*Mangifera indica* L. (Anacardiaceae)	fruits (pulp, seeds, peels, kernels)	ME, Water	TPC, DPPH, RP	Ribeiro et al., 2008
63.	*Mangifera indica* L. (Anacardiaceae)	seed kernels	95% ET	TPC, Metal chelating activity, DPPH, ABTS	Maisuthisakul and Gordon, 2009
64.	*Murraya koenigii* L. (Rutaceae)	leaves	HE, C, ET, Water	SO, HO, DPPH, ferric reducing, Metal Chelating	Ningappa et al., 2008
65.	*Musa paradisiaca* L. (Musaceae)	peels	70% A	DPPH,	Mokbel and Hashinaga, 2005
66.	*Nelumbo nuficera* Gaerth. (Nymphaeaceae)	rhizomes	PE, DM, A, ME, ET	TPC, DPPH, β carotene	Me et al., 2007
67.	*Nicotina tabacum* L. (Solanaceae)	leaves	80% ET	TPC, HO, SO, DPPH, RP	Wang et al., 2008a
68.	*Ocimum basilicum* L. (Lamiaceae)	leaves	ET	Total antioxidant activity, RP, HO, DPPH	Gulcin et al., 2007
69.	*Parmelia saxatilis* (L.) (Parmeliaceae)	prothallus	ME, Water	RP, SO, Ferric thiocynate, metal chelating, H_2O_2, TPC	Ozen and Kinalioglu, 2008

Table 1 Continued

70.	*Paullinia cupana* Mart. (Sapindaceae)	seeds	ME, 35% A, 60% ET, Water	TPC, β carotene assay, DPPH	Majhenic et al., 2007
71.	*Pedilanthus tithymaloides* L. Poit. (Euphorbiaceae)	stem, leaves	30 % ET	SO, HO, H_2O_2, NO, DPPH	Abreu et al., 2006
72.	*Perilla pankinesis* decne (Labiatae)	leaves	EA , 1% AA	Ferric thiocynate , RP, Metal chelating, H_2O_2, DPPH, SO	Gulcin et al., 2005
73.	*Peumus boldus* Mol. (Monimiaceae)	leaves	EA, ME, Water	DPPH, SO, Xanthine oxidase, TPC, TF	Schmeda-Hirschmann et al., 2003
74.	*Phoenix dactylifera* L. (Arecacea)	fruit	ME :Water (4:1)	ABTS, FRAP, TPC, TF	Biglari et al., 2008
75.	*Phyllanthus emblica* L. (Euphorbiaceae)	fruit	ME	DPPH, SO, HO, RP	Liu et al., 2008
76.	*Phyllanthus niruri* Linn. (Euphorbiaceae)	leaves , fruit	ME, Water	TPC, DPPH, SO	Harish and Shivanandappa, 2006
77.	*Piper nigrum* Linn. (Piperaceae)	seeds	ET, Water	DPPH, Total antioxidant activity, RP, Metal chelating H_2O_2, TPC	Gulcin, 2005
78.	*Piper nigrum* Linn. (Piperaceae)	fruit	PE, EA	SO, HO, DPPH, NO, TPC	Singh et al., 2008a
79.	*Polygonum paleaceum* Wall. ex Hook. f. (Polygonaceae)	rhizomes	BT, EA, A, Water	DPPH	Wang et al., 2005
80.	*Psidium guajava* L. (Myrtaceae)	leaves	50 % ET, Water	DPPH, TPC	He and Venant, 2004
81.	*Pyrrosia petiolosa* Ching (Polypodiaceae)	whole plant	50% ET	DPPH, SO, TPC, TF	Hsu, 2008
82.	*Ramaria flava* (Schaeff) Quel. (Ramariaceae)	prothallus	ET	DPPH, β carotene, TPC, TF	Gezer et al., 2006

Table 1 Continued

83.	*Randia hebecarpa* Benth. (Rubiaceae)	leaves	HE, EA, ME, Water	DPPH, Total antioxidant activity	Nazari et al., 2006
84.	*Rhus succedanea* L. (Anacardiaceae)	galls	Water	DPPH, NO	Baheti et al., 2005
85.	*Rosmarius officinalis* L. (Lamiaceae)	leaves	Essential oil	TPC, FRAP	Stefanovits-Banyai et al., 2003
86.	*Rubus ulmifolius* Schott (Rosaceae)	leaves	ME	TEAC, TPC, DPPH	Dall'Acqua et al., 2008
87.	*Rumex crispus* L. (Polygonaceae)	aerial parts	ME, Water	TPC, β carotene	Coruh et al., 2008
88.	*Rumex ecklonianus* Meissner (Polygonaceae)	whole plant	A, ME, Water	TPC, TF, ABTS, DPPH, FRAP	Jimoh et al., 2008
89.	*Salvia mirzayani* Rech. (Labiatae)	aerial parts	PE, C, BT, EA, ET, Water	RP, DPPH, β carotene, TPC	Moein et al., 2007
90.	*Salvia verbenaca* L. (Lamiaceae)	aerial parts	ME, Water	Total antioxidant activity, TPC	Khlifi et al., 2006
91.	*Sida cordifolia* Linn (Malvaceae)	root, stem, leaves, whole plants	90% ET	TPC, DPPH, RP, NO, SO	Dhalwal et al., 2005
92.	*Sideritis raeseri* Boiss et Heldr. subsp. raeseri (Lamiaceae)	aerial parts	BZ , C, ME	DPPH	Gabrieli et al., 2004
93.	*Smilax glyciphylla* Sm. (Smilaceae)	leaves, stem	Water	SO, TRAP, HO	Cox et al., 2005
94.	*Solanum pseudocapsicum* L. (Solanaceae)	leaves	ME	DPPH, NO, ABTS, HO, H_2O_2	Badami et al., 2005b
95.	*Soymida febrifuga* (Roxb.) A. Juss. (Meliaceae)	leaves	ME	TPC, DPPH	Reddy et al., 2008
96.	*Sphenocentrum jollyanum* Pierre (Menispermaceae)	leaves, stem, root, bark	ME	DPPH, DPPH (TLC)	Nia et al., 2004
97.	*Staphylea* sp. L. (Staphyleaceae)	leaves	PE, C,EA, Water	TPC, DPPH	Lacikova et al., 2007

Table 1 Continued

98.	*Tagetes mendocina* Phil. (Asteraceae)	aerial parts	HE, DM, ME	DPPH (TLC), DPPH, SO	Schmeda-Hirschmann et al., 2004
99.	*Tamarindus indica* L. (Fabaceae)	seed coat	ME	TPC, SO, Total antioxidant activity, DPPH, ABTS, FRAP	Siddhuraju, 2007
100.	*Tamus communis* L. (Dioscoreaceae)	root	ME, Water	TPC, TF, xanthine oxidase, TRAP	Boumerfeg et al., 2009
101.	*Uncaria tomentosa* Willd. DC. (Rubiaceae)	bark	Phosphate buffer (0.1M, pH 7.4), ET	TPC, TEAC, peroxyl radical trapping capacity	Pilarski et al., 2006
102.	*Urtica dioica* L. (Urticaceae)	nettle	Water	Total antioxidant activity, RP, SO, DPPH, Metal chelating, H_2O_2, TPC	Gulcin et al., 2004
103.	*Vaccinium stamineum* L. (Ericaceae)	fruit	80% A	TPC, TF, ORAC, ABTS, DPPH, SO, H_2O_2, HO	Wang and Ballington, 2007
104.	*Varthemia iphionoides* Boiss. (Asteraceae)	aerial parts	HE, EA, ET, Water	TPC, TF, DPPH, RP	Al-Dabbas et al., 2006

A – acetone, AA – acetic acid, BT – butanol, BZ – benzene, C – chloroform, DE – diethyl ether, DM – dichloromethane, EA – ethyl acetate, ET – ethanol, HE – hexane, ME – methanol, PE – petroleum ether

This review provides information on a number of plants which show promising antioxidant activity. It lists various methods for evaluating antioxidant activity along with different standards so it will be easy for the experimenter. It is also recommended to use at least two different types of assays for antioxidant activity. It emphasizes that *In vitro* antioxidant assays have been carried out for most of the plants, but *in vivo* remains to be done in majority of them. Methanol as a solvent has priority for extraction of plants for evaluating their antioxidant activity. Based on the above points, in the present work, methanol was used for extraction, part selected was leaves and five *in vitro* antioxidant assays were assayed for evaluating antioxidant capacity of *P. longifolia* leaves.

2.2 Review of literature for *in vivo* antioxidant activity

The body has an effective defence mechanism to prevent and neutralize the free radical-induced damage. This is accomplished by a set of endogenous antioxidant enzymes such as SOD, CAT and GPX. These enzymes constitute a mutually supportive team of defence against ROS (Venukumar and Latha, 2002). Regarding non-enzymatic antioxidants, Glutathione reduced (GSH) is a critical determinant of tissue susceptibility to oxidative damage and the depletion of hepatic GSH has been shown to be associated with an enhanced toxicity to chemicals (Hewawasam et al., 2003). Low levels of ROS are necessary in many biochemical processes, including intracellular messaging in the cell differentiation and cell progression or the arrest of growth, apoptosis, immunity, and defence against micro-organisms (Mate et al., 1999). In contrast, high doses and/or inadequate removal of ROS result in oxidative stress, which may cause severe metabolic malfunctions and damage to biological macromolecules (Chopra and Wallace, 1998).

Aerobic organisms possess antioxidant defence systems that deal with ROS produced as a consequence of aerobic respiration and substrate oxidation as shown in Fig. 3 (Mate et al., 1999).

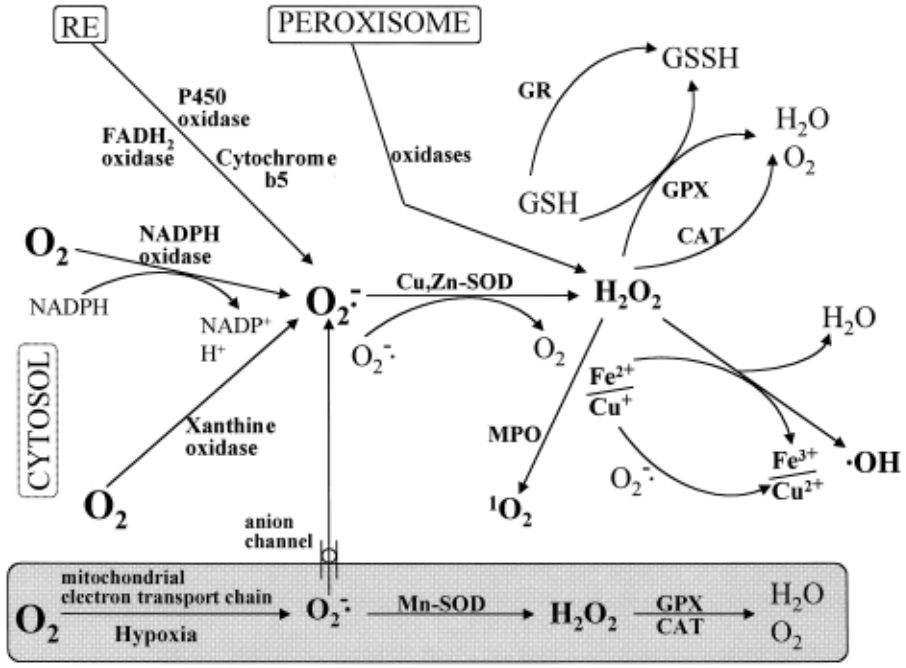


Fig. 3 Generation of reactive oxygen species and the defence mechanisms against damage by active oxygen. During hypoxia superoxide generated may be degraded into the mitochondria by Mn-SOD or, if it reaches the cytosol, by Cu, Zn-SOD. In the endoplasmic reticulum (RE), Nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P_{450} reductase can leak electrons onto O_2 generating $O_2^{\bullet -}$. Reduced flavin adenine dinucleotide ($FADH_2$) and cytochrome b_5 can also contribute to this system. Within peroxisomes, there are enzymes localized that produce H_2O_2 without intermediation of $O_2^{\bullet -}$. Contrarily to $O_2^{\bullet -}$, H_2O_2 is able to cross cell membranes and within the cells it can react with Fe^{2+} or Cu^{+} to form hydroxyl radicals via Fenton reaction. GR = glutathione reductase; MPO = myeloperoxidase; $^1O_2$: singlet oxygen.

2.2.1 Superoxide dismutase

Superoxide dismutase (EC 1.15.1.1) is the antioxidant enzyme that catalyses the dismutation of the highly reactive superoxide anion to O_2 and to the less reactive species H_2O_2. Peroxide can be destroyed by CAT or GPX reactions (Fridovich, 1995).

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^{+} \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

2.2.2 Catalase

Catalase (EC 1.11.1.6) is a primary component of the antioxidant system that defends against oxidative stress which is ubiquitously associated with pathologic conditions, including cancer, diabetes, cataracts, atherosclerosis, ischemic /reperfusion injury, arthritis, neurodegenerative disease, nutritional deficiencies and aging. Catalase decomposes H_2O_2, a major reactive oxygen species that in the presence of iron or other metal ions oxidizes cellular biochemicals to cause cytotoxicity (Vendemiale et al., 1999).

$$2H_2O_2 \xrightarrow{CAT} 2\,H_2O + O_2$$

2.2.3 Glutathione peroxidase

Glutathione peroxidase (EC 1.11.1.19) is a family of selenium containing antioxidant enzymes that catalyze the reduction of hydrogen peroxide in the presence of reduced glutathione. GPx plays an important role in the inhibition of lipid peroxidation and in the prevention of DNA and RNA damage (Mergener et al., 2009).

$$ROOH + 2GSH \xrightarrow{GPx} ROH + GSSG + H_2O$$

2.2.4 Glutathione reduced

Glutathione reduced is tripeptide and a super oxide radical scavenger and it protects thiol protein groups required for maintaining the integrity of cell against oxidation. GSH serves as the most important intracellular radical scavenger and is a substrate for the antioxidant enzyme GPx (Naito and Yoshikawa, 2002). GSH is present in the stomach at high concentration and plays an important role in maintaining the integrity of gastric mucosa (Altinkaynak et al., 2003).

2.2.5 Lipid peroxidation

Lipid peroxidation is probably the most extensively investigated process induced by free radicals and reactive oxygen species (Nakagawa et al., 1992). Lipid peroxidation is a free radical mediated chain reaction that can inactivate cellular components and are purportedly associated with various chronic disorders (Halliwell, 1991). Lipid peroxidation can be evaluated by the thiobarbituric acid reactive substances method (TBARS); this method evaluates the oxidative stress assayed for malondialdehyde, the last product of lipid breakdown caused by oxidative stress (Kowaltowski et al., 1996).

Table 2 lists some of the plants which show *in vivo* antioxidant activity. It lists various antioxidant assays for *in vivo* antioxidant activity. It also shows which part of the plant and solvent is used for *in vivo* antioxidant activity.

Table 2 List of medicinal plants, their family, parts and solvents used, assay employed for *in vivo* antioxidant activities

No.	Plants (family)	Parts used	Solvent	Assay	References
1.	*Ajuga iva* L. (Lamiaceae)	whole plant	Water	GSH, TBARS, SOD, GPx, GR	Chenni et al., 2007
2.	*Asparagus racemosus* Willd. (Liliaceae)	root	Crude powder	CAT, SOD, TBARS	Visavadiya and Narasimhacharya, 2005
3.	*Berberis tinctoria* Lesch (Berberidaceae)	leaves	PE, C, ME	GSH, CAT, SOD	Murugesh et al., 2005
4.	*Caesalpinia digyna* Rottler (Caesalpiniaceae)	root	PE, ME, Water	TBARS, CAT, SOD	Srinivasan et al., 2007
5.	*Caesalpinia sappan* L. (Caesalpiniaceae)	heartwood	PE, C, EA, ME, Water	CAT, SOD, TBARS	Badami et al., 2003a
6.	*Crataegus aronia* L. (Rosacea)	leaves, unripe fruits	Water	GSH, TBARS	Ljubuncic et al., 2005
7.	*Cytisus scoparius* L. (Leguminosae)	aerial parts	ET : Water 7:3	SOD, CAT, GPx, GR, GST	Raja et al., 2007
8.	*Ferula szovitsiana* DC (Umbelliferae)	aerial parts, root	HE, DE, EA, ME	CAT, SOD, TBARS	Dehghan et al., 2007
9.	*Hibiscus sabdariffa* L. (Malvaceae)	calyx	Water	TBARS	Hirunpanich et al., 2006
10.	*Melampyrum barbatum* L. (Scrophulariaceae)	leaves, flower	phosphate buffer (pH 7.0, 0.1M)	SOD, CAT, GPx, GSH, TBARS	Stajner et al., 2009
11.	*Momordica dioica* Roxb. (Cucurbitaceae)	leaves	PE, ET, Water	TBARS, SOD, CAT, GSH	Jain et al., 2008
12.	*Musa paradisiaca* L. (Musaceae)	fruit	HE, ME, Water	SOD, CAT, TBARS, Hydroperoxides	Vijayakumar et al., 2008
13.	*Nasturtium officinale* R. Br. (Brassicaceae)	aerial parts	ET: Water (7:1)	SOD, CAT, GPx, GSH, GR, TBARS	Yazdanparast et al., 2008
14.	*Nelumbo nucifera* Gaerth. (Nymphaeaceae)	seeds	50% ET	CAT, SOD, TBARS	Rai et al., 2006
15.	*Pfaffia glomerata* (Spreng.) (Amaranthaceae)	root	BT, ME, Water	TBARS, 4-hydroxynoneal	De Souza Daniel et al., 2005
16.	*Phyllanthus fraternus* L.(Euphorbiaceae)	whole plant	Water	TBARS, GSH	Sailaja and Setty, 2006
17.	*Rhizophora mangle* L. (Rhizophoraceae)	bark	Water	Myloperoxidase, GPx, SOD	Berenguer et al., 2006

Table 2 Continued

18.	*Rhodococcum vitis-idaea* L. (Ericaceae)	leaves	Water	GST, GSH, TBARS	Myagmar et al., 2004
19.	*Sargassum* Sp. (Sargassaceae)	prothallus	ME	GST	Patra et al., 2008
20.	*Sargassum polycystum* (Sargassaceae)	prothallus	ET	TBARS,GSH	Raghavendran et al., 2004
21.	*Scoparia dulcis* L. (Scrophulariaceae)	whole plant	Water	TBARS	Ratnasooriya et al., 2005
22.	*Scoparia dulcis* L. (Scrophulariaceae)	whole plant	Water	TBARS, GPx, SOD, CAT, GSH	Pari and Latha, 2004
23.	*Smilax china* L. (Liliaceae)	root	HE, BT, DM, EA, ME	CAT, GPx, SOD, TBARS	Lee et al., 2001
24.	*Solanum nigrum* Linn. (Solanaceae)	fruit	ME	SOD, CAT, TBARS, GPx, GSH, GST	Jainu and Shymala Devi, 2004
25.	*Striga orobanchioides* Benth (Scrophulariaceae)	whole plant	ET	CAT, SOD, TBARS	Badami et al., 2003b
26.	*Tephrosia purpurea* Linn. (Fabaceae)	aerial parts	HE, 95% ET	SOD, TBARS	Soni et al., 2006
27.	*Terminalia chebula* Retz. (Combretaceae)	fruit	Water	SOD, TBARS	Naik et al., 2004
28.	*Terminalia chebula* Retz. (Combretaceae)	fruit	PE, ET	SOD, CAT, GPx, TBARS	Senthilkumar and Subramanian, 2007
29.	*Teucrium polium* L. (Lamiaceae)	aerial parts	80% ET	TBARS	Hasani et al., 2007
30.	*Toona sinensis* Roem (Meliaceae)	leaves	Water	TBARS, SOD	Hseu et al., 2008
31.	*Trichilia emetica* Vahl. (Meliaceae)	root	Phenol HPLC analysis	TBARS	Germano et al., 2006
32.	*Vitis vinifera* L. (Vitaceae)	leaves	BT, EA, Water	GSH, TBARS	Orhan et al., 2006
33.	*Withania somnifera* (Dunal) (Solanaceae)	root	Crude powder	TBARS, CAT, SOD	Visavadiya and Narasimhacharya, 2007

BT – butanol, C - chloroform, DE – diethyl ether, DM – dichloromethane, EA – ethyl acetate, ET – ethanol, HE – hexane, ME – methanol, PE – petroleum ether, GR-Glutathione redutase, GST-glutathione s- transferase

In the present work, five *in vivo* antioxidant assays *viz.* lipid peroxidation, GSH, GPx, CAT and SOD were assayed for evaluating antioxidant capacity of *P. longifolia* leaves.

2.3 Review of literature for toxicity study

Since ancient times, plants have commonly been used in traditional medicine for treatment of various ailments. Traditional medicines are used by about 60% of world's population. These are not only used in rural areas in developing countries, but also in developed countries as well as where modern medicines are predominantly used. The World Health Organization (WHO) has recently defined traditional medicine (including herbal drugs) as comprising therapeutic practices that have been in existence, often for hundreds of years, before the development and spread of modern medicine and are still in use today. Traditional medicine is the synthesis of therapeutic experience of generations of practicing physicians of indigenous systems of medicine (Kamboj, 2000). The research on medicinal plants has experienced a huge increase in the last few years and some traditional species have been the starting point for discovery of many important drugs. According to WHO, approximately 20,000 plant species are employed for medicinal purposes around the world (Phillipson, 1994). The herbal medicine market in 1991 in the countries of the European Union was about $ 6 billion, with Germany accounting for $ 3 billion, France $ 1.6 billion and Italy $ 0.6 billion. Incidentally in Germany and France, herbal extracts are sold as prescription drugs and are covered by national health insurance. In 1996, the US herbal medicine market was about $ 4 billion and with the current growth rate may be more than double by the turn of century. Thus a reasonable guesstimate for current herbal medicine market worldwide may be around $ 30–60 billion. The Indian herbal drug market is about $ one billion and the export of herbal crude extracts is about $ 80 million (Kamboj, 2000). The practice of traditional medicine is widespread in China, India, Japan, Pakistan, Sri Lanka and Thailand. In China about 40% of the total medicinal consumption is attributed to traditional tribal medicines. In Thailand, herbal medicines make use of legumes encountered in the *Caesalpiniaceae*, the *Fabaceae*, and the *Mimosaceae*. In Japan, herbal medicinal preparations are more in demand than mainstream pharmaceutical products (Hoareau and DaSilva, 1999). The traditional medicines are derived from medicinal plants,

minerals and organic matter, but the herbal remedies are prepared from medicinal plants only (Seth and Sharma, 2004). There is limited scientific evidence regarding the safety and efficacy to support the continued therapeutic application of these herbal remedies. With increase in the use of herbal remedies, there is a need for thorough scientific evaluation of these medicinal plants. This will help to validate or invalidate their folkloric uses (Zhu et al., 2002).

Herbs are supposed to be safe but many unsafe and fatal side effects have recently been reported (Whitto et al., 2003; Izzo, 2004). These could be direct toxic effects, allergic reactions, effects from contaminants and/or interactions with drugs and other herbs (Seth and Sharma, 2004). Phytotherapeutic products are, many times, mistakenly regarded as less toxic because they are 'natural' (Gesler, 1992). Nevertheless, those products contain bioactive principles with potential to cause adverse effects (Bent and Ko, 2004). An adverse effect is defined as an abnormal, undesirable or harmful change following exposure to the potentially toxic substance. The ultimate adverse effect is death but less severe adverse effects may include altered food consumption, altered body and organ weights, visible pathological changes or simply altered enzyme levels (Duffus and Worth, 2006). Thus, all the "natural" products used in therapeutics must be submitted to efficacy and safety test by the same methods used for new synthetics drugs (Talalay and Talalay, 2001).

For clinical trials designed to study pharmacologic effects of candidate products, more extensive preclinical safety data would be needed to support the safety of such studies. The critical preclinical information required includes a two week toxicology study in sensitive species (usually rodents) plus toxicokinetics that should allow determination of the no observed adverse effect level (NOAEL). In addition to study in rodent species, additional studies in nonrodents (generally dogs) are conducted to confirm that the rodent is a sensitive species (Tammara and Jacobson-Kram, 2007). For some compounds and types of toxic effect there will clearly be a dose below which no effect or response is measurable. There is thus a threshold dose.

The concept of a threshold dose for the toxic effect is an important one in toxicology because it implies that there is a NOAEL. The NOAEL is usually based on animal toxicity studies. The NOAEL is important for setting exposure limits. For example,

the acceptable daily intake (ADI) is based on the NOAEL. This is a factor used to determine the safe intake for food additives and contaminants such as pesticides and residues of veterinary drugs and, hence, to establish the safe level in food (Timbrell, 2002).

Toxicology is the fundamental science of poisons. A poison is generally considered to be any substance that can cause severe injury or death as a result of a physicochemical interaction with living tissue. However, all substances are potential poisons since all of them can cause injury or death following excessive exposure. On the other hand, all chemicals can be used safely if exposure of people or susceptible organisms to chemicals is kept below defined tolerable limits (Duffus and Worth, 2006). There are three so-called laws that underline the science of toxicology. Paracelsus is usually considered to be the father of toxicology, having formulated the first law, which states that the dose makes the poison. The second law concerns the specific effects of individual chemicals, a specificity due to the unique chemicals structure of the agent and the laws of biology that govern the response. The third law is that humans are animals and that therefore the study of animals can provide useful insight into effects in humans (Goldstein and Gallo, 2001). Appropriate dose of a drug should be determined by preliminary studies of acute toxicity. Such studies are essential to prevent any overdose of drug which may interfere with results of experiment. The lethal dose (LD_{50}) is defined as the dosage of a substance which kills 50 per cent of the animals in a particular group, usually determined in an acute, single exposure study. There are three major sites for the absorption of foreign compounds: the skin, lungs and gastrointestinal tract. The gastrointestinal tract is the most important in toxicology as most foreign compounds are ingested orally. The lungs are clearly important for all airborne compounds whereas the skin is only rarely a significant site for absorption (Timbrell, 2002). They are also helpful in understanding toxicity profiles of the drug (Ozbek et al., 2004). The multiple dose study with a drug is also necessary. But, in order to choose the doses to be used in the study, the clinical observation of the acute assay is important along with pharmacological activity studies in animals and in humans (Da Silva et al., 2002; Alvarez et al., 2004). Daily clinical observation is of major importance as well as the final observation (Eaton and Klaassen, 1996). The doses to be evaluated in chronic toxicity in animals must be larger than that suggested for use in humans (Feres et al., 2006).

Table 3 lists some of the plants which show ethnomedicinal uses with botanical name, plant family, plant part(s) used and solvent used for extraction. Table 3 also provides information on toxicity study, route of administration and doses of plants. It lists toxicity studies *viz.* acute, sub-acute, chronic etc. with their doses, route of administration and LD_{50} values along with their safety profile. Determination of appropriate dose is a very important issue in the study of plant extracts. Therefore, before starting the study on plants, researchers should determine the dose of extract by referring the previous toxicity trials or do the toxicity workup by themselves.

Table 3 List of medicinal plants, their family, ethnomedicinal uses, parts and solvents used and toxicity study

No.	Plants (family)	Ethnomedicinal uses	Parts used	Solvent	LD_{50} (g/kg) b.w. (route of administration)	Toxicity study (experimental periods), Dose (g/kg, b.w.) and route of administration	Result	References
1.	*Acacia karroo* Hayne (Fabaceae)	Gum is an important food source	Stem bark	Water	-	**Acute** (48 h) 0.4, 0.8, 1.6 and 3.2 (p.o.) **Sub acute** (14 days) 0.8 (p.o.)	Toxic	Adedapo et al., 2008a
2.	*Acmela brasiliensis* DC (Asteraceae)	Respiratory infections and pain	Aerial parts	50% ET	-	**Acute** (24 h) 0.1, 0.5, 1, 2 and 4 (p.o.), **Sub acute** (15 days) 0.5, 1, 2 and 4 (p.o.)	Low toxicity	Burger et al., 2005
3.	*Aconium napeilus* Linn. (Ranunculaceae)	Pain, coldness, vertigo and general fatigue	Isolated alkaloid Aconitine	-	-	**Chronic** (22 days) 1 (p.o.)	Safe	Wada et al., 2006
4.	*Aframomum melegueta* (Roscoe) K. Schum. (Zingiberaceae)	Stomachache, diarrhea and snakebite	Seeds	95% ET	-	**Sub chronic** (28 days) 0.12, 0.45 and 1.5	Toxic (liver)	Ilic et al., 2010
5.	*Ajuga iva* (L.) schreber (Labiatea)	Hyper tension, diabetes, gastrointestinal disorders and anthelmintic	Whole plant	Water	3.6 (i.p.)	**Acute** (14 days) 2, 4, 6, 10 and 14 (p.o.) 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 and 5.5 (i.p.) **Chronic** (90 days) 0.1, 0.3, 0.6	Safe (p.o.) Toxic (i.p.)	Hilaly et al., 2004

Table 3 Continued

6.	*Anacardium occidentale* L. (Anacardiaceae)	Gastrointestinal disorders, mouth ulcer, throat problems and hypertension	Leaves	70% ET	> 2	**Acute** (14 days) 2 (p.o) **Sub acute** (30 days) 0.40, 0.70, 1 (p.o)	Moderate toxicity	Konan et al., 2007
7.	*Anacardium occidentale* L. (Anacardiaceae)	Diabetes mellitus and inflammation	Leaves	HE	16 (p.o.)	**Acute** (7 days) 2, 6, 10, 14, 18, 22, and 26 (p.o.) **Chronic** (56 days) 6, 10 and 14	Toxic at high dose (chronic)	Tedong et al., 2007
8.	*Anogeissus leiocarpus* (DC.) Guill. & Perr. (Combretaceae)	Helminthosis, schistosomiasis, leprosy, diarrhea and psoriasis	Leaves	Water	1.4 (i.p.)	**Acute** 72 h for p.o. and 24 h for i.p. 0.8, 1.2, 1.6, 2, 2.4, 2.8 (p.o. and i.p.)	Safe (oral) and low toxic (i.P.)	Agaie et al., 2007
9.	*Artemisia afra* (Jacq. Ex. Willd) (Asteraceae)	Cough, colds, sore throat, heart burns, hemorrhoids, fever, malaria, asthma and diabetes mellitus	Leaves	Water	8.96 (p.o.) and 2.45 (i.p.)	**Acute** (14 days) 2, 4, 6, 8, 10, 12, 16, 20, and 24 (p.o.) 1.5, 2.5, 3.5, 4.5 and 5.5 (i.p.) **Chronic** (3 months) 0.01, 0.1, 1 (p.o.)	Safe	Mukinda and Syce, 2007
10.	*Asiasari radix* (Aristolochiaceae)	Neuroprotective	Whole plant	70% ME	-	**Acute** (14 days) 0.1, 0.3, 0.5 (p.o.)	Safe	Ramesh et al., 2007
11.	*Asparagus pubescens* Bak (Liliaceae)	Used as remedy for liver and kidney disorders	Roots	ME	-	**Acute** (24 h) 0.25 and 1 (p.o.)	Safe	Nwafor et al., 2004
12.	*Aspilia africana* (Pers) C.D. Adams (Compositae)	Stop bleeding, remove corneal opacities, anemia and various stomachs complains	Leaves	Water	6.6 (p.o.)	**Acute** (7 days) 2, 4, 8, 12 and 16 (p.o.) **Sub acute** (26 days) 0.5 and 1 (every 48 h) (p.o.)	Low toxicity	Taziebou et al., 2007

Table 3 Continued

13.	*Basella alba* L. (Basellaceae)	Diarrhea, laxative and anemia	Leaves	Water	-	**Sub acute** 0.06, 0.08 and 0.1 (p.o.)	Safe	Bamidele et al., 2010
14.	*Bixa orellana* L. (Bixaceae)	Used in food industries	Isolated compound from annatto	-	-	**Sub acute** (28 days) 2 (p.o.)	Safe	Bautista et al., 2004
15.	*Boerhavia diffusa* L.(Nyctaginaceae)	Inflammatory disorders, bacterial infection, heart diseases, corneal ulcers, antiviral and hepatic disorders	Leaves	Water	-	**Sub chronic** (30 days) 0.5, 1 and 2 (p.o.)	Safe	Orisakwe et al., 2003
16.	*Boswellia dalzielii* Hutch.(Frankincense)	Wound healing, induce vomiting	Stem bark	Water	-	**Acute** (48 h) 3 (p.o.) **Sub chronic** (28 days) 0.9, 1.8 and 2.7 (p.o.)	Safe (acute) Toxic at high dose (sub chronic)	Etuk et al., 2006
17.	*Bryophyllum calycinum* Salisb. (Crassulaceae)	Antiviral, antimicrobial, antitumor, antioxidant, diuretic, antiulcer, anti-inflammatory, anti-diabetic	Leaves	ME and water	-	**Acute** (24 h) 0.5 to 3 (p.o.), 0.35 to 2.60 (i.p.)	Safe	Devbhuti et al., 2008
18.	*Calendula officinalis* L. (Asteraceae)	Anti-inflammatory, wound healing and antiviral	Flowers	70% ET	-	**Acute** (14 days) 0.625, 1.25, 2.5 and 5 (p.o.) **Sub acute** (30 days) 0.025, 0.25, 0.5 and 1 (p.o.)	Safe	Silva et al., 2007
19.	*Calycopteris floribunda* Lam. (Combertaceae)	Dysentery, fever, emesis and ulcer	Leaves	ME, water	ME 0.38 (p.o)	**Acute** (14 days) 0.10, 0.20, 0.40, 0.60 (p.o)	ME was toxic while water extract was safe	Sreekanth et al., 2006

Table 3 Continued

20.	*Camellia sinensis* (L.) Kuntze (Theaceae)	Antioxidant, anti-allergic, antiangiogenic, anti-inflammatory and hypolipidemic	Catechins	-	-	**Sub chronic** (90 days) 0.3, 1.25 and 5% (w/w) fed in diet	Safe up to 1.25 %	Takami et al., 2008
21.	*Careya arborea* Roxb. (Myrtaceae)	Anthelmintic, epileptic fits, bronchitis and astringents	Stem bark	ME	-	**Acute** (72 h) 0.1 to 1.6 (p.o.)	Safe	Samdath Kumar et al., 2008
22.	*Carica papaya* L. (Caricaceae)	Anti-fertility	Seeds	ME	-	**Acute** (14 days) 2 (p.o) **Sub chronic** (28 and 90 days) 0.05, 0.1, 0.25 and 0.5 (p.o.)	Safe	Lohiya et al., 2006
23.	*Carrica papaya* L. (Caricaceae)	Digestive agent, wound healing, ulcer, boils and induce menstruation	Unripe fruit	Water	2.52 (p.o.)	**Acute** (24 h) 0.4, 0.8, 1.6 and 3.2 (p.o.) **Chronic** (42 days) 0.05, 0.1, 0.15, 0.2 and 0.25 (p.o.)	Safe	Oduola et al., 2007
24.	*Cassia fistula* L. (Caesalpiniaceae)	Mild, pleasant purgative action, antifungal, antiviral, menstrual disorders and fever	Pod	Water	6.60 (i.p.)	**Acute** (48 h) 0.8, 1.6, 3.2, 6.4 and 12.8 (i.p.) **Sub chronic** (6 weeks) 0.25, 5 and 1 (i.p.)	Low toxic	Akanmu et al., 2004
25.	*Cassia sieberiana* DC (Caesalpiniaceae)	Urinogenital infection, antimicrobial and dysentery	Pod pulp	Water	1.95 (p.o.)	**Acute** (24 h) 1, 1.5, 2, 2.5 and 3 (p.o.) **Sub acute** (5 weeks) 0.2, 0.4, 0.8 and 1.6 (p.o.)	Toxic at high dose (sub acute)	Toma et al., 2009
26.	*Cassytha filiformis* R.Br. (Lauraceae)	Diabetes mellitus, ulcer and cough	Stems and leaves	Water	-	**Sub chronic** (28 days) 0.25, 0.5, 1 (p.o.)	Safe	Babayi et al., 2007

Table 3 Continued

27.	*Ceiba pentandra* L. (Bombacacease)	Antiameobic and antibacterial	Leaves	40% ME	-	**Acute** (24 h) 0.01 to 5 (p.o.) **Chronic** (21 days) 0.25 to 5 (p.o.)	Safe	Sarkiyayi et al., 2009
28.	*Centaurium erythraea*(L.) Rafn. (Gentianaceae)	sedative, antipyretic, asthma, jaundice, intestinal parasitic infestation, rheumatism, wounds and sores, blood pressure, edema and digestive disorders	Whole Plant	Water	0.12 (i.p.)	**Acute** (14 days) 1, 3, 5, 7, 9, 11, 13 and 15 (p.o.), 2, 4, 6, 8, 10, 12, 13 and 14 (i.p.) **sub-chronic** (90 days) 0.1, 0.6 and 1.2 (p.o.)	Safe	Tahraoui et al., 2010
29.	*Chiococca alba* (L.) Hitchc (Rubiaceae)	Rheumatic disorders, emetic, antidiarrheic, purgative, diuretic, antipyretic, tonic and delayed menstruation	Roots	ET	-	**Acute** (14 days) 0.062, 0.125, 0.25, 0.5, 1 and 2 (p.o.), 0.062, 0.125, 0.25 and 0.5 (i.p. and s.c.) **Repeated** (14 days) 0.5, 1 and 2 (p.o.), 0.015, 0.013, 0.062 and 0.125 (i.p.)	Low toxicity (oral) Toxic (parenteral)	Gazda et al., 2006
30.	*Cissampelos pareira* L. var hirsute (Menispermaceae)	Menstruation problems, pain reliever and used as remedy to control fertility temporarily	Roots	50% ET	-	**Acute** (13 days) 2 (p.o.) **Sub acute** (4 weeks) 1 and 2 (p.o.)	Safe	Amresh et al., 2008
31.	*Crateva nurvala* Buch.-Ham. (Capparidaceae)	Digest, laxative, anthelmintic, antilithic, expectorant and antipyretic	Stem bark	PE, BZ,C, 95% ET, water	> 5 (p.o.)	**Acute** (14 days) 0.05 to 5 (p.o.)	Safe	Sikarwar et al., 2009
32.	*Cucrbita maxima* Duch. (Cucurbitaceae)	Stomach pain, anti-inflammatory and antipyretic	Seeds	50% ET	> 5 (p.o.)	**Acute** (24h) 0.1, 0.5, 1 and 5 (p.o.) **Sub acute** (30 days) 1 (p.o.)	Safe	Cruz et al., 2006

Table 3 Continued

33.	*Cylicodiscus gabunensis* (Taub.) Harms (Mimosaceae)	Diarrhea and gastrointestinal disorders	Stem bark	EA	11 (p.o) for female and 14.5 (p.o.) for male	**Acute** (7 days)- 4, 8, 12 and 16 (p.o) **Sub acute** (6 weeks) 0.75, 1.5, 3 and 6 (p.o.)	Toxic at high dose	Kouitcheu et al., 2007
34.	*Datura stramonium* L.(Solanaceae)	Asthma, gastric pain, anti-inflammatory, stimulation of central nervous system and skin infection	Leaves	60% ET	-	**Chronic** (5 weeks) 0.05 and 0.2 (p.o.)	Safe	Gidado et al., 2007
35.	*Delphinium denudatum* Wall (Ranunculaceae)	Anticonvulsion, anti-sterss, hepatoprotection, cardioprotection and antimicrobial	Roots	Water	16.1 (p.o.)	**Acute** (24 h) 14, 15, 16, 17, 18 and 24 (p.o)	Safe up to 16 g/kg b.w.	Zafar et al., 2003
36.	*Dimorphandra mollis* Benth (Caesalpiniaceae)	Antioxidant, antiviral, anti-inflammatory, anti-tumor and anti platelets	Fruits	20% ET	-	**Acute** (13 days) 0.5, 2, 3.5 and 5 (p.o.)	Safe	Feres et al., 2006
37.	*Drimys angustifolia* Miers (Winteraceae)	Analgesic, antiulcer and anti-inflammatory	Leaves and stem bark	ET	-	**Acute** (48 h) 1.75, 3.5 and 5.25 (p.o.)	Toxic at high dose	Witaicenis et al., 2007
38.	*Elephantorrhiza elephantina* (Burch.) Skeels. (Fabaceae)	diarrhoea, coughing, pneumoni and tick-borne diseases	Rhizomes	Water	-	**Acute** (24 h) 0.4, 0.8, 1.6 (p.o.) **Sub acute** (14 days) 0.2, 0.4, 0.8 (p.o.) **Chronic** (35 days) 0.05, 0.1, 0.2, 0.4 (p.o.)	Toxic at high dose	Maphosa et al., 2010
39.	*Entada africana* Guill. and Perr. (Mimosaceae)	Antileishmanial, anti-inflammatory, hepato-protective, respiratory tract disorders and wound healing	Stem bark and leaves	ME	Stem bark 0.146 and leaves 0.249 (i.p.)	**Acute** (72 h) 0.05 to 0.4 (i.p.)	Moderate toxic	Tibiri et al., 2007

Table 3 Continued

40.	*Erythrina senegalensis* DC (Pailionaceae)	Bronchial infection, cough, and throat infection	Stem bark	C	0.526 (i.p.)	**Acute** (24 h) 0.1, 0.2, 0.4, 0.6 and 1.2 (i.p.) **Chronic** (12 weeks) 0.25, 0.5 and 1 (fed in diet)	Toxic at high dose	Udem et al., 2010
41.	*Euphorbia hirta* L (Euphorbiaceae)	Inflammation, respiratory tract and asthma	Leaves	ET, HE, EA, ME	-	**Repeated** (14 days) 0.4, 0.8 and 1.6 (p.o.)	Toxic	Adedapo et al., 2005
42.	*Ficus exasperata* (Vahl) (Moraceae)	Stimulant, ring worm and chest complications	Leaves and stems	98% ET		**Repeated** (3 days) 0.5, 0.1 and 0.5 (p.o)	Leaves were toxic while stems were safe	Irene and Iheanacho, 2007
43.	*Ficus exasperata* (Vahl) (Moraceae)	Chest pain, eye troubles and stomach pains and to arrest bleeding	Leaves	Water	0.54 (i.p.)	**Acute** (24 h) 2.5, 5, 10 and 20 (p.o.) 0.1, 0.2, 0.4, 0.8 and 1 (i.p.)	Safe	Bafor and Igbinuwen, 2009
44.	*Galega officinalis* L. (Papilionaceae)	Antidiabetic and increasing lactation	Aerial parts	Crude powder	-	**Acute** (14 days) 0.5, 1, 2.5 and 5 (p.o.) **Sub chronic** (90 days) 0.15%, 1.5 %, and 3% (w/w) fed in diet	Safe (acute) Toxic (for liver and lung in Sub chronic)	Rasekh et al., 2008
45.	*Galphinia glauca* Cav. (Malpighiaceae)	Mental disorders, diminishing nervous excitement	Leaves	Water, ME, ET	-	**Sub chronic** (28 days) 2.5 (p.o.)	Safe	Santamaria et al., 2007
46.	*Garcinia haburyi* Hook. f. (Guttiferae)	Cytotoxic and anticancer activity	Gambogi c acid (resin)	-	-	**Chronic** (13 weeks) 0.03, 0.06 and 0.12 (p.o.)	Safe	Qi et al., 2008
47.	*Glinuus lotoides* L. (Molluginaceae)	Anthelmintic	Seeds	60 % ME	-	**Acute** (14 days) 1 and 5 (p.o.) **Repeated** (28 days) 0.25, 0.5 and 1 (p.o.)	Safe	Demma et al., 2007

Table 3 Continued

48.	*Gynura procumbens* (Merr.) (Compositae)	Eruptive fiver, rash, migraines, constipation, hypertension, diabetes mellitus, kidney diseases, and cancer	Leaves	ME	-	**Acute** (14 days) 1.25, 2.5 and 5 (p.o.) **Sub chronic** (90 days) 0.125, 0.25 and 0.5 (p.o.)	Safe	Rosidah et al., 2009
49.	*Helicteres isora* L. (Sterculiaceae)	Diabetes mellitus, colic, gastropathy, diarrhea and dysentery	Bark	Water	-	**Acute** (12 days) 2 (p.o.) **Repeated** (28 days) 0.5 (p.o.)	Safe	Kumar G et al., 2007
50.	*Ipomoea batatas* L. (Convolvulaceae)	Isolated compound ipomeamarone	Tuber	-	-	**Acute** (48 h) 0.25 and 0.5 (p.o.)	Toxic for liver	Pandey, 2008
51.	*Jatropha curcus* L. (Euphorbiaceae)	Biodiesel	Phorbol (Isolated)	-	0.027 (p.o.)	**Acute** (19 days) 0.036, 0.032, 0.29, 0.026, 0.023 and 0.021 (p.o.)	Toxic	Li CY et al., 2010
52.	*Kielmeyera coriaceae* Mart. (Clusiaceae)	Schistosomiasis, malaria, fungal and bacterial infections	Stems	DM	1.50 (p.o.) and 0.538 (i.p.)	**Acute** (14 days) 0.05, 0.2, 0.4, 0.8, 1.2, 1.8, 2 and 2.2 (p.o.) 0.05, 0.2, 0.4, 0.5, 0.6 and 0.8 (i.p.) **Repeated** (90 days) 0.005, 0.025 and 0.125 (p.o)	Safe	Obici et al., 2008
53.	*Kyllinga brevifolia* Rottb. Hassk (Cyperaceae)	Diuretic, sedative and antispasmodic properties	Rhizomes	70 % ET	0.575 (i.p.)	**Acute** 0.001, 0.01 and 0.1 (i.p.)	Safe	Hellion-Ibarrola et al., 1999
54.	*Laportea crenulata* Gaud (Urticaceae)	Weakness, asthma, gout, mumps, chronic fever	Roots	PE, C, ME	> 1 (i.p.)	**Acute** (24 h) 0.2 to 1 (i.p.)	Safe	Khan et al., 2007
55.	*Lonicera japonica* Thunb. (Caprifoliaceae)	Antipyretic, antibacterial, antiviral and antioxidant	Leaves	95% ET	-	**Acute** (14 days) 5 (p.o.) **Sub acute** (14 days) 1 (p.o.)	Safe	Thanabhorn et al., 2006

Table 3 Continued

56.	*Macrothelypteris torresiana* (Gaud.) Ching (Thelypteridaceae)	Hydropsy and traumatic bleeding	Root	EA	2.76 (p.o.), 0.87 (p.o.)	**Acute** (14 days) 6.67, 5, 3.75, 2.81, 2.11, 1.58, 1.19 and 0.89 (p.o.) 2.14, 1.57, 1.18, 0.89, 0.67, 0.50, 0.37 and 0.28 (p.o.) **Sub acute** (14 days) 6, 60, 600 and 1200 (p.o.), 4, 40, 400 and 800 (p.o.)	Low toxicity	Huang et al., 2010
57.	*Magnistipula butayei* Subsp. montana (Hauman) F. white (Chrysobalanceae)	Trunk bark and root used as decoction and leaves and fruit used as killing wild animals (rats, dogs and other animals)	Trunk bark	Water	0.37 (p.o.)	**Acute** (3 days) 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 (p.o.)	Toxic	Karangwa et al., 2006
58.	*Mammea africanna* Sabine (Guttiferae)	Hypercholesterolemia, internal heat, microbial infection	Stem bark	Water	0.387 (i.p.)	**Acute** (24 h) 0.05 to 1 (i.p.) **Sub acute** (21 days) 0.03, 0.06 and 0.09 (p.o.)	Low toxic	Antia et al., 2006
59.	*Manihot esculenta* Crantz (Euphorbiaceae)	Human and animal nutrition and raw material for industrial products	Cassava	-	-	**Acute** (14 days) 5 ml/kg b.w. **Sub chronic** (28 days) 25%, 50%, and 100% ml/kg b.w.	Safe	Avancini et al., 2007
60.	*Mitragyna inermis* (Willd.) O.Kuntze (Rubiaceae)	Malaria and fever	Leaves	60% ET	-	**Acute** (4 days) 0.30 and 3 (p.o.) **Chronic** (28 days) 0.30 to 3 (p.o.)	Toxic at high dose	Monjanel-Mouterde et al., 2006

Table 3 Continued

61.	*Mitragyna speciosa* Korth (Rubiaceae)	analgesic, antipyretic, antidiarrheal and local anesthetic	Leaves	ME	-	**Acute** (14 days) 0.1, 0.5 and 1 (p.o.)	Severe hepatotoxic and mild nephrotoxic	Harizal et al., 2010
62.	*Monascus purpureus* MTCC 410 (red mould rice)	Lowering blood pressure and blood cholesterol	-	-	-	**Acute** (14 days) 0.5, 1, 2.5 and 5 (fed in diet) **Sub chronic** (14 weeks) 2, 4, 8 and 12% w/w (fed in diet)	Safe	Mohan Kumari et al., 2009
63.	*Murraya koenigii* (L.) spreng (Rutaceae)	Tonic, stomachic, anti-vomiting, dysentery, diarrhoea, hypoglycemic, febrifuge, antifungal and antiperiodic	Leaves	ME	0.316 (p.o.)	**Acute** (72 h) 0.2, 0.5, 1 and 2 (p.o.) **Sub chronic** (14 days) 0.25, 0.35 and 0.45 (p.o.)	Toxic at high dose	Adebajo et al., 2006
64.	*Musanga cecropioides* R.Br. (Cecreopiaceae)	Rheumatism, leprosy and chest infection	Stem bark	Water	-	**Acute** (12 days) 3 (p.o.) **Repeated** (28 days) 0.75 (p.o.)	Safe	Adeneye et al., 2006
65.	*Ocimum suav*e wild. (Lamiaceae)	Ulcers, anticathartic, mosquito repellent and analgesic	Leaves	Water	-	**Acute** (7 days) 2 and 8 (p.o.) **Sub acute** (6 weeks) 0.25, 0.5 and 1 (p.o.)	Safe	Tan et al., 2008
66.	*Polygala fruticos* (P.J. Bergius) (Polygalaceae)	Chronic ulcer, poor circulation, intestinal sores, gonorrhoea and the snuff to improve sinusitis	Leaves	Water	10.8 (p.o.)	**Acute** (14 days) 2, 4, 8, 12, 16 and 20 (p.o.) **Sub chronic** (31 days) 0.1 to 1 (p.o.)	Toxic at high dose	Mukinda and Eagles, 2010
67.	*Pongamia pinnata* (L.) Merr. (Papilionaceae)	Anticonvulsant, hypotensive effects, bronchitis, chronic fever, whooping cough, and skin diseases	Pongamol (isolated)	-	-	**Sub acute** (14 days) 300 µg/0.3 ml (i.p.)	Safe	Baki et al., 2007

Table 3 Continued

68.	*Portulaca grandiflora* Hook. (Portulaceae)	Sore throat, skin rash and detoxification	Aerial part	Water	-	**Chronic** (6 months) 0.01, 0.1 and 1 (p.o.)	Safe	Chavalittumrong et al., 2004
69.	*Pothomorphe umbellate* L. Miq. (Piperaceae)	Liver and inflammation disorders	Root	50% ET	-	**Acute** (14 days) 1, 2, and 5 (p.o) **Sub chronic** (40 days) 0.5 (p.o.)	Safe	Barros et al., 2005
70.	*Salacia oblonga* Wall. (Celastaceae)	Used as remedy for diabetes	Whole plant	Water	-	**Sub chronic** (90 days) 0.25, 1.25 and 2.5 (p.o)	Safe up to 0.25 g/kg	Flammang et al., 2007
71.	*Salvia przewalskii* Maxim (Labiatae)	coronary heart diseases, myocardial infarction and atherosclerosis, angina pectoris and liver diseases	Rhizome	ET	2.54 (p.o.), 0.90 (i.m.), 0.78 (i.p.)	**Acute** (14 days) 1.72, 1.98, 2.27, 2.62, 3.01 and 3.46 (p.o.), 0.288, 0.412, 0.58, 0.84, 1.2 and 1.71 (i.m.) 0.5, 0.625, 0.781, 0.977, 1.22 and 1.52 (i.p.) **Sub acute** (30 days) 0.05 and 0.25 (p.o.)	Safe	Li X et al., 2010
72.	*Salvia scutellarioides* Kunth (Lamiaceae)	Antihypertensive and diuretic properties	Bark and leaves	Water	-	**Acute** (14 days) 2 (p.o.) **Sub acute** (4 weeks) 1 and 2 (p.o.)	Safe	Ramirez et al., 2007
73.	*Schinus molles* var areira (Anacardiaceae)	Antibacterial, antiviral, antiseptic, astringent, digestive, purgative, diuretic, tooth ache, wound healer, menstrual disorders and rheumatism	Fruits	ET	-	**Acute** (14 days) 2 (p.o) **Sub-acute** (14 days) 1 (p.o)	Safe	Ferrero et al., 2007

Table 3 Continued

74.	*Schinus terebinthifolius* Raddi (Anacardiaceae)	ulcers, gout, tumors, respiratory problems, wounds, rheumatism, diarrhea, skin ailments, arthritis, antiseptic, anti-inflammatory, balsamic and haemostatic	Stem bark	70% ET	-	**Acute** (14 days) 0.625, 1.25, 2.5 and 5 (p.o.) **Sub acute** (45 days) 0.25, 0.625 and 1.5625	Safe	Limaa et al., 2009
75.	*Semecarpus anacrdium* L. (Anacardiaceae)	Asthma, piles digestive disorders, cardiac tonic, antimicrobial, anticancer and anti-inflammatory	Fruits (oil)	-	-	**Sub acute** (7 days) 0.25, 0.5 and 0.75 (p.o.) (21 days) 0.083 and 0.166 (p.o.)	Toxic	Choudhari and Deshmukh, 2007
76.	*Senna alata* (L.) Roxb. (Ceasalpiniaceae)	Hepatitis and skin diseases	Leaves	Water and ET	18.5 (p.o.)	**Acute** (8 days) 4, 8, 12, 16 and 20 (p.o.) **Sub acute** (28 days) 0.5 and 1 (every 48 h) (p.o.)	Safe	Pieme et al., 2006
77.	*Sida cordifolia* L. (Malvaceae)	Stomatitis of asthma and nasal congestion	Leaves	70% ET	2.64 (i.p.)	**Acute** (48 h) 0.5 to 5 (p.o.), 0.5 to 3 (i.p.)	Safe (p.o.) Toxic at high dose (i.p.)	Franco et al., 2005
78.	*Sida rhombifolia* L. (Malvaceae)	Antiseptic, wound-healing activity, diarrhea, cough, ulcer	Whole plant	Water / ME	-	**Acute** (24 h) 4, 8, 12 and 16 (p.o.)	Toxic at high dose	Assam et al., 2010
79.	*Smilax kraussiana* (Liliaceae)	Inflammation	Leaves	ME	0.24 (i.p.)	**Acute** (24 h) 0.01 to 1 (i.p.)	Safe up to 0.24 g/kg b.w. (i.p.)	Nwafor et al., 2006
80.	*Sphenocentrum Jollyanum* Pierre (Menispermaceae)	Vermifuge, chronic wound, cough and anti-inflammatory	Leaves	ET	1.44 (i.p.)	**Acute** (24 h) 11 (p.o.) 0.25 to 4 (i.p.) **Sub chronic** (120 days) 0.05, 0.1 and 0.2 (p.o.)	Safe	Mbaka et al., 2010

Table 3 Continued

81.	*Stachtarpheta cayennensis* (Verbanaceae)	Stomachic, febrifuge and chronic liver diseases	Leaves	Water, 50 % ET, BT, EA	50% ET extract 0.09 (i.p.) in mice	**Acute** (72 h) **Rat-** 0.25, 0.5 and 1 (i.p.) **Mice-** 0.005 to 0.1 (i.p.) and 0.05 to 0.25 (p.o.)	50% ET extract was toxic in mice other extract were safe	Akanmu et al., 2005
82.	*Striga hermonthica* (Del.) Benth (Scrophulariaceae)	Dermatosis, leprosy, jaundice and antibacterial	Leaves, flower and stem	80% A	17.53 (i.p.)	**Acute** (24h) 15.5, 16.5, 17.5, 18.5, 21.5 and 23 (i.p.)	Safe up to 17.53 g/kg b.w.	Kiendrebeogo et al., 2005
83.	*Strychnos potatorum* L (Loganiaceae)	Astringent, refrigerant, emetic, antithelmintic, diuretic, digestive, tonic, stomachic, ophthalmic, appetizer, water purifier and relive colic	Seed	Water	-	**Acute** (72 h) 0.05, 0.3 and 2 (p.o.) **Chronic** (90 days) 0.1 and 0.2 (p.o.)	Safe	Sanmugapriya and Venkataraman, 2006
84.	*Stryphnodendron adstringens* (Martius) coville (Leguminosae)	Anti-inflammatory, analgesic and gastric mucosa	Stem bark	Water	-	**Acute** (7 days) 2 (p.o.) **Sub acute** (30 days) 0.80 and 1.60 (p.o.)	Safe (acute) Toxic (sub acute)	Rebecca et al., 2002
85.	*Syzygium cumini* L. (Myrtaceae)	Diabetes, high blood pressure, diarrhea and fever	Stem bark, leaves	70% ME	Leaves 0.387 (p.o.) Stem bark > 5 (p.o.)	**Acute** (24 h) 0.01, 0.1 and 1 (p.o.)	Safe	Ugbabe et al., 2010
86.	*Syzygium cumini* L.(Myrtaceae)	Hypoglycemic, anti-HIV, antibacterial and anti-diarrheal	Seeds	EA, ME		**Acute** (14 days) 0.05, 0.3 and 2 (p.o)	Safe	Kumar A et al., 2007
87.	*Tamarindus indica* L. (Fabaceae)	Cold, jaundice, stomach disorders, diarrhea, fever and skin cleanser	Pulp	Water	-	**Sub acute** (28 days) 0.9, 1.8, 2.7, 3.6 and 4.5 (p.o.)	Safe	Abukakar et al., 2008

Table 3 Continued

88.	*Tanacetum vulgare* L. (Asteraceae/compositae)	Menstrual irregularities, anthelmic, carminative, antispasmodic, stimulant and tonic properties	Leaves	Water	9.9 (p.o.) and 2.8 (i.p.)	**Acute** (14 days) 1 to 13 (p.o.) 1, 1.5, 2, 2.5, 3, 3.5, 4 and 4.5 (i.p.) **Chronic** (90 days) 0.1, 0.2 and 0.6 (p.o.)	Safe	Lahlou et al., 2008
89.	*Tetrapleura tetraptera* (Schumach. & Thonn.) Taub. (Mimosaceae)	Convulsion, leprosy, inflammation, jaundice, rheumatism, flatulence	Fruit	80% ET	-	**Sub acute** (10 days) 0.05, 0.1 and 0.15 (p.o.)	Toxic	Odesanmi et al., 2010
90.	*Tithonia diversifolia* (Hemsl) (Asteraceae)	Malaria, menstrual pains, diabetes mellitus, sore throat, liver and measles	Leaves	Water	-	**Repeated** (7 days) 0.1 and 0.2 (p.o.)	Toxic (liver, heart and kidney)	Adebayo et al., 2009
91.	*Tithonia diversifolia* (Hemsl.) (Asteraceae)	Malaria, diarrhea, bacterial and parasitic infection	Leaves	Water	0.12 (p.o)	**Acute** (24 h) 0.05, 0.08, 0.1, 0.12 and 0.14 (p.o.)	Toxic	Oyewole et al., 2007
92.	*Toona sinensis* Roemor (Meliaceae)	Carminative enteritis and dysentery	Leaves	Water	-	**Acute** (14 days) 5 (p.o.) **Sub acute** (28 days) 1 (p.o.)	Safe	Liao et al., 2007
93.	*Vernonia amygdalina* Del (Compositae)	Antimalaria, anticancer, antimicrobial, as laxative herbs and anthelmintics	Leaves	95% ME HE, EA, ME	-	**Sub acute** (6-fraction) (28 days) 0.08, 0.16 and 0.32 (p.o.)	Safe	Akah et al., 2009
94.	*Vernonia condensate* Baker (Asteraceae)	Gastro intestinal disorders, headache, diarrhea and protection against snakebites	Leaves	Water	-	**Acute** (0.30, 0.45, 0.67, 1, 1.5, 2.25, 3.4 and 5 (p.o. and i.p.)	Safe	Monteiro et al., 2001
95.	*Zingiber zerumbet* Smith. (Zingiberaceae)	Anticancer and cytotoxic activity	Zerumbone from rhizomes	-	1.84 (i.p.)	Acute (48 h) 0.1, 0.2, 0.5, 1, 1.5, 2, 2.5 and 3 (i.p.)	Toxic at high dose	Ibrahim et al., 2010

A – acetone, BT – butanol, BZ – benzene, C – chloroform, DM – dichloromethane, EA – ethyl acetate, ET – ethanol, HE – hexane, ME – methanol, PE – petroleum ether, p.o. – per oral, i.p. – intraperitoneally, s.c. – subcutaneous, i.m. – intramuscular

In the above review, it is seen that in acute toxicity study, the dose is single but observations are carried out for 14 days, but it varied from 24 h to 19 days. In repeated dose studies, the dose is given daily or on alternate days. If done for 21-28 days it was called sub-acute toxicity study if continued for more days up to 90 days or more it was called chronic or sub-chronic toxicity study. The table also lists a number of plants and its toxicity profile so it becomes easy to carry out further work. It also helps in dose and route selection. The most common route was oral or i.p. Such review helps in knowing the toxicity level of different plants. If any pharmacological activity is done or to be done, this toxicity data will help to decide if that particular plant is safe or not. Considering the above review and OECD guide lines, in the present work, acute and sub-acute toxicity studies were done for methanol extract of *P. longifolia* for 14 days and 21 days respectively. The route used was oral.

CHAPTER 3: MATERIAL AND METHODS

3.1 Collection and identification of the plant material

Fresh leaves of *P. longifolia* were collected in the year 2007, Rajkot, Gujarat, India. The plant was compared with voucher specimen (voucher specimen No. PSN 4) deposited at Department of Biosciences, Saurashtra University, Rajkot, Gujarat, India. The fresh and mature leaves were used for pharmacognostic study.

3.2 Pharmacognostic analysis

3.2.1 Macroscopic characteristics

The plant was macroscopically examined for shape, size, surface characteristics, texture, color, consistency, odour, taste, etc. (Khandelwal, 2008).

3.2.2 Microscopic characteristics

Free hand sections of the fresh leaf sample of *P. longifolia* were taken. Sections were cleared with chloral hydrate and then stained with phloroglucinol and hydrochloric acid and mounted with glycerin. Same procedure was followed for microscopic characteristics of powdered material of leaf of *P. longifolia* (Khandelwal, 2008).

3.3 Extraction of plant material

The leaves were separated, washed thoroughly with tap water, shade dried, homogenized to fine powder and stored in air tight bottles. The dried powder was defatted with hexane and extracted in methanol for 24 h on a rotary shaker by cold percolation method (Parekh and Chanda, 2007a; Vaghasiya and Chanda, 2007). The methanolic extract was concentrated using rotary evaporator to get the solid mass. The yield obtained was 8.62%. The methanolic extract was used for the entire study.

3.4 Physicochemical analysis

The following physicochemical parameters were carried out (WHO, 2002; The Ayurvedic Pharmacopoeia of India 2008; Vaghasiya et al., 2008) in dried leaf powder and or methanolic extract of *P. longifolia*

- Loss on drying
- Total ash
- Acid insoluble ash
- Water soluble ash
- Petroleum ether soluble extractive
- Alcohol soluble extractive
- Methanol soluble extractive
- Water soluble extractive
- Solubility test
- pH
- Melting and Boiling point
- Heavy metal analysis

3.4.1 Loss on drying

Two grams of crude powder of *P. longifolia* (leaf) was taken in an evaporating dish and then dried in an oven at 105 ºC till constant weight was obtained. The weight after drying was noted and loss on drying was calculated. The percentage was calculated on the basis of sample taken initially.

3.4.2 Total ash

Two grams of dried powder of *P. longifolia* (leaf) was taken in a silica crucible and ignited it by gradually increasing the heat to 500 °C in a muffle furnace until it was white, indicating the absence of carbon. Ash was cooled in a desiccator and weighed without delay. The percentage was calculated on the basis of sample taken initially.

3.4.3 Acid insoluble ash

To the crucible containing total ash, 25 ml of hydrochloric acid (HCl, ~70g/l) was added; it was covered with a watch-glass and boiled gently for 5 minutes. The watch-glass was rinsed with 5 ml of hot water and this liquid was added to the crucible. The insoluble matter was collected on an ashless filter paper and it was washed with hot water until the filtrate was neutral. The filter paper containing the insoluble matter was transferred to the original crucible; it was dried on a hot plate and ignited to constant weight. The residue was allowed to cool and then weighed without delay. The percentage was calculated on the basis of sample taken initially.

3.4.4 Water soluble ash

To the crucible containing the total ash, 25 ml of water was added and boiled for 5 minutes. The insoluble matter was collected on an ashless filter paper. It was washed with hot water and ignited in a crucible for 15 minutes. The residue was allowed to cool and then weighed without delay. Weight of insoluble matter was subtracted from the weight of total ash. The percentage was calculated on the basis of sample taken initially.

3.4.5 Determination of petroleum ether soluble extractive

Five grams of dried leaf powder of *P. longifolia* was taken in 100 ml of petroleum ether in a conical flask, plugged with cotton wool and then kept on a rotary shaker at 120 rpm for 24 h. Thereafter, it was filtered and the filtrate was evaporated to dryness at 105 °C till constant weight was obtained. The percentage of extractable matter was calculated with reference to the sample taken initially.

3.4.6 Determination of alcohol soluble extractive

Five grams of dried leaf powder of *P. longifolia* was taken in 100 ml of alcohol in a conical flask, plugged with cotton wool and then kept on a rotary shaker at 120 rpm for 24 h. Thereafter, it was filtered and the filtrate was evaporated to dryness at 105

°C till constant weight was obtained. The percentage of extractable matter was calculated with reference to the sample taken initially.

3.4.7 Determination of methanol soluble extractive

Five grams of dried leaf powder of *P. longifolia* was taken in 100 ml of methanol in a conical flask, plugged with cotton wool and then kept on a rotary shaker at 120 rpm for 24 h. Thereafter, it was filtered and the filtrate was evaporated to dryness at 105 °C till constant weight was obtained. The percentage of extractable matter was calculated with reference to the sample taken initially.

3.4.8 Determination of water soluble extractive

Five grams of dried leaf powder of *P. longifolia* was taken in 100 ml of water in a conical flask, plugged with cotton wool and then kept on a rotary shaker at 120 rpm for 24 h. Thereafter, it was filtered and the filtrate was evaporated to dryness at 105 °C till constant weight was obtained. The percentage of extractable matter was calculated with reference to the sample taken initially.

3.4.9 Solubility

The quantitative solubility test of methanolic extract of *P. longifolia* was determined in different solvents. 5 mg of extract was weighed for solubility test for different solvents. The extract was added in each solvent until saturated solution developed. Solubility was calculated in mg/ml.

3.4.10 Determination of pH

The methanolic extract of *P. longifolia* was dissolved in distilled water and was kept in a water bath for 20 min. It was then filtered and the pH of the filtrate was noted with the help of a Systronic pH meter (pH system 361).

3.4.11 Determination of melting and boiling point

The melting and boiling point of the methanolic extract of *P. longifolia* were done at Department of Chemistry, Saurashtra University, Rajkot, Gujarat, India by open capillary method (Sukhwal et al., 1995).

3.4.12 Determination of heavy metals

The analysis for heavy metals like arsenic, chromium, cadmium, lead, and mercury for methanolic extract of *P. longifolia* were done at Choksi Laboratory Limited, Vadodara.

3.5 Phytochemical analysis

3.5.1 Qualitative phytochemical analysis

Preliminary chemical tests were carried out for crude powder and methanolic extract to identify different phyto-constituents (Harborne, 1973; Parekh and Chanda 2007b).

3.5.1.1 Alkaloids

The crude powder and methanolic extract of *P. longifolia* was dissolved in 2 N HCl. The mixture was filtered and the filtrate was divided into 3 equal portions. One portion was treated with few drops of Mayer's reagent; one portion was treated with equal amount of Dragondroff reagent and the other portion was treated with equal amount of Wagner's reagent. The creamish precipitate, orange precipitate and brown precipitate indicated the presence of respective alkaloids. A (+) score was recorded if the reagent produced only a slight opaqueness; a (++) score was recorded if a definite turbidity but no flocculation was observed and a (+++) score was recorded if heavy precipitate or flocculation was observed (Salehi-Surmaghi et al., 1992).

3.5.1.2 Flavonoids

3.5.1.2.1 Shinoda test

The presence of flavonoids was estimated by Shinoda test. The crude powder and methanolic extract of *P. longifolia* were treated with few drops of concentrated HCl and magnesium ribbon. The appearance of pink or tomato red colour within few minutes indicated the presence of flavonoids (Somolenski et al., 1972).

3.5.1.2.2 Alkaline reagent test

The crude powder and methanolic extract of *P. longifolia* was treated with few drops of diluted sodium hydroxide (NaOH) separately. Formation of intense yellow color which turned colorless on addition of few drops of diluted HCl indicated presence of flavonoids.

3.5.1.3 Tannins

The crude powder and methanolic extract of *P. longifolia* was treated with alcoholic ferric chloride ($FeCl_3$) reagent. Blue color indicated the presence of tannins (Segelman et al., 1969).

3.5.1.4 Phlobatanins

The crude powder and methanolic extract of *P. longifolia* was boiled with 1% aqueous HCl. Deposition of red precipitate was taken as evidence for the presence of phlobatanins (Harborne, 1973).

3.5.1.5 Triterpenes

Chloroform extract of the crude powder and methanolic extract of *P. longifolia* was treated with concentrated sulphuric acid (H_2SO_4). Appearance of reddish brown ring indicated the presence of triterpenes (Harborne, 1973).

3.5.1.6 Steroids

Liebermann-Burchard reaction was performed for the presence of steroids. A chloroformic solution of the crude powder and methanolic extract of *P. longifolia* was treated with acetic anhydride and few drops of concentrated H_2SO_4 were added down the sides of test tube. A blue green ring indicated the presence of steroids.

3.5.1.7 Saponins

The presence of saponins was determined by Frothing test. The crude powder and methanolic extract of *P. longifolia* was vigorously shaken with distilled water and was allowed to stand for 10 minutes and classified for saponin content as follows: no froth indicates absence of saponins and stable froth for more than 1.5 cm indicated the presence of saponins (Kapoor et al., 1969).

3.5.1.8 Cardiac glycosides

Keller-kiliani test was performed for the presence of cardiac glycosides. The crude powder and methanolic extract of *P. longifolia* each was treated with 1ml mixture of 5% $FeCl_3$ and glacial acetic acid (1:99 v/v). To this solution, few drops of concentrated H_2SO_4 were added. Appearance of greenish blue color within few minutes indicated the presence of cardiac glycosides (Ajaiyeobu, 2002).

3.5.2 Quantitative phytochemical analysis

3.5.2.1 Total phenolic content

The amount of total phenol content was determined by Folin-Ciocalteu reagent method (McDonald et al., 2001). 0.5 ml of extract and 0.1 ml of Folin-Ciocalteu reagent (0.5 N) was mixed and incubated at room temperature for 15 min. Then 2.5 ml saturated sodium carbonate was added. The mixture was allowed to stand for 30 min at room temperature and the absorbance of the reaction mixture was measured at 760 nm. The total phenolic content is expressed in terms of gallic acid equivalent (mg/g of extracted compound).

3.5.2.2 Total flavonoid content

The amount of total flavonoid content was determined by Aluminium chloride method (Chang et al., 2002). The reaction mixture consisted of 1.0 ml extract, 1 ml methanol, 0.5 ml aluminium chloride (1.2%) and 0.5 ml potassium acetate (120 mM). The mixture was allowed to stand for 30 min at room temperature and the absorbance of the reaction mixture was measured at 415 nm. The flavonoid content is expressed in terms of quercetin equivalent (mg/g of extracted compound).

3.5.2.3 Extraction of crude alkaloid

One gram of dried leaf powder was taken in 100 ml flask and 40 ml 20% glacial acetic acid in methanol was added to it and was allowed to stand for 4 h at room temperature. This was filtered and the extract was concentrated on a water bath to one quarter of the original volume. Concentrated ammonium hydroxide (25%) was added drop wise to the extract until the precipitation was complete. Then it was taken in a separating funnel and equal volume of chloroform was added. It was washed with distilled water three times to make pH neutral. Sodium sulphate (Na_2SO_4) was added to remove moisture. It was filtered and dried. Crude alkaloid content was collected and weighed. Crude alkaloid content is expressed in mg/g of dried leaf powder (Harbone, 1973; Djilani et al., 2006).

3.5.2.4 Extraction of crude saponin

Ten grams of dried leaf powder was defatted with petroleum ether for 1 h at 40 ºC in a water bath and then extracted with methanol for 1 h with mild heating. It was then filtered and centrifuged at 5000 g for 10 min. In order to get the crude saponin extract, the methanolic extract was dissolved in mixture of methanol and acetone (1:5 v/v) to precipitate the saponins. It was filtered and dried. Crude saponin was collected and weighed. Crude saponin content is expressed in mg/g of dried leaf powder (Yan et al., 1996).

3.5.3 HPTLC fingerprinting

The HPTLC fingerprinting of the methanolic extract of *P. longifolia* leaf was carried out at Gujarat Ayurved University, Jamnagar. Analysis report was given by Pharmaceutical Chemistry Laboratory, Gujarat Ayurved University, Jamnagar, Gujarat, India. The thin layer chromatographic plates were developed using the solvent system Toluene: Ethyl acetate: glacial acetic acid (14: 6: 0.6).

3.5.3.1 Detection and scanning of HPTLC

Thin layer chromatographic plates were examined with ultraviolet (UV) lamp operating at a wavelength of 366 nm (long wavelength) and 254 nm (short wavelength). Number of peaks and peaks height of the resolved bands were recorded.

3.5.4 UV-visible spectral analysis

The methanolic extract was analyzed in UV-Visible range between 200-800 nm using UV-Visible Spectrophotometer (UV-1601, Shimadzu). This method is useful for analyzing organic compounds *viz*. ketones, dienes etc. For analysis, 3 mg/ml of methanolic extract in methanol was used.

3.5.5 IR spectral analysis

Infrared spectroscopy is one of the powerful analytical techniques which offer the possibility of chemical identification. The technique is based on the simple fact that chemical substance shows selective absorption in the infrared region. After absorption of IR radiations, the molecules vibrate, giving rise to absorption spectrum. It is an excellent method for the qualitative analysis because except optical isomers, the spectrum of compound is unique. It is most useful for the identification of purity and gross structural details. This method is useful in the field of natural products, forensic chemistry and in industrial analysis of competitive products. The IR spectra of methanolic extract of *P. longifolia* was scanned on FT-IR-Shimadzu-8400 over the frequency range from 4000-400 cm^{-1}. IR spectral analysis was done at Department of Chemistry, Saurashtra University, Rajkot, Gujarat, India.

3.5.6 ^{1}H, ^{13}C NMR spectral analysis

NMR spectroscopy is useful in the field of structure determination. It is an essential tool in almost every area of chemical analysis and fundamental research. This technique has also been employed successfully for the quantitative determination of water in food products and agricultural materials. In the presence of an externally applied magnetic field, a spinning nucleus can only assume a limited number of stable orientations. In a magnetic field, a spinning nucleus in lower energetic orientations absorbs sufficient electromagnetic radiation and is excited to a higher energetic orientation. This results in nuclear magnetic resonance. The NMR spectra were scanned on ^{1}H, ^{13}C NMR, Buker 300 Mhz by using methanol as a solvent and TMS as an internal standard (chemical shift in a ppm) at SAIF, Chandigarh, Punjab, India.

3.5.7 GC-MS spectral analysis

GC-MS analysis of methanolic extract was performed using GC-MS instrument (GCMS-QP-2010) equipped with glass column SGE BPX5 and capillary dimension 30 m x 0.25 mm x 0.25 µ. The oven temperature was programmed from 80-260 °C. Inlet and interface temperature were 250 °C and 200 °C respectively. Carrier gas was helium at a flow rate of 1.0 ml/min. Ion source temperatures were maintained at 200 °C and spectra were measured. GC-MS spectral analysis was dose at Central Salt and Marine Chemicals Research Institute (CSMCRI), Bhavnagar, Gujarat, India.

3.6 *In vitro* antioxidant assays

3.6.1 Determination of 2, 2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) free radical scavenging activity

DPPH free radical scavenging activity was measured by the modified method of McCune and Johns (2002). The reaction mixture (3.0 ml) consisted of 1.0 ml DPPH in methanol (0.3 mM), 1.0 ml methanol and 1.0 ml different concentrations of the methanolic extract was incubated in dark for 10 min, after which the absorbance was measured at 517 nm against blank. For control, 1.0 ml of methanol was used in place

of extract. Ascorbic acid was used as positive control (Blois, 1958). Percentage of inhibition was calculated using the formula:

$$\text{Inhibition (\%)} = (A_0 - A_1 / A_0) \times 100$$

Where, A_0 is the absorbance of control and A_1 is the absorbance of sample.

3.6.2 Determination of hydroxyl ($^{\bullet}OH$) radical scavenging activity

Hydroxyl radical scavenging activity was measured by the method of Kunchandy and Rao (1990). The reaction mixture (1.0 ml) consisted of 0.1 ml of 2-deoxy-D-ribose (28 mM in 20 mM KH_2PO_4 -KOH buffer, pH 7.4), 0.5 ml of methanolic extract, 0.2 ml of premixed ethylenediaminetetraacetic acid (EDTA, 1.04 mM) and 0.2 mM $FeCl_3$ (1:1 v/v), 0.1 ml of H_2O_2 (1.0 mM) and 0.1 ml of ascorbic acid (1.0 mM) was incubated at 37 ºC for 1 h. 1.0 ml of thiobarbituric acid (TBA, 1%) and 1.0 ml of trichloroacetic acid (TCA, 2.8%) were added and incubated at 100 ºC for 20 min. After cooling, absorbance was measured at 532 nm, against blank. For control, 0.5 ml of water was used in place of the extract. Gallic acid was used as positive control (Kunchandy and Rao, 1990). Percentage of inhibition was calculated using the formula:

$$\text{Inhibition (\%)} = (A_0 - A_1 / A_0) \times 100$$

Where, A_0 is the absorbance of control and A_1 is the absorbance of sample.

3.6.3 Determination of superoxide anion ($O_2^{\bullet -}$) radical scavenging activity

The superoxide radical scavenging activity was measured by the method of Robak and Gryglewski (1988). The reaction mixture (3.0 ml) consisted of 0.5 ml of nitro blue tetrazolium (NBT) (0.3 mM), 0.5 ml of Tris-HCl buffer (16 mM, pH-8), 0.5 ml of nicotinamide adenine dinucleotide reduced (NADH) (0.93 mM), 0.5 ml of phenazine methosulphate (PMS) (0.12 mM) and 1.0 ml of different concentrations of methanolic extract. The superoxide radical generating reaction was started by the addition of PMS solution to the mixture. The reaction mixture was incubated at 25 ºC

for 5 min and then the absorbance was measured at 560 nm against a blank. For control, 1.0 ml of water was used in place of extract. Gallic acid was used as a positive control (Robak and Gryglewski, 1988). Percentage of inhibition was calculated using the formula:

$$\text{Inhibition (\%)} = (A_0 - A_1 / A_0) \times 100$$

Where, A_0 is the absorbance of control and A_1 is the absorbance of sample.

3.6.4 Determination of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical scavenging activity

ABTS radical cation ($ABTS^{\bullet+}$) decolouration test is a spectrophotometric method widely used for assessment of the antioxidant activity of various substances. The $ABTS^{\bullet+}$ scavenging activity was measured by the method of Re et al. (1999). ABTS was dissolved in water to a 7 mM concentration. $ABTS^{\bullet+}$ was produced by reacting ABTS stock solution with 2.45 mM potassium persulphate (final concentration) and allowing the mixture to stand in the dark at room temperature for 16 h before use. The radical was stable in this form for more than 2 days when stored in dark at room temperature. The $ABTS^{\bullet+}$ solution was diluted with methanol to an absorbance of 0.850 ± 0.05 at 734 nm. 3.0 ml of this $ABTS^{\bullet+}$ solution was added to 1.0 ml of different concentrations of the methanolic extract and incubated for 4 min at room temperature. Absorbance was measured spectrophotometrically at 734 nm. For control, 1.0 ml of methanol was used in place of extract. Ascorbic acid was used as a positive control (Alzoreky and Nakahara, 2001). Percentage of inhibition was calculated using the formula:

$$\text{Inhibition (\%)} = (A_0 - A_1 / A_0) \times 100$$

Where, A_0 is the absorbance of control and A_1 is the absorbance of sample.

3.6.5 Reducing capacity assessment

The reducing capacity assessment was measured by the modified method of Athukorala et al. (2006). 1.0 ml of different concentrations of methanolic extract was mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of $K_3Fe(CN)_6$ (30 mM). The mixture was then incubated at 50 ºC for 20 min. There after 2.5 ml of TCA (600 mM) was added to the reaction mixture and then centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (6 mM) and absorbance was measured at 700 nm against blank. Ascorbic acid was used as positive control (Oyaizu, 1986).

3.7 Animals

Wistar albino rats of both sexes were obtained from the animal house of Sarabhai Research Center (SRC), Vadodara. Animal colonies were maintained at Department of Biosciences, Saurashtra University, Rajkot. Animals were fed with commercial rat and mice food supplied by Pranav Agro Industries Ltd., Amrut Brand, Vadodara and water ad-libitum. They were maintained in a 12 h light/dark cycle at 25 ± 2º C. The study was approved by CPCSEA approved local ethical committee.

3.8 Selection of the doses for antioxidant activity and toxicity study

The dose considered for the experiment on rats was obtained from conversion of human dose of *Polyalthia longifolia* (3 g/kg). The conversion factor of human dose (per 200 g body weight) is 0.018 for rats (Ghosh, 1984). Hence the calculated dose for the rats is 270 mg/kg. Thus, *in vivo* antioxidant activity and sub-acute toxicity studies were done at two different doses 270 and 540 mg/kg body weight. Acute toxicity was done at four different doses 540, 1080, 2160 and 3240 mg/kg body weight.

3.9 *In vivo* antioxidant study

3.9.1 CCl_4 induced stress model

The stress was induced by CCl_4 (carbon tetrachloride) according to the method described by Jain et al. (2006) with few modifications. Wistar albino rats were divided into five groups (n = 6). Group I served as normal control and received distilled water (10 ml/kg, p.o.) daily for 5 days and received olive oil (2 ml/kg, i.p.) on days 3 and 4. Group II served as CCl_4 (toxin) control received distilled water (10 ml/kg, p.o.) daily for 5 days and received CCl_4:olive oil (1:1, 2 ml/kg bw, i.p.) on days 3 and 4. Groups III – IV were treated with methanolic extract of *P. longifolia* at doses of 270 and 540 mg/kg (p.o.) respectively for 5 days and received CCl_4:olive oil (1:1, 2 ml/kg, i.p.) on days 3 and 4, 1 h after administration of extract. Group V received standard drug Silymarin (100 mg/kg, p.o.) daily for 5 days and received CCl_4:olive oil (1:1, 2 ml/kg, i.p.) on days 3 and 4, 1 h after administration of standard drug Silymarin. Animals were sacrificed 48 h after last injection of CCl_4 under mild ether anesthesia. The blood was collected and allowed to stand for 30 min at 37°C and then centrifuged to separate the serum to estimate various biochemical parameters.

3.9.2 Water immersion stress model

The stress was induced by water immersion according to method described by Basile et al. (1990) with few modifications. Wistar albino male rats were divided into three groups (n = 5). Group I served as swim stress control and received water (10 ml/kg, p.o.) daily for 7 days. Groups II – III were treated with methanolic extract of *P. longifolia* at doses of 270 and 540 mg/kg (p.o.) respectively for 7 days. Animals were fasted for 24 h prior to water immersion stress induction. Stress was induced by forced swimming in the transparent plastic cylinder (height 27 cm, diameter 15.5 cm) containing water to the height of 17 cm maintained at 25ºC for 12 h. After 12 h of water immersion stress, the animals under mild ether anesthesia, were sacrificed and the blood was collected and allowed to stand for 30 min at 37°C and then centrifuged to separate the serum to estimate various biochemical parameters.

In both the models, various *in vivo* antioxidant parameters were estimated from liver. The liver was quickly removed and perfused immediately with ice-cold saline (0.9% NaCl). A portion of the liver was homogenized in chilled Tris-HCl buffer (0.025 M, pH 7.4) using a homogenizer. The homogenate obtained was centrifuged at 5,000 rpm for 10 min, supernatant collected and used for analysis.

3.10 Biochemical analysis from serum

The absorbance of all the biochemical parameters was measured in a UV–VIS Spectrophotometer (Shimadzu, Tokyo, Japan).

3.10.1 Estimation of total protein content

The total serum protein was estimated by modified Biuret method (Gornall et al., 1949) using the total protein test kit (Span Diagnostics Ltd.).

3.10.1.1 Reagents

Reagent I: Biuret Reagent (Copper sulphate, 7 mM/L; sodium hydroxide 200 mM/L; sodium potassium tartrate 20 mM/L; surfactant)

Reagent II: Protein Standard (BSA, 6.5 g/dl)

3.10.1.2 Procedure

3.0 ml of Reagent I was added to all the test tubes. Thereafter, 0.03 ml serum was added for the test and 0.03 ml Reagent II was added for the standard, while in blank 0.03 ml of D/W was added. They were then mixed well and incubated at 37 ºC for 5 minutes. The absorbance was read at 578 nm.

3.10.2 Estimation of albumin content

The serum albumin was estimated by the method given by Corcoran and Durnan (1977) using albumin test kit (Span Diagnostics Ltd.).

3.10.2.1 Reagents

Reagent I: Albumin reagent (Succinic acid, 37mM/L; Bromocresol green, 0.15 mM/L; NaOH, 1mM; Buffer pH 3.68)

Reagent II: Albumin standard (BSA, 4 g/dl)

3.10.2.2 Procedure

3.0 ml of albumin reagent (Reagent I) was added to all the test tubes. Thereafter, 0.03 ml serum was added for the test and 0.03 ml Reagent II was added for the standard, while in blank 0.03 ml of D/W was added. They were then mixed well and incubated at room temperature for 1 min. The absorbance was read at 630 nm.

3.10.3 Estimation of urea content

The serum urea was estimated by Enzymatic Urease (Berthelot) method (Fawcett and Scott, 1960) using Urea Berthelot test kit (Span Diagnostics Ltd.).

3.10.3.1 Reagents

Reagent I- Urea enzyme reagent (Phosphate buffer, 20 mM, Urease, >20000 U/L, Sodium Nitroprusside 3.2 mM/L, Sodium Salicylate, 60 mM/L)

Reagent II- Urea chromogen reagent [Sodium hypochlorite (0.2 %)]

Reagent III- Urea standard 50 mg dL^{-1}

Reagent IV- Purified water

Solution I: To Reagent I add 50 ml Reagent IV and mix gently.

Solution II: Dilute the contents of Reagent II, with 160 ml reagent IV

3.10.3.2 Procedure

1.5 ml Solution I was added to clean test tubes. 0.01 ml serum was added for the test and 0.01 ml Reagent III was added for the standard. It was then mixed well and incubated at 37 ºC for 3 min; then 1.5 ml of Solution II was added. It was then mixed

well and incubated at 37 ºC for 5 min. The absorbance was read at 578 nm against reagent blank.

3.10.4 Estimation of cholesterol content

Serum cholesterol was estimated by the method of Trinder (1969) using test kit (Span Diagnostic Ltd.)

3.10.4.1 Reagents

Reagent I: Cholesterol reagent (Good's buffer, pH 6.7, 50 mmol/L; cholesterol esterase, ≥ 100 U/L; cholesterol oxidase and peroxidase, ≥ 50 U/L and ≥ 3 KU/L; 4-aminoantipyrine, 0.4 mmol/L)

Reagent II: Cholesterol standard, 200 mg/dl

3.10.4.2 Procedure

3.0 ml of cholesterol reagent (Reagent I) was added to all the test tubes. Thereafter, 0.03 ml serum was added for the test and 0.03 ml Reagent II was added for the standard, while in blank 0.03 ml of D/W was added. They were then mixed well and incubated at 37 ºC for 10 min. The absorbance was read at 505 nm.

3.10.5 Estimation of alkaline phosphatase activity

Alkaline phosphatase activity was estimated by the method of Kind and King (1954) using ALP test kit (Span Diagnostics Ltd.).

3.10.5.1 Reagents

Reagent I: Buffered substrate, pH 10.0

Reagent II: Chromogen reagent

Reagent III: Phenol standard, 10 mg%

Working solution: Reconstitute one vial of reagent I, buffered substrate with 2.2 ml of purified water

3.10.5.2 Procedure

All the test tubes were marked properly as blank (B), standard (S), control (C), and test (T). 0.5 ml of working buffered substrate was added in clean tubes. 1.5 ml of purified water was added in all the tubes. They were mixed well and incubated at 37 °C for 3 min. 0.05 ml of serum was added in test (T), 0.05 ml of reagent III (Phenol standard) was added in standard (S) and 0.05 ml of purified water was added in blank (B) tubes. All the tubes were mixed well and incubated at 37 °C for 15 min. 1 ml of reagent II was added in all the tubes. 0.05 ml of serum was added in control (C). All the tubes were mixed well and O.D. was measured at 510 nm. Serum alkaline phosphatase activity is expressed as KA units.

3.10.6 Estimation of glutamate oxaloacetate transaminase activity

Serum glutamate oxaloacetate transaminase was estimated by the method of Reitman and Frankel (1957) using GOT test kit (Span Diagnostics Ltd.).

3.10.6.1 Reagents

Reagent I: Buffered Aspartate α-KG substrate, pH 7.4
Reagent II: DNPH colour reagent
Reagent III: Sodium Hydroxide, 4 N
Reagent IV: Working Pyruvate Standard, 2 mM
Solution I: Dilute 1 ml of Reagent III up to 10 ml of D/W

3.10.6.2 Procedure

0.25 ml of Reagent I was added in clean test tubes and incubated at 37ºC for 5 minutes. 0.05 ml of serum was added in the test, 0.05 ml Reagent IV was added in standard and 0.05 ml distilled water was added in the blank. They were mixed well and incubated at 37 ºC for 60 minutes. Thereafter, 0.25 ml of Reagent II was added to all the tubes, mixed well and allowed to stand at room temperature (15-30 ºC) for 20 minutes. Then 2.5 ml of Solution I was added to all the tubes, mixed well and allowed

to stand at room temperature (15-30 ºC) for 10 min. The absorbance of blank, standard and test were read at 505 nm.

3.10.7 Estimation of glutamate pyruvate transaminase activity

Serum glutamate pyruvate transaminase was estimated by the method of Reitman and Frankel (1957) using GPT test kit (Span Diagnostics Ltd.).

3.10.7.1 Reagents

Reagent I: Buffered Alanine α-KG substrate, pH 7.4
Reagent II: DNPH colour reagent
Reagent III: Sodium Hydroxide, 4 N
Reagent IV: Working Pyruvate Standard, 2 mM
Solution I: Dilute 1 ml of Reagent III up to 10 ml of D/W

3.10.7.2 Procedure

0.25 ml of Reagent I was added in clean test tubes and incubated at 37 ºC for 5 minutes. 0.05 ml of serum was added in the test, 0.05 ml Reagent IV was added in the standard and 0.05 ml distilled water was added in the blank. They were mixed well and incubated at 37 ºC for 30 minutes. Thereafter, 0.25 ml of Reagent II was added to all the tubes, mixed well and allowed to stand at room temperature (15-30 ºC) for 20 minutes. Then 2.5 ml of Solution I was added to all the tubes, mixed well and allowed it to stand at room temperature (15-30 ºC) for 10 min. The absorbance of blank, standard and test were read at 505 nm.

3.11 Antioxidant parameters from liver homogenate

3.11.1 Estimation of total protein content

The total protein content was measured by the method of Lowry et al. (1951). The assay mixture contained 0.1 ml of liver homogenate, 0.9 ml of NaOH (0.1 N) and 5.0 ml of alkaline copper sulphate reagent. The reaction mixture was incubated for 15 min

at room temperature and then 0.5 ml of Folin Ciocalteau reagent (1 N) was added. The reaction mixture was further incubated for 30 min at room temperature. The absorbance was measured at 660 nm. Bovine serum albumin was used as standard.

3.11.2 Estimation of lipid peroxidation assay

Thiobarbituric acid reactive substance, the last product in lipid peroxidation pathways was estimated by the methods of Niehaus and Samuelsson (1968). 0.2 ml of liver homogenate was treated with 4 ml of TBA-TCA-HCl reagent (1:1:1ratio, 0.37% TBA, 0.25 N HCl and 15% TCA) and placed in boiling water bath for 15 min, cooled and centrifuged for 5 min at 5000 rpm. The absorbance of clear supernatant was measured against blank at 535 nm. The values were calculated using molar extinction coefficient of chromophore (1.56×10^5 $M^{-1}cm^{-1}$).

3.11.3 Estimation of glutathione reduced level

The glutathione reduced was estimated by the method of Ellman (1959). 1.0 ml of liver homogenate in Tris-Hcl buffer (25 mM, pH 7.4) was added to 1.0 ml of TCA (10 %) and mixed thoroughly. The mixture was then centrifuged at 5000 rpm for 5 min at room temperature. The assay mixture consisted of 1.0 ml of this supernatant, 0.5 ml of Ellman's reagent (0.02% of 5,5' dithio(bis)nitrobenzoic acid (DTNB) in 1% tri sodium citrate) and 3 ml of phosphate buffer (200 mM, pH 8.0). The yellow colour developed was immediately measured at 412 nm. The values were calculated using molar extinction coefficient of chromophore (1.36×10^4 $M^{-1}cm^{-1}$).

3.11.4 Estimation of glutathione peroxidase activity

The glutathione peroxidase activity was measured by the method described by Rotruck et al. (1973) and Mills (1959). The reaction mixture contained 1.0 ml of Tris-HCl buffer (400 mM, pH 7.0), 0.5 ml of sodium azide (10 mM), 0.5 ml liver homogenate, 0.5 ml glutathione (4mM) and the reaction was started by addition of 1.0 ml of H_2O_2 (1.25 mM). The reaction mixture was incubated for 3 min at 37 ºC. The reaction was stopped by addition of 1.5 ml TCA (10%) and centrifuged for 5 min at 5000 rpm. GSH in the protein free filtrates was determined by mixing 1.0 ml of

supernatant with 2.0 ml of phosphate buffer (200 mM, pH 8.0) and 1.0 ml of DTNB solution (0.02% DTNB in 1% tri sodium citrate). The absorbance was read at 412 nm. Glutathione peroxidase activity is expressed as μM GSH utilized / min / mg protein. For control, 0.5 ml of Tris-HCl buffer (25 mM, pH 7.4) was used in place of the liver homogenate.

3.11.5 Estimation of catalase activity

The catalase was colorimetrically assayed as described by Sinha (1972). The reaction mixture (1.5 ml) contained 1.0 ml of phosphate buffer (10 mM, pH 7.0), 0.1 ml of liver homogenate and the reaction was started by addition of 0.4 ml of H_2O_2 (2000 mM). The reaction mixture was incubated for 3 min at room temperature. The reaction was stopped by addition of 2.0 ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio), was incubated at 100 °C for 2 min. The absorbance was measured at 620 nm. Catalase activity is expressed as μM H_2O_2 consumed / min / mg protein. For control, 0.1 ml of Tris-HCl buffer (25 mM, pH 7.4) was used in place of the liver homogenate.

3.11.6 Estimation of superoxide dismutase activity

The superoxide dismutase activity was analysed by the method described by Kakkar et al., (1984). The assay mixture contained 0.1 ml of liver homogenate, 1.2 ml of sodium pyrophosphate buffer (52 mM, pH 8.3), 0.15 ml of PMS (0.186 mM), 0.35 ml of NBT (0.3 mM), 1.0 ml of distilled water, 0.25 ml of NADH (0.75 mM). Reaction was started by addition of NADH. After incubation at 30 ºC for 90s, the reaction was stopped by addition of 1.0 ml of glacial acetic acid. Reaction mixture was stirred vigorously and shaken with 4.0 ml of n-butanol. The mixture was centrifuged for 5 min at 5000 rpm and butanol layer was taken out. Color intensity of chromogen in butanol layer was measured at 560 nm. A single unit of SOD is expressed as 50% inhibition of nitro blue tetrazolium reduction / min / mg protein. For control, 0.1 ml of Tris-HCl buffer (0.025 M, pH 7.4) was used in place of the liver homogenate.

3.12 Acute toxicity study

Acute oral toxicity (Ryu et al., 2004), study was performed as per OECD-404 guidelines (1987). 10 rats/group (5 males and 5 females) were used for the study. Group 1 was control group and other four groups were that of methanolic extract at different doses (540, 1080, 2160 and 3240 mg/kg body weight). Single dose of the extract was administrated orally to each animal. Signs of toxicity, body weight, feed and water consumption of each animal was observed every day for 14 days.

3.13 Sub-acute toxicity study

Sub-acute oral toxicity (Khan et al., 2002), study was performed. Ten rats/group (5 males and 5 females) were used for the study. Group 1 was control group and other two groups were that of methanolic extract at different doses (270 and 540 mg/kg body weight). Repeated dose of the extract was administrated daily orally to each animal for 21 days. Signs of toxicity, body weight, feed and water consumption of each animal were observed every day for 21 days.

3.14 Cage side observations

In acute toxicity study, the animals were observed prior to dosing. Thereafter, observations were made at every hour, for five hours and then at 24 h and then every day for 14 days. All observations were systematically recorded, with individual records being maintained for each animal. Cage side observations included evaluation of skin and fur; eyes; respiratory effect; autonomic effects, such as salivation, diarrhea, urination; and central nervous system effects, including tremors and convulsions, straub tail, ptosis, relaxation, changes in the level of activity, gait and posture, reactivity to handling, altered strength and stereotypy (Demma et al., 2007, Nair et al., 2009). In sub-acute toxicity study, the observation continued up to 21 days.

3.15 Feed and water consumption and body weight measurement

The animals were monitored daily for mortality, feed and water consumption and changes in body weight for 14 days in acute toxicity study and for 21 days in sub-acute toxicity study. Feed intake was calculated as g/animal/day. Water intake was calculated as ml/animal/day. Individual animal body weight was recorded daily till the end of the experiment.

3.16 Biochemical analysis in acute and sub-acute toxicity studies

At the end of the study, all animals were fasted for 12 h and then under mild ether anesthesia, animals were sacrificed and blood samples were collected. The biochemical parameters *viz.* total protein, albumin, urea, cholesterol, alkaline phosphatase, glutamate oxaloacetate transaminase and glutamate pyruvate transaminase were estimated from serum using kits (Span Diagnostic Ltd., India). The absorbance of all the biochemical parameters was measured in a UV–VIS Spectrophotometer (Shimadzu, Tokyo, Japan).

3.17 Hematological analysis in acute and sub-acute toxicity studies

At the end of the study, all animals were fasted for 12 h and then under mild ether anesthesia, animals were sacrificed and blood samples were collected. Blood was collected immediately into tubes containing EDTA for analysis of hematological parameters *viz.* hemoglobin, total red blood cells (R.B.C.), packed cell volume, mean cell volume (M.C.V.), mean cell hemoglobin (M.C.H.), mean cell hemoglobin concentration (M.C.H.C.), total white blood cells (W.B.C.), neutrophils, lymphocytes, eosinophils, monocytes, basophiles, total platelet count (Theml et al., 2004) using hematology analyzer Sysmex XS800i (Sysmex corporation, USA).

3.18 Relative organ weight (ROW) analysis in acute and sub-acute toxicity studies

Thymus glands, lungs, heart, liver, spleen, kidneys, adrenal glands, testes, uterus and ovaries were mopped with filter paper, weighed and the relative weights were calculated and expressed as g/100g b.w.

$$ROW = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rats on sacrifice day (g)}} \times 100$$

3.19 Histopathological studies in sub-acute toxicity study

Histopathology study of liver, kidney, heart, spleen and thymus glands was performed.

3.19.1 Methods for histopathological studies

The organs (Liver, Heart, Kidney, Spleen and Thymus glands) were transferred to 4% formalin solution for fixation and later on processed for histopathological studies following the standard procedure described by Raghuramulu et al. (1983). The microtome sections were cut processed and stained with haematoxylin and eosin. The section thus obtained was scanned in Trinocular Carl-Zeiss microscope (Germany) under different magnifications. Changes if any in the cytoarchitecture were noticed.

3.19.1.1 Fixation

Fixation is the process of preserving, hardening and preventing postmortem changes of the tissues. The tissues were excised out immediately after sacrificing, cleaned of extraneous matter, cut in to pieces of such thickness that the fixative readily penetrated throughout the tissue to be fixed. Tissue was transferred to the 4% formaldehyde solution and allowed to remain in it till they were taken up for processing.

3.19.1.2 Tissue processing

Tissue processing involves dehydration, clearing and infiltration of the tissue with paraffin. The usual dehydrating agent is ethyl alcohol; acetone and isopropyl alcohol can also be used. Following dehydration, the tissue was transferred to a paraffin solvent, which is miscible with the dehydrating agent as well. These are known as clearing agents such as chloroform and xylene. Tissue were thoroughly washed by placing them under running tap water and then conveyed through a series of the following solvents as per schedule for dehydration, clearing and paraffin infiltration.

Alcohol 70%	-	20 minutes
Alcohol 80%	-	20 minutes
Alcohol 90%	-	20 minutes
Alcohol 95% (2 changes)	-	20 minutes each
Isopropyl alcohol	-	20 minutes
Acetone (2 changes)	-	20 minutes each
Chloroform (3 changes)	-	20 minutes each
Melted paraffin wax (60 ºC) (3 changes)	-	30 minutes each

Then the tissues were embedded in paraffin wax to prepare tissue blocks, which were oriented so that sections could be cut in desired plane of the tissue. Tissues were then fixed to metal object holder after trimming them to suitable size.

3.19.1.3 Section cutting

A smear of 5% Mayer's egg albumin was prepared and smeared onto the slide and dried. The tissue sections of 6 µm thickness were cut with the help of Spencer type rotating microtome. The tissue sections were put on slide and then section were floated in water on slide at 55-60 °C, water drained off and slide dried on hot plate at about 50 °C for 30 minutes. This section was ready for staining.

3.19.1.4 Staining procedure

3.19.1.4.1 Reagents

1). Mayer's heamotoxyline stain
2). Eosin stain, 2% w/v in alcohol

After fixing the sections on slides, they were stained by serially placing them in the following reagents:

Xylol (2 changes)	-	3 minutes
Acetone	-	3 minutes
Alcohol 95%	-	3 minutes
Haematoxyline stain	-	20 minutes
Running water	-	20 minutes
Eosin stain	-	5 minutes
Alcohol 95% (3 changes)	-	3 minutes each
Acetone (2 changes)	-	3 minutes each
Xylol (2 changes)	-	3 minutes each

After passing through all the above reagents and stains, the slides were mounted with D.P.X. (Diphenyl Phthalate Xylene) and cover slip was placed. Care was taken to avoid air bubbles while mounting the slide. The slides were viewed under binocular research Carl Zeiss microscope (Germany) at various magnifications to note down the changes in the microscopic features of the tissues studied.

3.20 Statistical analysis

The results are expressed as mean ± SEM; data was analyzed using Student's t-test and results are considered significant when $P < 0.05$.

CHAPTER 4: RESULTS

4.1 Macroscopic characteristics

P. longifolia is a tall tree up to 25 m tall, with a straight trunk and horizontal branches, dense crown and hairless twigs. The leaves were ovate-oblong to narrowly lance-shaped, 11-22 cm X 2-5 cm size, wedge-shaped to round at base, wavy at margins and 4-8 mm long petiole (Fig. 4).

4.2 Microscopic characteristics

Transverse section of leaf is shown in Fig. 5. Leaf is dorsiventral. The upper and lower epidermis was single layered. The palisade tissue was two layered. Prismatic crystals of calcium oxalate were found. The mesophyll was small, circular, spherical cell with plenty of air spaces. The collenchymas were of 2-3 layers. Oil glands were found in palisade tissue and in lower midrib (Figs. 5, 6). The lower epidermis had more number of trichomes than the upper epidermis. The anomocytic stomata were present in lower epidermis. The stomata were surrounded by small subsidiary cells, whereas the guard cells were comparatively larger in size and each stoma was surrounded by 4-5 subsidiary cells. There was a large vascular bundle covered with sclerenchymatous ring.

The crude powder of *P. longifolia* leaf was light green in color with characteristic odour. The powder characteristics are shown in Fig. 7. The characteristics determined from the powder study were epidermis, mesophyll, multicellular blunt tip trichome, and phloem and spiral xylem vessel.

4.3 Physicochemical properties

The results of the physicochemical parameters are given in Table 4. The average values are expressed as percentage of air-dried material. The crude powder of *P. longifolia* showed 12% loss on drying. It contained 5.8% total ash, 0.4% acid insoluble ash and 2.5% water soluble ash. The percent extractive yield of crude powder extracted in petroleum ether was 4.60%; that extracted in alcohol was 20.11%, extracted in methanol was 20.16% and extracted in water was 15.25%. The pH of methanolic extract was 5.51. The extract was acidic in nature. The melting

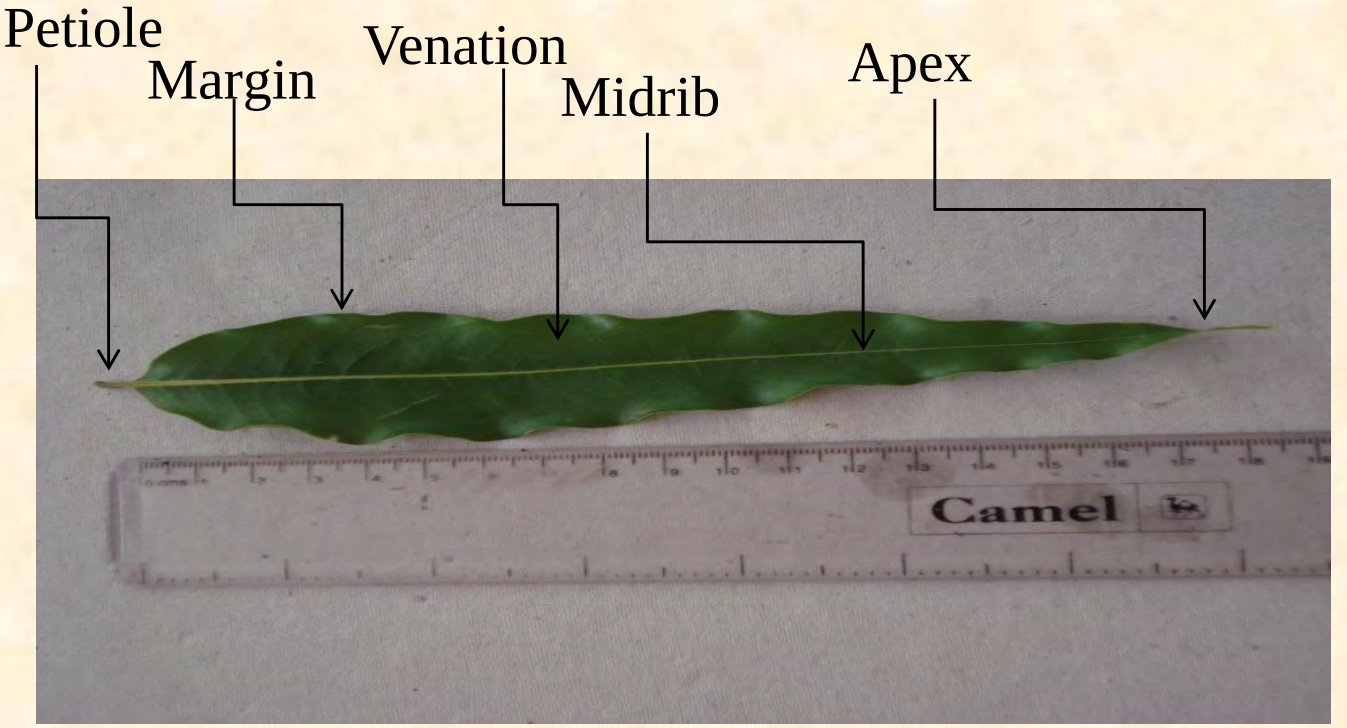
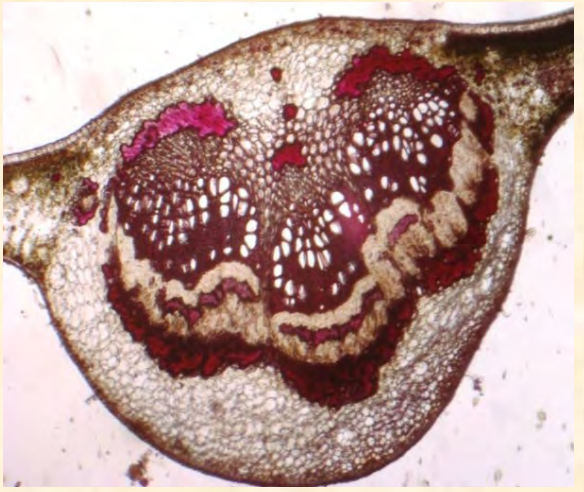
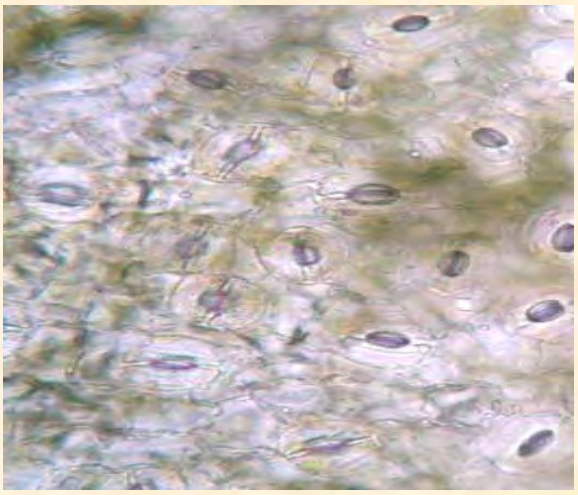


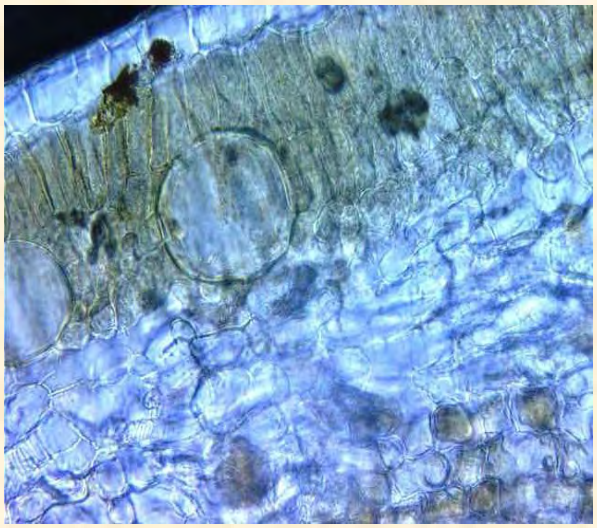
Fig. 4 Macroscopic study of *P. longifolia* leaf



T.S. of *P. longifolia* leaf

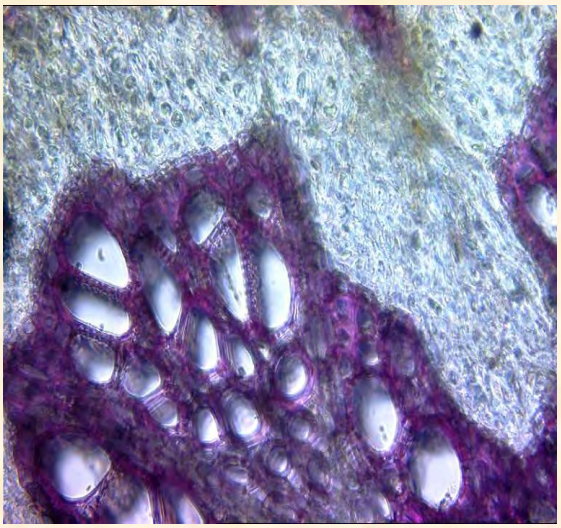


Stomata



Oil gland in palisade tissue

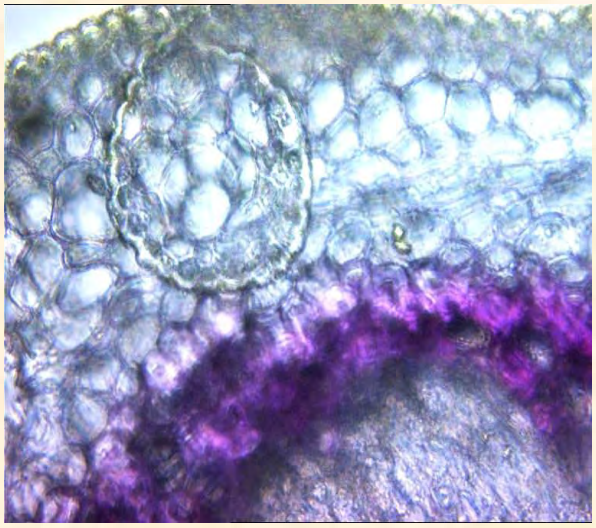
Lamina



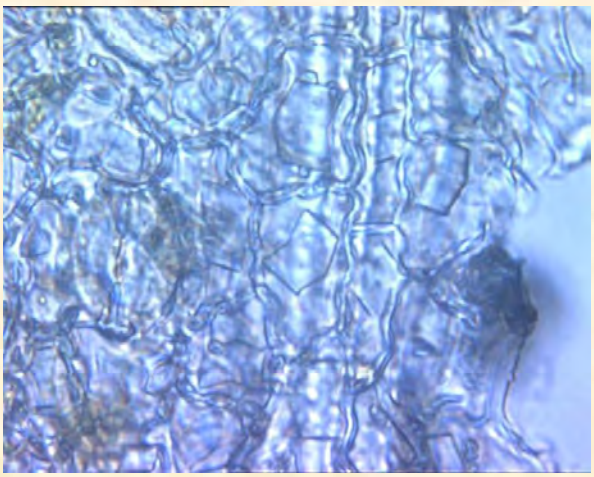
Xylem and Phloem

Fig. 5 Microscopic study of *P. longifolia* leaf

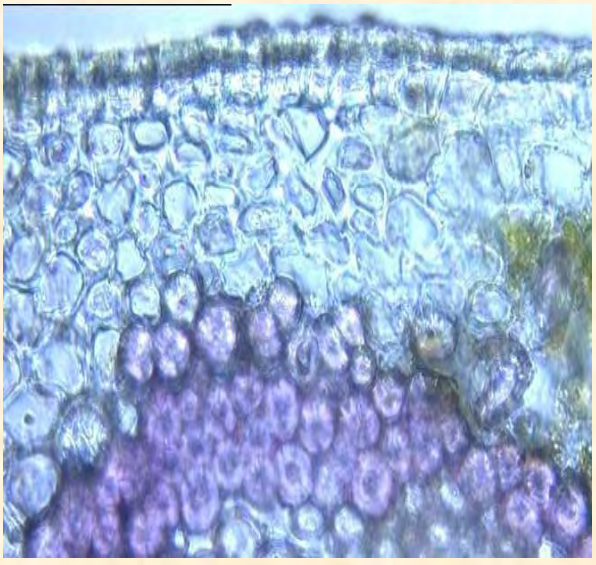
Trichome



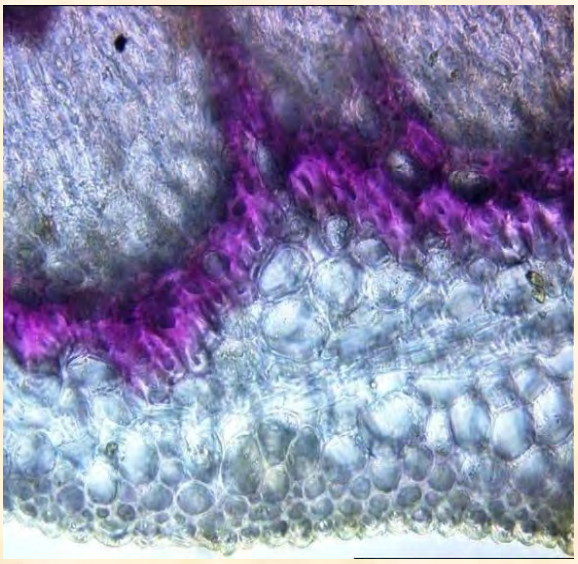
Oil gland in lower midrib



Calcium oxalate crystal

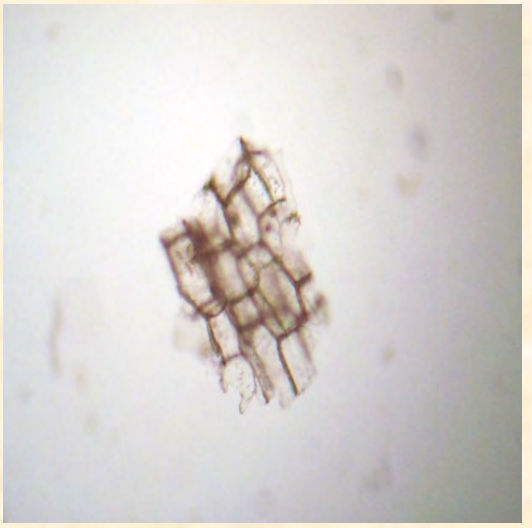


Upper midrib

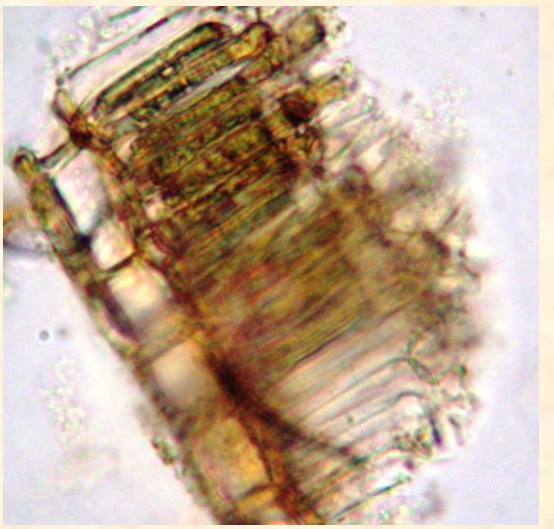


Lower midrib

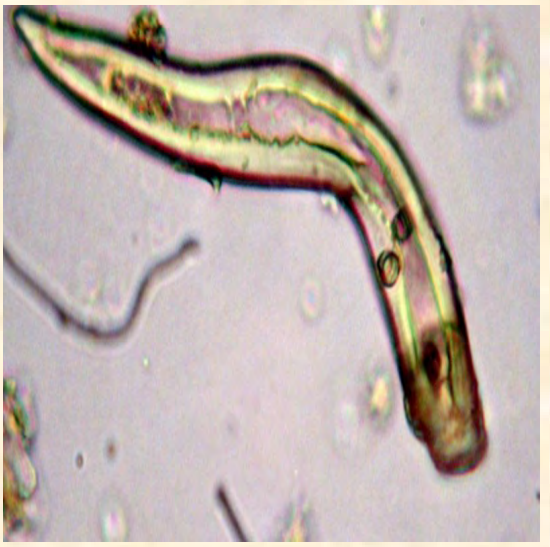
Fig. 6 Microscopic study of *P. longifolia* leaf



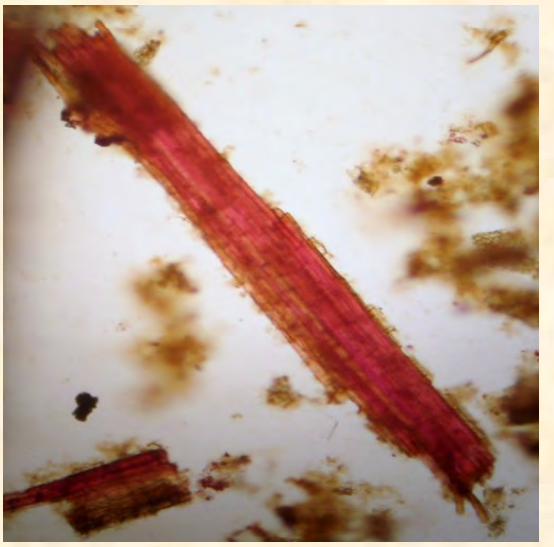
Epidermis



Mesophyll

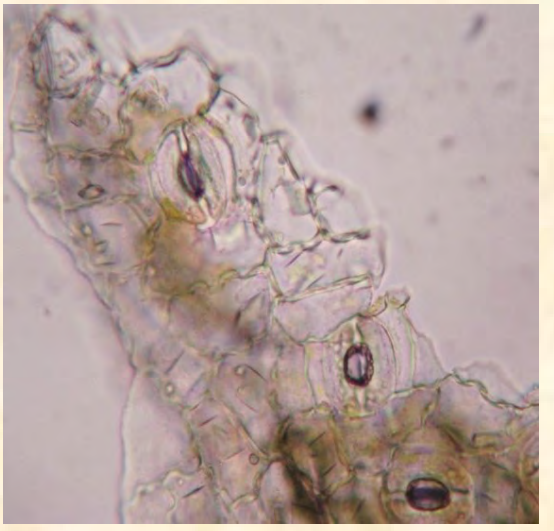


Trichome



Phloem

Xylem vessel



Anomocytic stomata

Fig. 7 Microscopic study of powder of *P. longifolia* leaf

point and boiling point of methanolic extract were 90 °C and 93 °C respectively (Table 4).

Table 4 Physicochemical parameters of leaves of *P. longifolia*

Parameters	**Value**
Total ash	5.8 % (w/w)
Acid insoluble ash	0.4 % (w/w)
Water soluble ash	2.5 % (w/w)
Loss on drying	12 % (w/w)
Petroleum ether soluble extractive	4. 60 % (w/w)
Alcohol soluble extractive	20.11 % (w/w)
Methanol soluble extractive	20.16 % (w/w)
Water soluble extractive	15.25 % (w/w)
pH of methanolic extract	5.51
Melting point of methanolic extract	90 ºC
Boiling point of methanolic extract	93 ºC

4.3.1 Solubility

The methanolic extract of *P. longifolia* was evaluated for qualitative solubility test for 8 solvents with varied polarities (Table 5)**.** The extract was maximum soluble in methanol (25 mg/ml) followed by dimethylsulphoxide (23 mg/ml) while the extract was less soluble in acetone (5 mg/ml) followed by chloroform (6.2 mg/ml). The extract was moderate soluble in other solvents such as petroleum ether (10 mg/ml), hexane (13 mg/ml), ethyl acetate (9.9 mg/ml) and 1, 4, dioxan (9.8 mg/ml).

Table 5 Solubility of methanolic extract of *P. longifolia* in different solvents

Solvents	Solubility (mg/ml)
Petroleum ether	10
Hexane	13
Chloroform	6.2
Ethyl acetate	9.87
1,4, Dioxan	9.85
Acetone	5
Dimethyl sulfoxide	23
Methanol	25

4.3.2 Heavy metal analysis

Lead, chromium, arsenic and cadmium were present in the methanolic extract with varying values (Table 6). The extract contained 3.2 ppm lead, 10.2 ppm chromium, 7.3 ppm arsenic and 0.3 ppm cadmium. Mercury was absent in the extract. Although, there was minor presence of some heavy metals but the extracts did not exceed the limit given according to the WHO guidelines (1998).

Table 6 Heavy metals in methanolic extract of *P. longifolia*

Heavy metal	Results (ppm)
Mercury	Not detected
Lead	3.2
Chromium	10.2
Arsenic	7.3
Cadmium	0.3

4.4 Qualitative phytochemical analysis

The results of qualitative phytochemical analysis of the crude powder and the methanolic extract of *P. longifolia* are shown in Table 7. The extract contained maximum amount of cardiac glycosides and alkaloids. In the crude powder and the methanolic extract of *P. longifolia*, flavonoid, tannins, triterpenes and saponins were present in moderate amount while steroids and phlobatannins were absent. In three different alkaloids test, alkaloids were present in higher amount with Dragendroff's test followed by Wagner's test while alkaloids were present in lesser amount with Mayer's test in crude powder and the extract. Flavonoids were present in moderate amount with alkaline reagent test in crude powder and the extract.

Table 7 Qualitative phytochemicals analysis of crude powder and methanolic extract of *P. longifolia* leaves

Phytochemicals		**Crude powder**	**Methanolic extract**
Alkaloids	**Dragendroff**	++	+++
	Mayer's	+	+
	Wagner's	+	++
Flavonoids	**Shinoda test**	-	++
	Alkaline reagent test	++	++
Tannins		++	++
Phlobatannins		-	-
Triterpenes		++	++
Steroids		-	-
Saponins		+	++
Cardiac glycosides		+++	+++

− : absence, + : less presence, ++ : moderate presence,

+++ : high presence

4.5 Total phenol and flavonoid content

Total phenol and flavonoid content of methanolic extract of *P. longifolia* are shown in Fig. 8. The total phenol content was more than that of total flavonoid content in methanolic extract. The amount of total phenol was 85.31 ± 0.61 mg/g, while the amount of total flavonoid was 23.82 ± 0.46 mg/g.

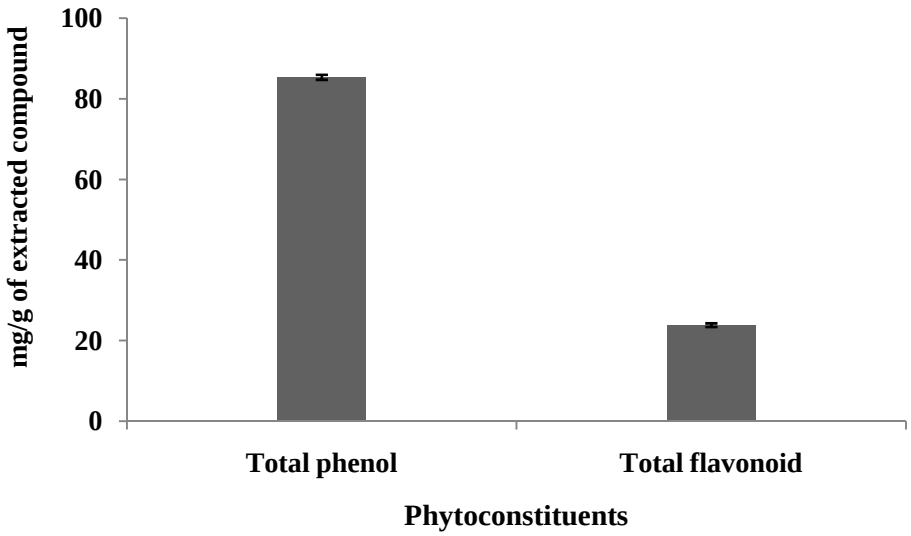


Fig. 8 Total phenol and flavonoid content of methanolic extract of *P. longifolia*

4.6 Crude alkaloid and saponin

Crude alkaloid and saponin content of methanolic extract of *P. longifolia* are shown in Fig. 9. The crude alkaloid content was more than that of crude saponin content in methanolic extract. The amount of crude alkaloid was 151 mg/g, while the amount of crude saponin was 56 mg/g.

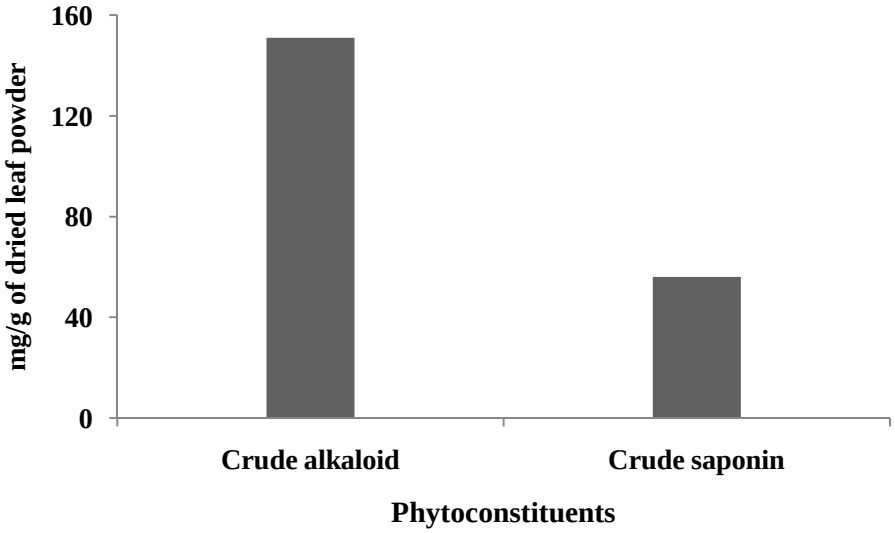


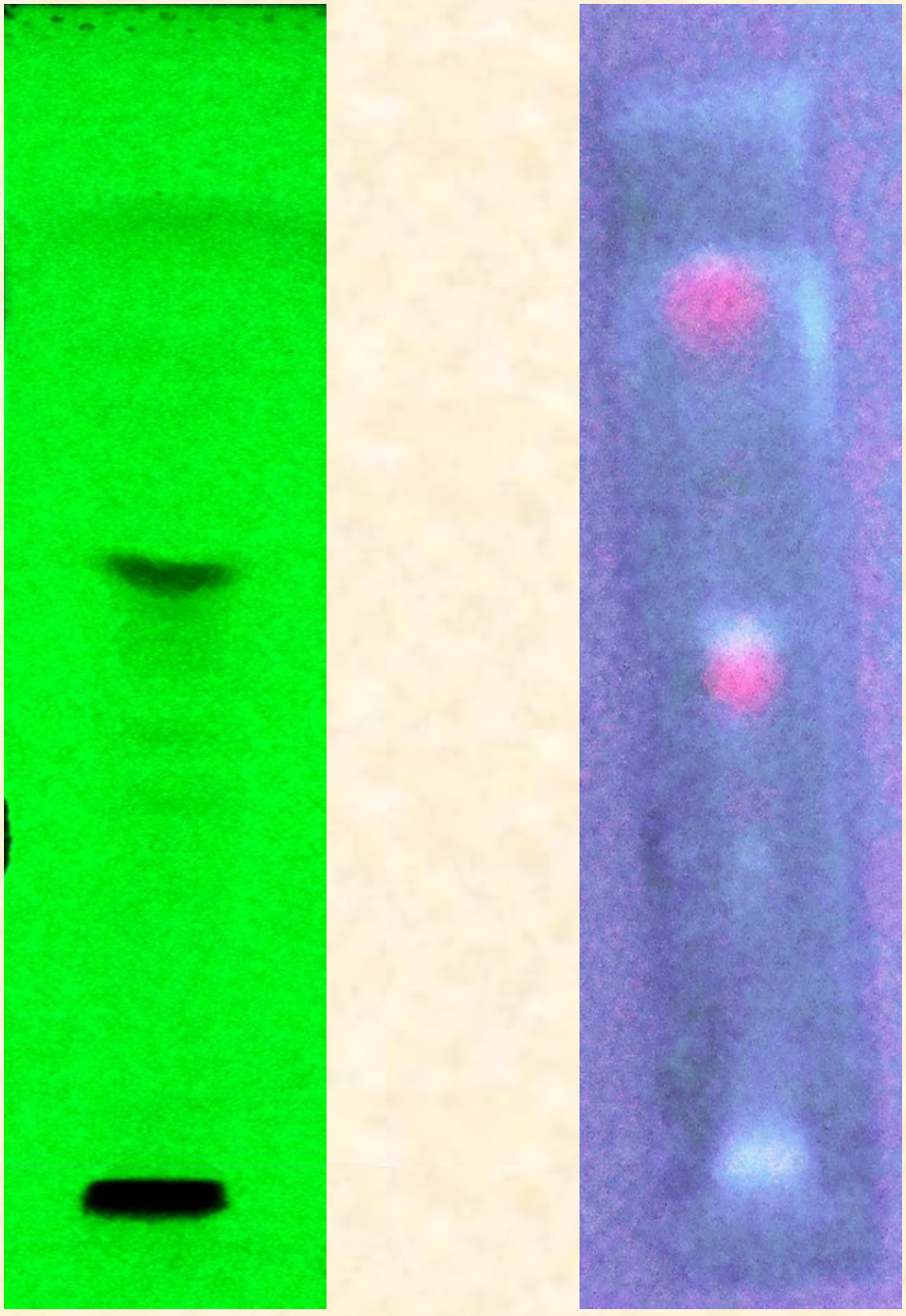
Fig. 9 Crude alkaloid and saponin content of methanolic extract of *P. longifolia*

4.7 HPTLC fingerprinting

The HPTLC fingerprinting of the methanolic extract are shown in Figs. 10, 11. The thin layer chromatography of methanolic extract is shown at 254 and 366 nm in Fig. 10. The extract showed 8 peaks in 200-500 nm spectral range. The maximum percentage area was covered in extract by peak no. 5 and 6 (Rf value 0.56) in Fig. 11.

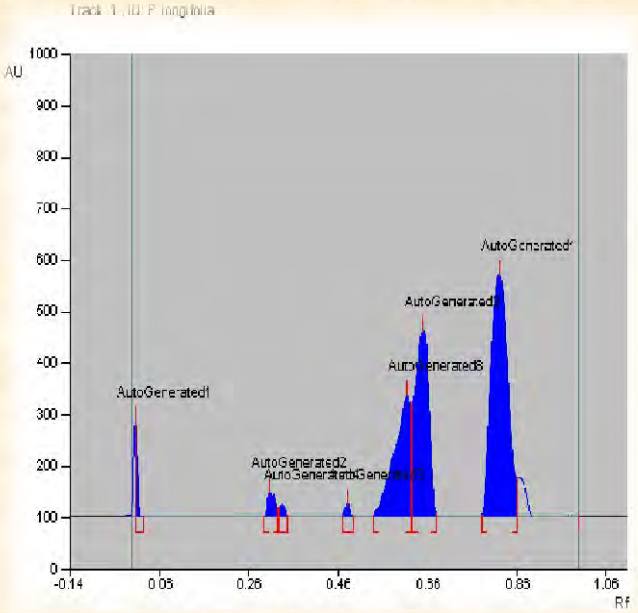
4.8 Spectral analysis

UV-Visible spectra is shown in Fig. 12. Maximum absorbance was found between the range 200-400 nm. IR spectra is shown in Fig. 13. The mid-infrared, approximately 4000–400 cm^{-1} (2.5–25 μm) may be used to study the fundamental vibrations and associated rotational-vibrational structure. Hydroxyl group stretching vibration (-OH) was obtained at 3537.573 cm^{-1}. Alken group stretching vibration (-CH) was obtained at 3167.22, 3130.57 and 2947 cm^{-1}. Kiton (C=O) stretching vibration (may be cyclic of benzene) was obtained at 1741.78 cm^{-1}. Simple C=O ring skeleton banding vibration was obtained at 1608.69 cm^{-1}. In GC-MS analysis m/z like 294, 316, 347, 325, 336, 375, 389, 349, 351 and 331 were obtained from the extract. NMR and GC-MS are shown in Figs. 14, 15a, 15b and 15c.

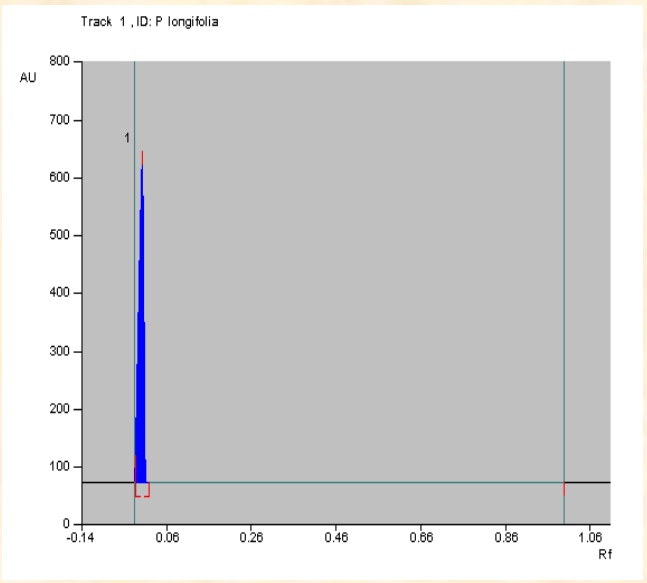


UV 254 nm **UV 366 nm**

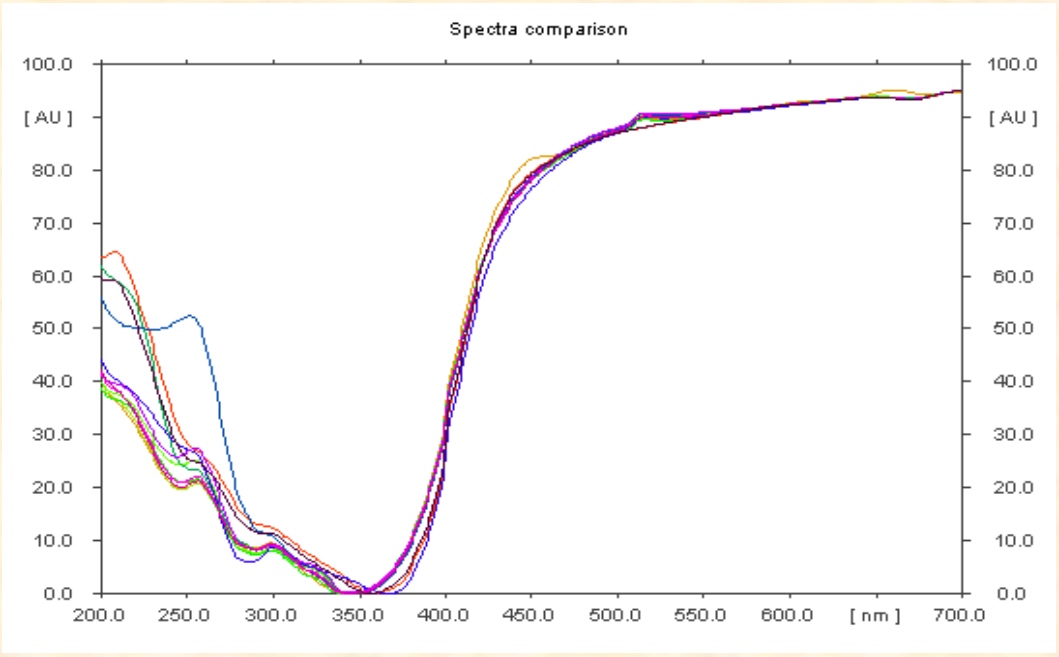
Fig. 10 Thin layer chromatography of methanolic extract of *P. longifolia*

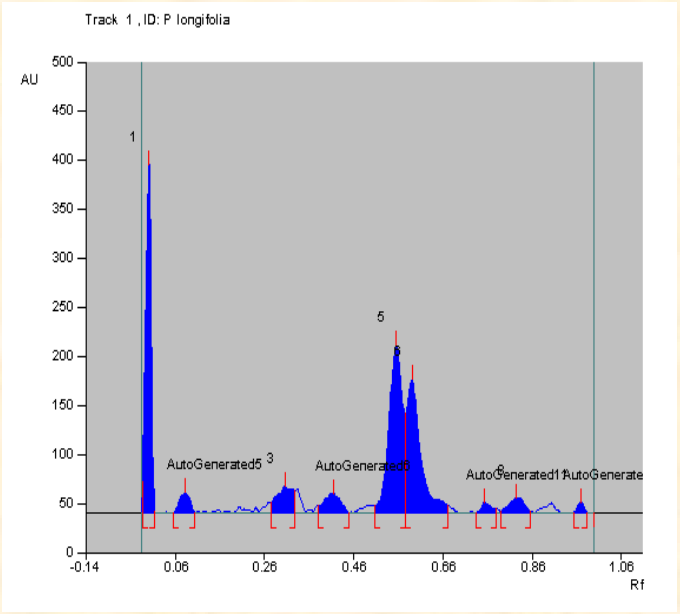


200 nm

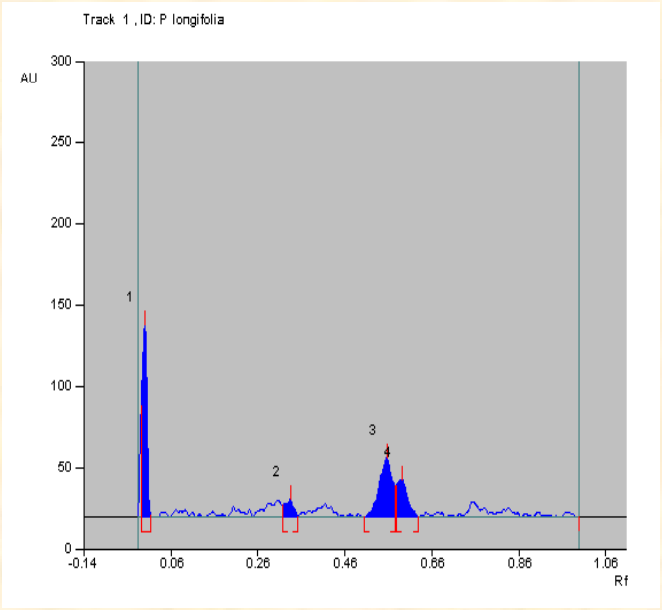


300 nm





400 nm



500 nm

Fig. 11 HPTLC spectra of methanolic extract of *P. longifolia*

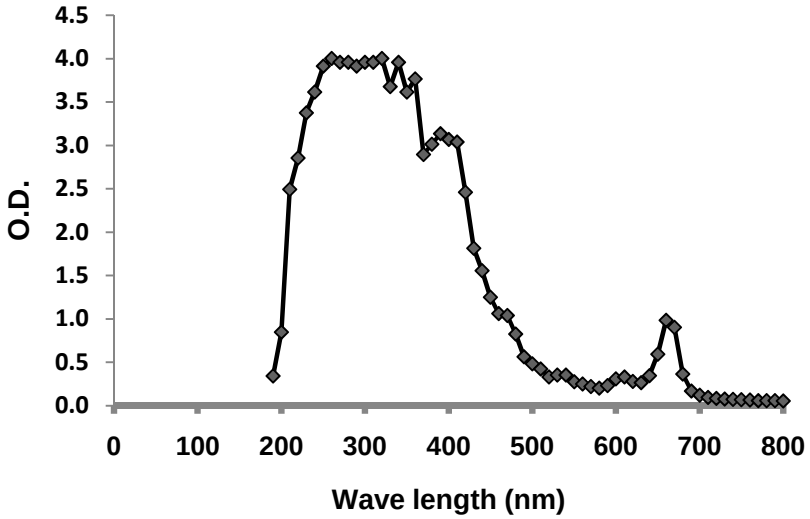


Fig. 12 UV-Visible spectra of methanolic extract of *P. longifolia*

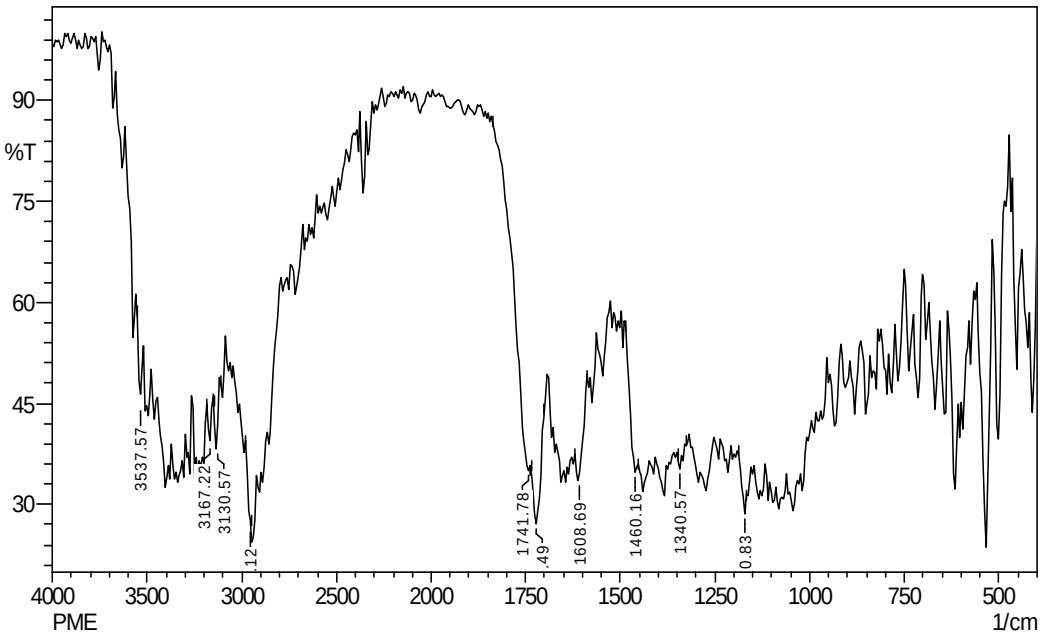


Fig. 13 IR spectra of the methanolic extract of *P. longifolia*

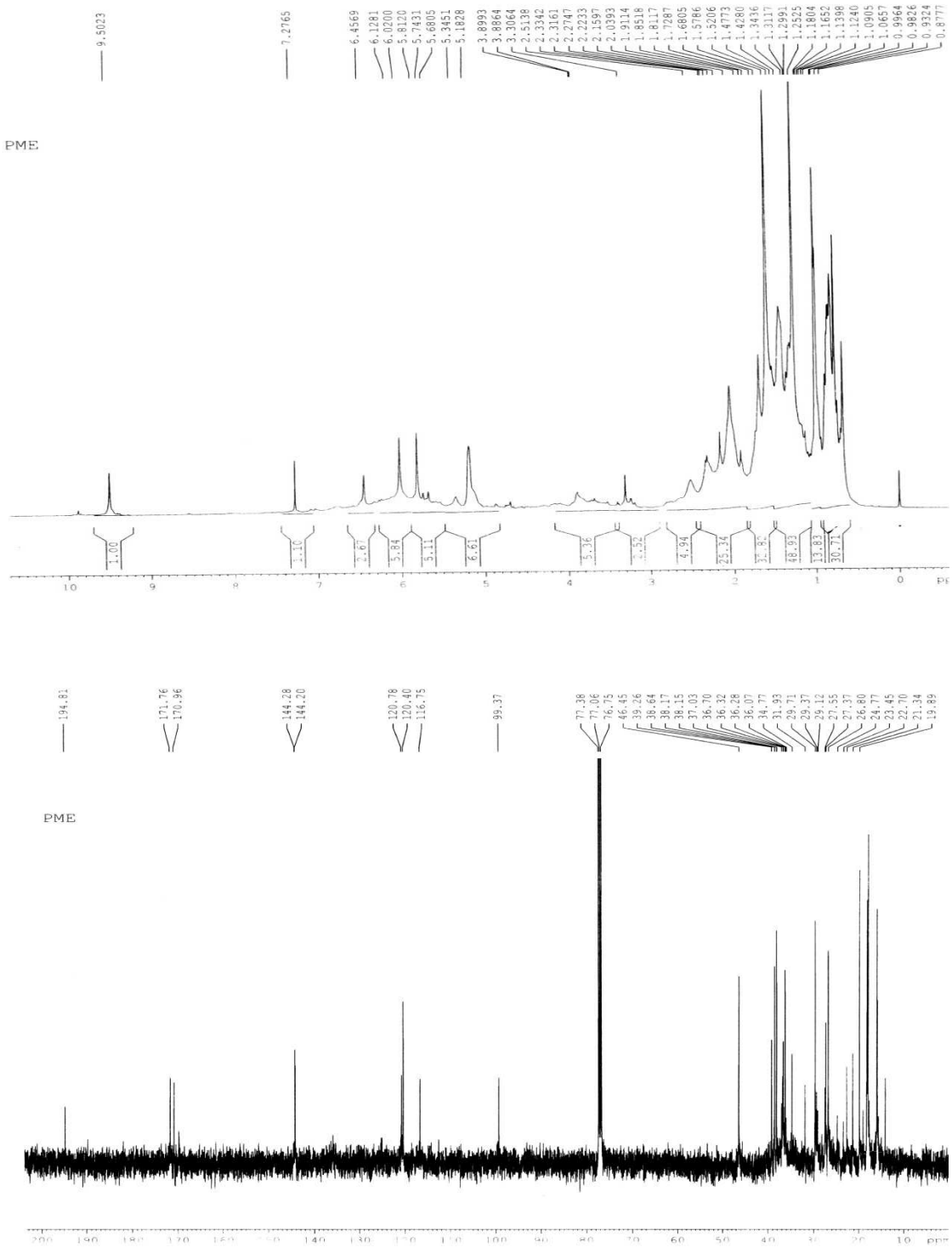
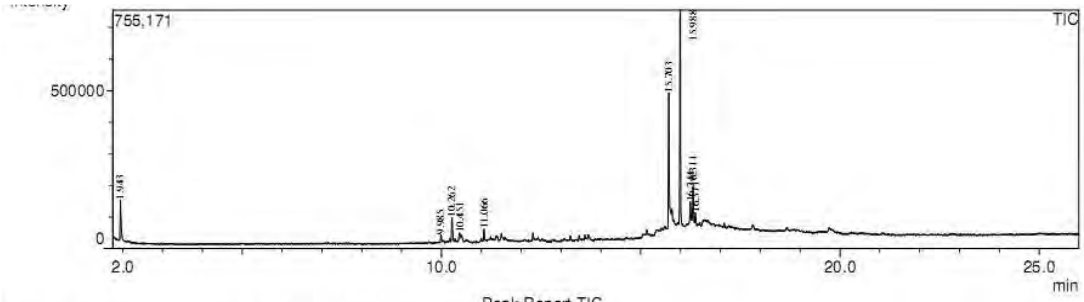
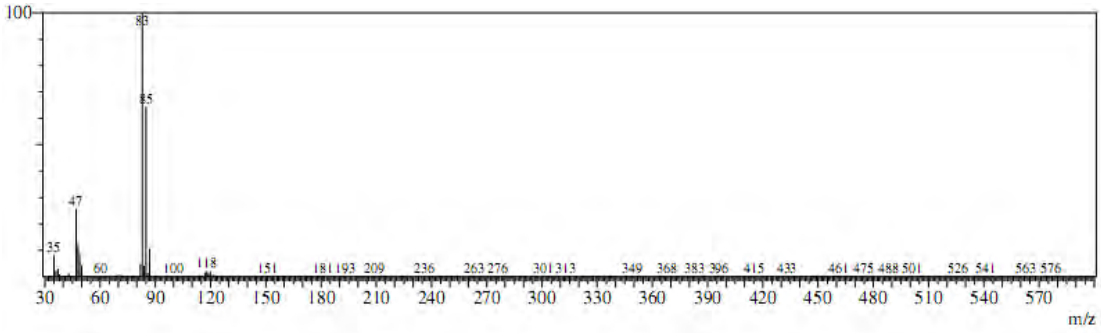


Fig. 14 ^{1}H and ^{13}C NMR spectra of the methanolic extract of *P. longifolia*

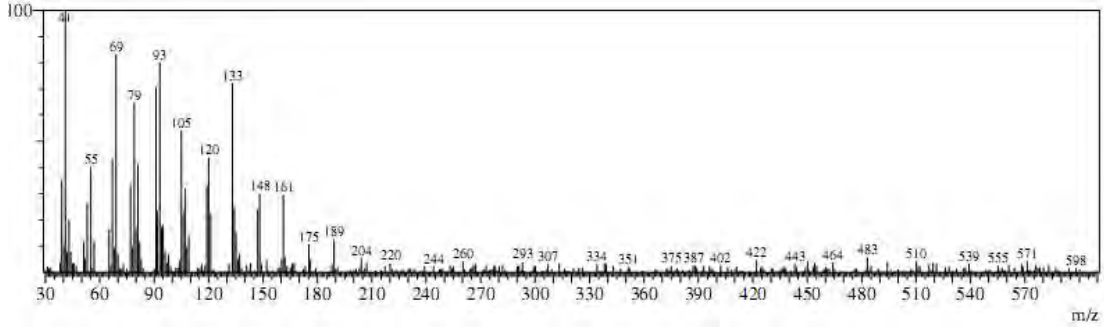


Peak Report TIC

Peak#	R.Time	Area	Area%	Height	Height%	Name
1	1.943	216472	8.54	126474	7.81	
2	9.985	40632	1.60	23979	1.48	
3	10.262	133696	5.28	76449	4.72	
4	10.451	48798	1.93	23720	1.46	
5	11.066	40943	1.62	35166	2.17	
6	15.703	756635	29.85	431194	26.63	
7	15.988	950489	37.50	681801	42.10	
8	16.244	98187	3.87	66367	4.10	
9	16.311	182786	7.21	113593	7.01	
10	16.371	65856	2.60	40728	2.51	
		2534494	100.00	1619471	100.00	

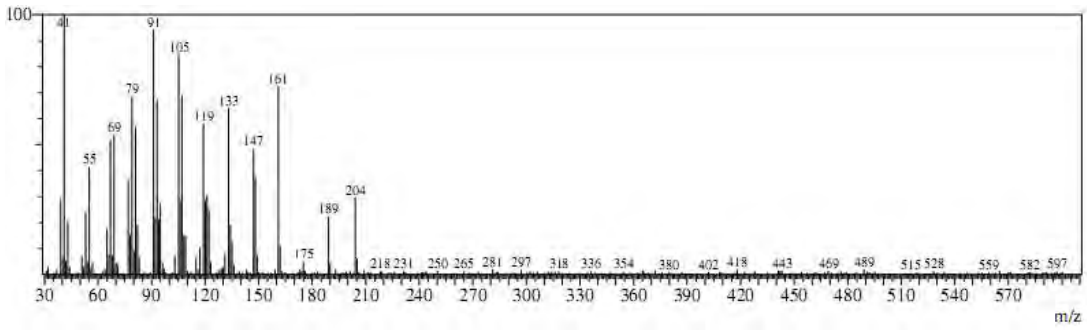


Mass peaks: 294

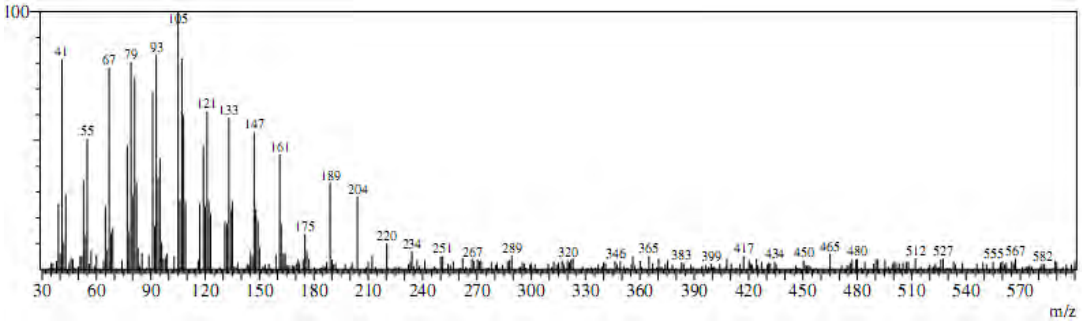


Mass peaks: 316

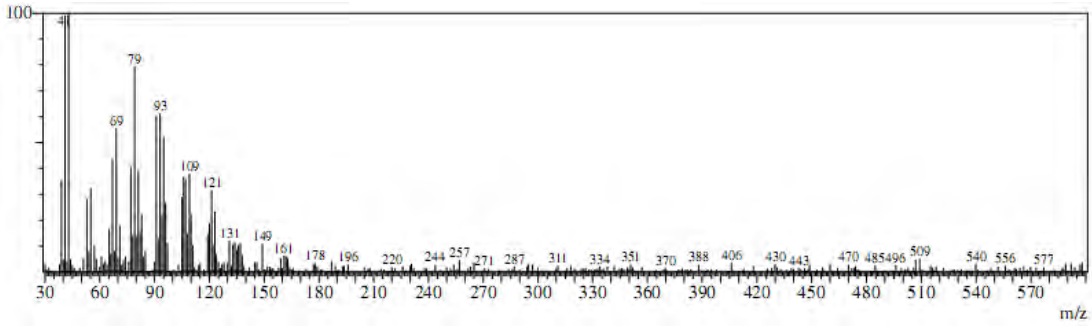
Fig. 15a GC-MS spectra of the methanolic extract of *P. longifolia*



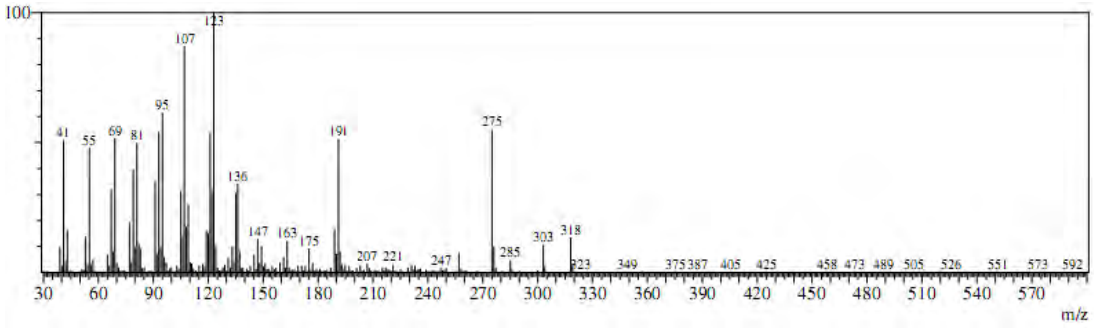
Mass peaks: 347



Mass peaks: 325

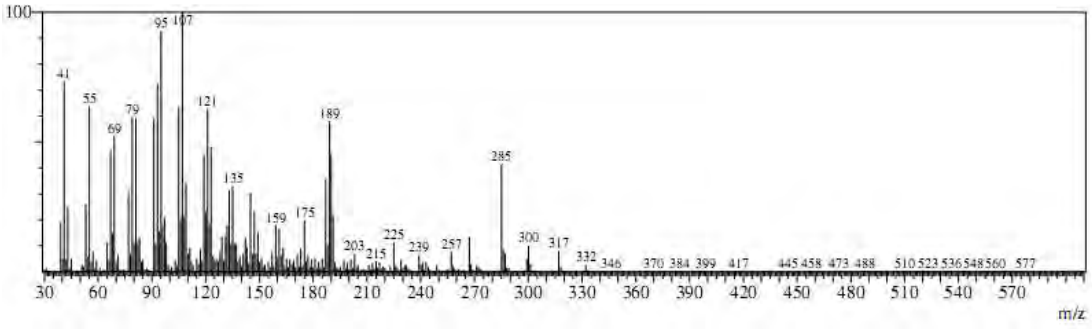


Mass peaks: 336

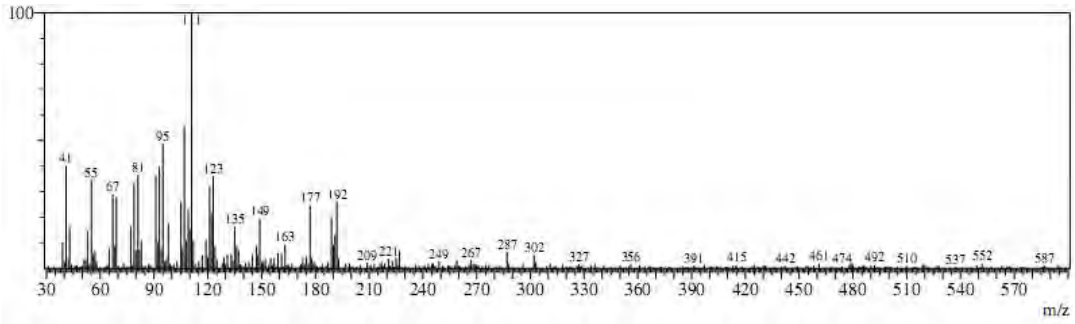


Mass peaks: 375

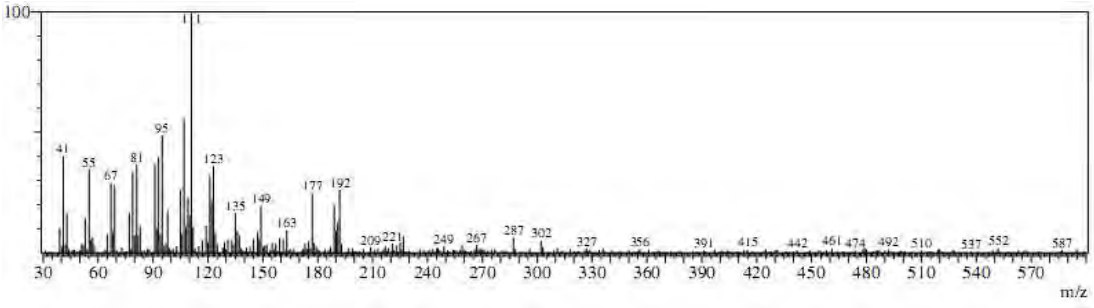
Fig. 15b GC-MS spectra of the methanolic extract of *P. longifolia*



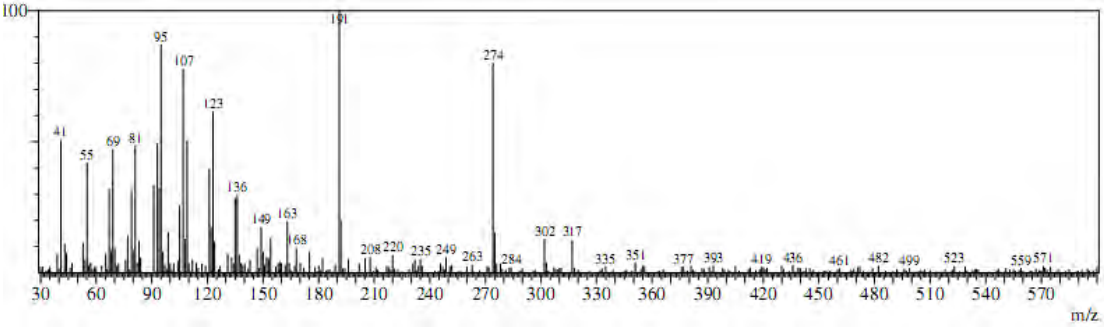
Mass peaks: 389



Mass peaks: 349



Mass peaks: 351



Mass peaks: 331

Fig. 15c GC-MS spectra of the methanolic extract of *P. longifolia*

4.9 *In vitro* antioxidant methods

4.9.1 DPPH free radical scavenging activity

DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The DPPH radical scavenging activity has been widely used as a model system to investigate the scavenging activity of natural compounds (Yokozawa et al., 1998). The reduction capability of the DPPH radical is determined by its absorbance decreases at 517 nm, as a discoloration from purple to yellow. The IC_{50} value (the concentration required to inhibit radical formation by 50%) of methanolic extract of *P. longifolia* was >1000 µg/ml. The IC_{50} value of standard ascorbic acid was 11.4 µg/ml (Data not shown).

4.9.2 Hydroxyl free radical scavenging activity

Hydroxyl radical is one of the potent reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell (Halliwell and Gutteridge, 1981). The model used is ascorbic acid-iron-EDTA model of $^{\bullet}OH$ generating system. This is a totally aqueous system in which ascorbic acid, iron and EDTA conspire with each other to generate hydroxyl radicals. The hydroxyl radical scavenging activity of methanolic extract of *P. longifolia* and standard gallic acid are shown in Figs. 16a and 16b respectively. In methanolic extract of *P. longifolia*, hydroxyl radical scavenging activity was concentration dependent and 50% inhibition was achieved at 1000 µg concentration in the reaction mixture, while in standard gallic acid, 50% inhibition was achieved at 200 µg concentration. The IC_{50} value of methanolic extract of *P. longifolia* and standard gallic acid were 610 µg/ml and 140 µg/ml respectively.

4.9.3 Superoxide anion radical scavenging activity

In the PMS-NADH-NBT system, superoxide anions were derived from dissolved oxygen by the PMS-NADH coupling reaction, which then reduced to NBT. The decreased absorbance at 560 nm with antioxidants indicates consumption of the superoxide anion in the reaction mixture (Robak and Gryglewski, 1988). The

superoxide anion radical scavenging activity of methanolic extract of *P. longifolia* and standard gallic acid are shown in Figs. 17a and 17b respectively. In methanolic extract of *P. longifolia*, superoxide anion radical scavenging activity was concentration dependent and 50 % inhibition was achieved at 1000 µg concentration in the reaction mixture, while in standard gallic acid, 50% inhibition was achieved at 225 µg concentration. The IC_{50} value of methanolic extract of *P. longifolia* and standard gallic acid were 810 µg/ml and 185 µg/ml respectively.

4.9.4 ABTS radical scavenging activity

The pre-formed radical monocation of $ABTS^{\bullet+}$ is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of such hydrogen-donating antioxidants (Re et al., 1999). The ABTS radical cation scavenging activity of methanolic extract of *P. longifolia* and standard ascorbic acid are shown in Figs. 18a and 18b respectively. In methanolic extract of *P. longifolia*, ABTS radical cation scavenging activity was concentration dependent and 50% inhibition was achieved at 100 µg concentration in the reaction mixture, while in standard ascorbic acid, 50 % inhibition was achieved at 10 µg concentration. The IC_{50} value of methanolic extract of *P. longifolia* and standard ascorbic acid were 75 µg/ml and 6.5 µg/ml respectively.

4.9.5 Reducing capacity assessment

The reducing capacity assessment of compounds may serve as significant indicators of its potential antioxidant activity. Many reports have revealed that there is a direct correlation between antioxidant activities and reducing power of components of some plants (Yildirim et al., 2001b). In the reducing power assay, the presence of antioxidants in the extract result in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. The amount of Fe^{2+} can then be monitored by measuring the formation of blue color at 700 nm. The reducing capacity assessment of methanolic extract of *P. longifolia* and ascorbic acid are shown in Fig. 19. In methanolic extract of *P. longifolia*, there was concentration dependent increase in the absorbance of reaction mixture and standard ascorbic acid. Higher absorbance of a reaction mixture indicated greater reducing power.

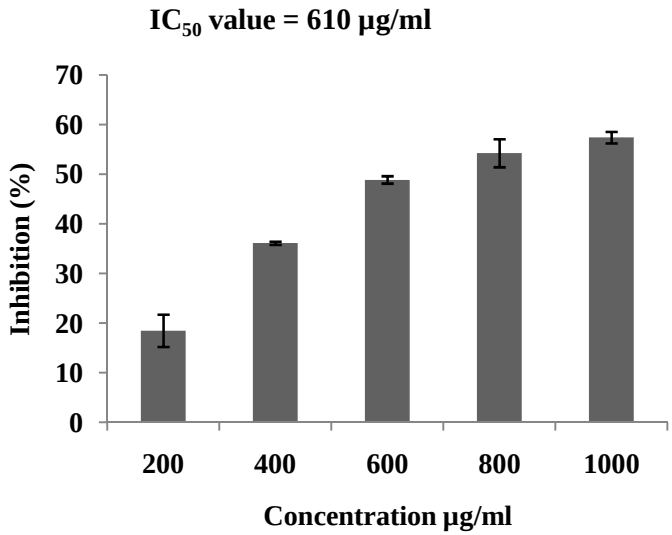


Fig. 16a Hydroxyl radical scavenging activity of methanolic extract of *P. longifolia*

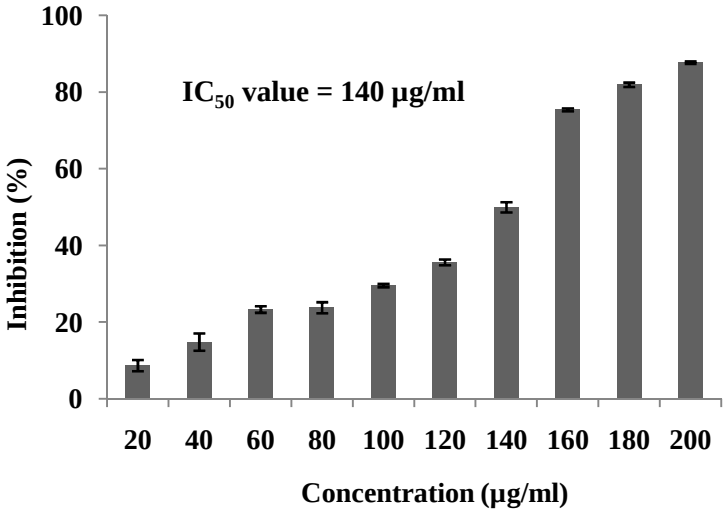


Fig. 16b Hydroxyl radical scavenging activity of standard gallic acid

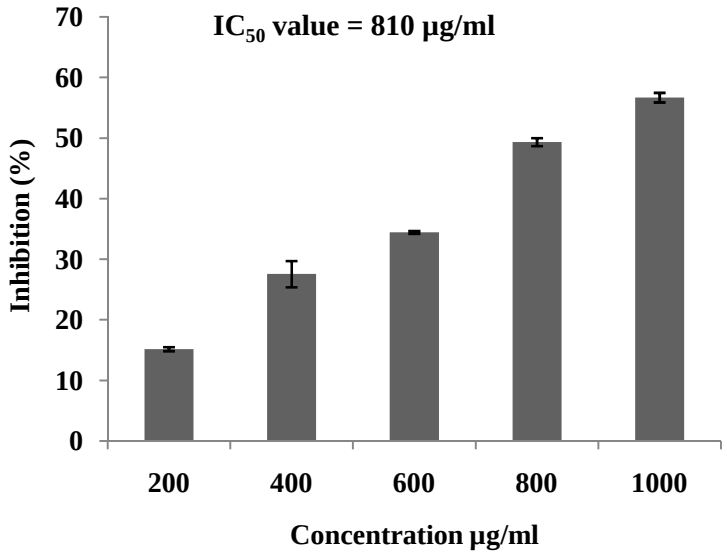


Fig. 17a Superoxide anion radical scavenging activity of methanolic extract of *P. longifolia*

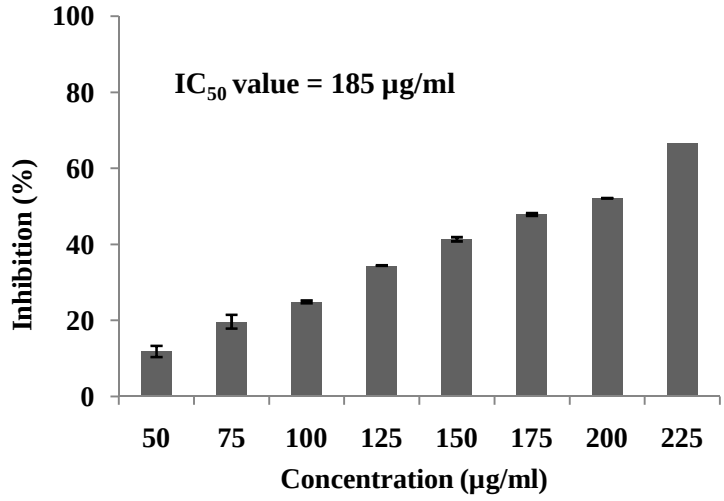


Fig. 17b Superoxide anion radical scavenging activity of standard gallic acid

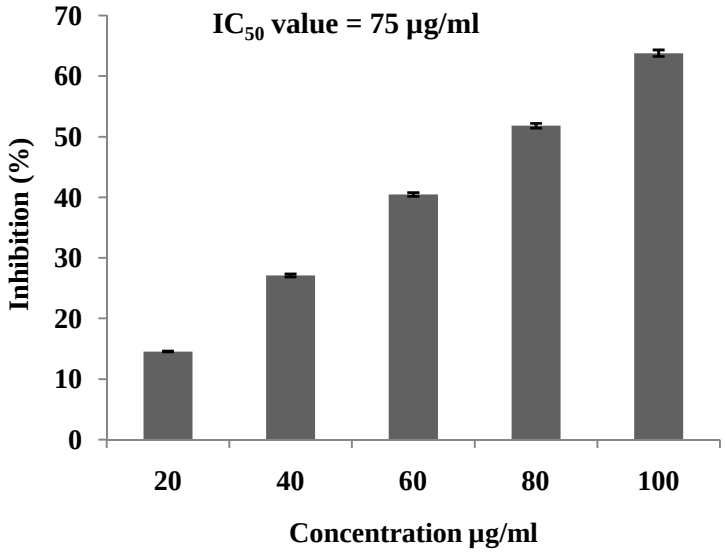


Fig. 18a ABTS radical cation scavenging activity of methanolic extract of *P. longifolia*

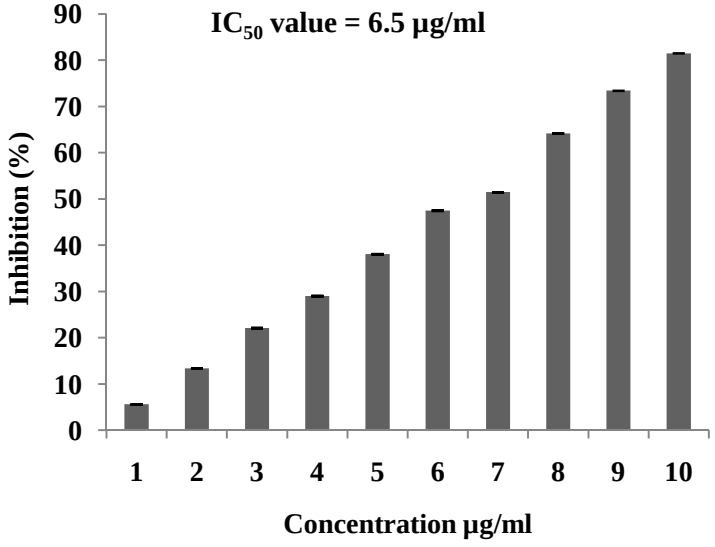


Fig. 18b ABTS radical cation scavenging activity of standard ascorbic acid

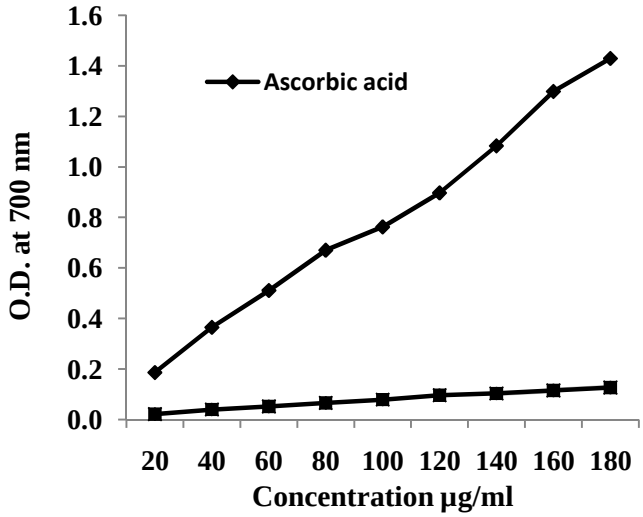


Fig. 19 Reducing capacity assessment of methanolic extract of *P. longifolia*

4.10 *In vivo* CCl_4 induced stress model

4.10.1 Biochemical analysis from serum

4.10.1.1 Total protein content

The total protein content in serum significantly ($P < 0.05$) decreased in CCl_4 treated rats as compared to normal group. Treatment with methanolic extract (270 and 540 mg/kg b.w.) showed significant ($P < 0.05$) increase in the level of total protein content as compared to CCl_4 treated group. The animals treated with standard drug Silymarin (100 mg/kg b.w.) showed significant ($P < 0.01$) increase in the level of total protein content as compared to CCl_4 treated group (Table 8).

4.10.1.2 Albumin content

The albumin content in serum significantly ($P < 0.05$) decreased in CCl_4 treated rats as compared to normal group. The low dose (270 mg/kg b.w.) of methanolic extract of *P. longifolia* showed significant ($P < 0.05$) increase in the level of albumin content whereas, the high dose (540 mg/kg b.w.) showed non-significant increase in the level of albumin content as compared to CCl_4 treated group. The animals treated with standard drug Silymarin (100 mg/kg b.w.) showed non-significant increase in the level of albumin content as compared to CCl_4 treated group (Table 8).

4.10.1.3 Urea content

The urea level in serum non-significantly increased in CCl_4 treated rats as compared to normal group. Treatment with methanolic extract (270 and 540 mg/kg b.w.) showed significant ($P < 0.05$) decrease in the level of urea content as compared to CCl_4 treated group. The animals treated with standard drug Silymarin (100 mg/kg b.w.) showed non-significant decrease in the level of urea content as compared to CCl_4 treated group (Table 8). Urea content was restored to normal level by both dose levels of methanolic extract of *P. longifolia*.

4.10.1.4 Blood urea nitrogen content

The blood urea nitrogen level (BUN) level in serum non-significantly increased in CCl_4 treated rats as compared to normal group. Treatment with methanolic extract (270 and 540 mg/kg b.w.) showed significant ($P < 0.05$) decrease in the level of BUN content as compared to CCl_4 treated group. The animals treated with standard drug Silymarin (100 mg/kg b.w.) showed non-significant decrease in the level of BUN content as compared to CCl_4 treated group (Table 8). Moreover, BUN content was restored to normal level by both dose levels of methanolic extract of *P. longifolia*.

4.10.1.5 Alkaline phosphatase activity

The level of alkaline phosphatase in serum non-significantly increased in CCl_4 treated rats as compared to normal group. Treatment with methanolic extract (540 mg/kg b.w.) showed significant ($P < 0.01$) decrease in level of ALP activity as compared to CCl_4 treated group. The animals treated with standard drug Silymarin (100 mg/kg b.w.) showed significant ($P < 0.01$) decrease in the level of ALP activity as compared to CCl_4 treated group (Table 8). However, low dose of methanolic extract (270 mg/kg b.w.) did not affect the level of ALP activity as compared to CCl_4 treated group.

4.10.1.6 Glutamate oxaloacetate transaminase activity

The level of glutamate oxaloacetate transaminase in serum significantly ($P < 0.001$) increased in CCl_4 treated rats as compared to normal group. Treatment with methanolic extract (270 and 540 mg/kg b.w.) showed significant ($P < 0.001$) decrease in level of GOT activity as compared to CCl_4 treated group. The animals treated with standard drug Silymarin (100 mg/kg b.w.) showed significant ($P < 0.01$) decrease in the level of GOT activity as compared to CCl_4 treated group (Table 8). Administration of methanolic extract of *P. longifolia* at the doses of 270 and 540 mg/kg remarkably prevented this rise level of GOT activity in a dose dependent manner.

4.10.1.7 Glutamate pyruvate transaminase activity

The level of glutamate pyruvate transaminase in serum significantly ($P < 0.001$) increased in CCl_4 treated rats as compared to normal group. Treatment with methanolic extract (540 mg/kg b.w.) showed significant ($P < 0.001$) decrease in level of GPT activity as compared to CCl_4 treated group. The animals treated with standard drug Silymarin (100 mg/kg b.w.) showed non-significant decrease in the level of GPT activity as compared to CCl_4 treated group (Table 8). However, low dose of methanolic extract (270 mg/kg b.w.) did not affect the level of GPT activity as compared to CCl_4 treated group.

4.10.2 Antioxidant parameters from liver homogenate

4.10.2.1 Total protein content

The total protein content significantly ($P < 0.001$) decreased in liver homogenate of CCl_4 treated group as compared to normal group. The high dose (540 mg/kg b.w.) of methanolic extract of *P. longifolia* showed non-significant increase in the level of total protein content as compared to CCl_4 treated group. The animals treated with standard drug Silymarin (100 mg/kg b.w.) showed significant ($P < 0.05$) increase in the level of total protein content as compared to CCl_4 treated group (Table 9). However, there was no significant change in total protein content at low dose level (270 mg/kg b.w.) as compared to CCl_4 treated group.

4.10.2.2 Lipid peroxidation level

The malondialdehyde (MDA) level significantly ($P < 0.05$) increased in liver homogenate of CCl_4 treated group as compared to normal group. Treatment with methanolic extract (270 mg/kg b.w.) showed significant ($P < 0.01$) decrease while the high dose (540 mg/kg b.w.) showed non-significant decrease the MDA level when compared to CCl_4 treated group. The animals treated with standard drug Silymarin (100 mg/kg b.w.) showed significant ($P < 0.05$) decrease in the level of MDA as compared to CCl_4 treated group (Table 9). Moreover, the rise in MDA level was found to attain near normal levels in low dose and Silymarin treated animals.

4.10.2.3 Glutathione reduced level

The reduced form of glutathione content non-significantly decreased in liver homogenate of CCl_4 treated group as compared to normal group. Treatment with methanolic extract (270 and 540 mg/kg b.w.) showed significant ($P < 0.001$, $P < 0.01$) increase in the glutathione content as compared to CCl_4 treated group. The animals treated with standard drug Silymarin (100 mg/kg b.w.) showed non-significant increase in glutathione content as compared to CCl_4 treated group (Table 9). Glutathione content was restored to normal level by both dose levels of methanolic extract of *P. longifolia*.

4.10.2.4 Glutathione peroxidase activity

The glutathione peroxidase activity significantly ($P < 0.001$) decreased in liver homogenate of CCl_4 treated group as compared to normal group. Treatment with methanolic extract (270 and 540 mg/kg b.w.) showed significant ($P < 0.001$) increase in the glutathione peroxidase activity as compared to CCl_4 treated group. The animals treated with standard drug Silymarin (100 mg/kg b.w.) showed non-significant increase in glutathione peroxidase activity as compared to CCl_4 treated group (Table 9). Glutathione peroxidase activity was restored to normal level by both the doses levels of methanolic extract of *P. longifolia*.

4.10.2.5 Catalase activity

The catalase activity significantly ($P < 0.05$) decreased in liver homogenate of CCl_4 treated group as compared to normal group. Treatment with methanolic extract (270 mg/kg b.w.) showed significant ($P < 0.001$) increase in the catalase activity while the high dose (540 mg/kg b.w.) showed non-significant increase in the catalase activity when compared to CCl_4 treated group (Table 9). Catalase activity was restored to normal level by low dose of methanolic extract of *P. longifolia*. However, standard drug Silymarin (100 mg/kg b.w.) did not affect catalase activity as compared to CCl_4 treated group.

Table 8 Changes in biochemical parameters of rats treated with methanolic extract of *P. longifolia* in CCl_4 induced stress model

Groups	Dose (mg/kg)	Total protein (g/dl)	Albumin (g/dl)	Urea (mg/dl)	BUN (mg/dl)	ALP (KA units)	GOT (IU/L)	GPT (IU/L)
Normal Control	-	6.42 ± 0.17	4.33 ± 0.16	48.32 ± 6.36	22.56 ± 2.94	31.99 ± 4.14	104 ± 2.75	58 ± 2.58
Toxin Control [a]	-	5.59 ± 0.20*	3.50 ± 0.23*	52.10 ± 5.66	24.33 ± 2.64	37.83 ± 3.94	147.67 ± 8.09***	95.67 ± 2.84***
Methanolic extract [b]	270	6.78 ± 0.33*	4.10 ± 0.05*	32.77 ± 2.87*	15.30 ± 1.34*	38.25 ± 7.05	62.67 ± 12.39***	93.32 ± 19.2
Methanolic extract [b]	540	6.30 ± 0.19*	3.74 ± 0.29	36.20 ± 1.95*	16.91 ± 0.91*	19.66 ± 1.92**	44.83 ± 10.79***	44.83 ± 6.78***
Silymarin [b]	100	6.28 ± 0.04**	3.70 ± 0.04	44.04 ± 4.55	20.57 ± 2.12	22.63 ± 2.55**	102.67 ± 6.97**	88 ± 3.01

Value are expressed as mean ± S.E.M., n = 5, *P < 0.05, **P < 0.01, ***P < 0.001, [a] compared to normal control, [b] compared to toxin control

Table 9 Changes in *in vivo* antioxidant parameters of rats treated with methanolic extract of *P. longifolia* in CCl_4 induced stress model

Groups	Dose (mg/kg)	Total protein (mg/g of tissue)	Lipid peroxidation (µM MDA formed/mg protein)	GSH (µM/mg protein)	GPx (µM GSH utilized/min/mg protein)	CAT (µM H_2O_2 consumed/min /mg protein
Normal Control	-	107.45 ± 1.79	0.077 ± 0.008	4.16 ± 0.11	0.117 ± 0.002	170.68 ± 8.21
Toxin Control [a]	-	97.40 ± 0.98***	0.133 ± 0.020*	4.12 ± 0.12	0.104 ± 0.001***	142.82 ± 5.43*
Methanolic extract [b]	270	94.69 ± 2.86	0.045 ± 0.010**	6.14 ± 0.43**	0.413 ± 0.012***	220.78 ± 8.36***
Methanolic extract [b]	540	102.85 ± 4.23	0.116 ± 0.015	5.39 ± 0.18***	0.383 ± 0.016***	169.04 ± 13.63
Silymarin [b]	100	108.82 ± 4.97*	0.070 ± 0.010*	4.53 ± 0.15	0.115 ± 0.006	133.38 ± 8.56

Value are expressed as mean ± S.E.M., n = 5, *P < 0.05, **P < 0.01, ***P < 0.001, [a] compared to normal control, [b] compared to toxin control

4.11 Water immersion stress model

Rats which were placed in the water were initially highly active, vigorously swimming around, scrabbling at the walls or diving to the bottom apparently searching for an exit. After 2-3 min their activity began to subside, being interspersed with phases of immobility of increasing length. The rats were considered exhausted when they sank and could not return to the surface by themselves.

4.11.1 Biochemical analysis from serum

4.11.1.1 Total protein content

The total protein content in serum significantly ($P < 0.05$) increased in methanolic extract at high dose of 540 mg/kg b.w. as compared to stress group. However the low dose showed significant decrease in the level of total protein content as compared to stress group (Table 10).

4.11.1.2 Albumin content

The methanolic extract did not affect the level of albumin in serum as compared to stress group. Albumin content in serum significantly ($P < 0.05$) decreased in methanolic extract at both the doses of 270 and 540 mg/kg b.w. as compared to stress group (Table 10).

4.11.1.3 Urea content

The methanolic extract of *P. longifolia* at the dose of 540 mg/kg b.w. showed non-significant decrease in the level of urea content in serum as compared to stress group. However, the low dose showed significant increase in the level of urea content in serum as compared to stress group (Table 10).

4.11.1.4 Blood urea nitrogen content

The methanolic extract of *P. longifolia* at the dose of 540 mg/kg b.w. showed non-significant decrease in the level of blood urea nitrogen content in serum as compared to stress group. However, the low dose showed significant increase in the level of BUN content in serum as compared to stress group (Table 10).

4.11.1.5 Cholesterol content

The methanolic extract of *P. longifolia* at the dose of 540 mg/kg b.w. showed non-significant decrease in the level of cholesterol in serum as compared to stress group. However the low dose did not affect the level of cholesterol in serum as compared to stress group (Table 10).

4.11.1.6 Alkaline phosphatase activity

The methanolic extract of *P. longifolia* at the dose of 270 mg/kg b.w. showed significant ($P < 0.05$) decrease in the level of ALP activity in serum as compared to stress group. However, the high dose showed non-significant increase in the level of ALP activity in serum as compared to stress group (Table 10).

4.11.1.7 Glutamate oxaloacetate transaminase activity

The methanolic extract of *P. longifolia* at the dose of 270 mg/kg b.w. showed significant ($P < 0.05$) decrease in the level of GOT activity in serum as compared to stress group. However, the high dose showed non-significant increase in the level of GOT activity in serum as compared to stress group (Table 10).

4.11.1.8 Glutamate pyruvate transaminase activity

The methanolic extract did not affect significantly in the level of GPT activity in serum as compared to stress group. Administration of methanolic extract at both the doses showed non-significant increase in the level of GPT activity as compared to stress group (Table 10).

4.11.2 Antioxidant parameters from liver homogenate

4.11.2.1 Total protein content

The methanolic extract of *P. longifolia* at the dose of 270 mg/kg b.w. showed significant increase in the level of total protein content in liver homogenate as compared to stress group. The high dose (540 mg/kg b.w.) showed non-significant increase in the level of total protein content in liver homogenate as compared to stress group (Table 11).

4.11.2.2 Glutathione reduced level

The methanolic extract of *P. longifolia* at the doses of 270 and 540 mg/kg b.w. showed non-significant increase in the level of glutathione content in liver homogenate as compared to stress group (Table 11).

4.11.2.3 Glutathione peroxidase activity

The methanolic extract of *P. longifolia* at the doses of 270 and 540 mg/kg b.w. showed significant ($P < 0.001$) increase in the level of glutathione peroxidase activity in liver homogenate as compared to stress group (Table 11).

4.11.2.4 Catalase activity

The methanolic extract of *P. longifolia* at the dose of 540 mg/kg b.w. showed non-significant increase in the level of catalase activity in liver homogenate as compared to stress group. The low dose (270 mg/kg b.w.) showed non-significant decrease in the level of catalase activity in liver homogenate as compared to stress group (Table 11).

4.11.2.5 Superoxide dismutase activity

The methanolic extract of *P. longifolia* at the dose of 270 mg/kg b.w. showed significant ($P < 0.05$) increase in the level of superoxide dismutase activity in liver homogenate as compared to stress group. The high dose (540 mg/kg b.w.) showed non-significant increase in the level of superoxide dismutase activity in liver homogenate as compared to stress group (Table 11).

Table 10 Changes in biochemical parameters of male rats treated with methanolic extract of *P. longifolia* in water immersion stress model

Groups	Dose (mg/kg)	Total protein (g/dl)	Albumin (g/dl)	Urea (mg/dl)	BUN (mg/dl)	Cholesterol (g/dl)	ALP (KA units)	GOT (IU/L)	GPT (IU/L)
Stress (swim)	-	6.15 ± 0.081	4.35 ± 0.126	46.30 ± 2.51	21.62 ± 1.17	106.19 ± 7.069	15.71 ± 1.03	120 ± 2.18	26 ± 2.16
Methanolic extract [a]	270	5.85 ± 0.015**	3.96 ± 0.079*	77.25 ± 12.30*	36.08 ± 5.74*	106.63 ± 2.31	11.54 ± 0.21**	113.20 ± 1.11*	28.40 ± 3.027
Methanolic extract [a]	540	6.64 ± 0.194*	3.95 ± 0.104*	44.58 ± 4.21	20.82 ± 1.96	105.55 ± 1.24	21.63 ± 4.60	134 ± 5.48	31.60 ± 8.74

Value are expressed as mean ± S.E.M., n = 5, *P < 0.05, ** P < 0.01, [a] Compared to stress (swim) control

Table 11 Changes in *in vivo* antioxidant parameters of male rats treated with methanolic extract of *P. longifolia* in water immersion stress model

Groups	Dose (mg/kg)	Total protein (mg/g of tissue)	GSH (µM/mg protein)	GPx (µM GSH utilized/min/mg protein)	CAT (µM H_2O_2 consumed/min/mg protein)	SOD (Unit/mg protein)
Stress (swim)	-	99.08 ± 1.60	1.74 ± 0.08	0.126 ± 0.002	188.62 ± 11.64	0.442 ± 0.098
Methanolic extract [a]	270	106.69 ± 1.62*	1.78 ± 0.17	0.382 ± 0.005***	171.31 ± 10.32	0.754 ± 0.031*
Methanolic extract [a]	540	102.68 ± 1.35	1.83 ± 0.22	0.382 ± 0.006***	192.10 ± 10.03	0.513 ± 0.125

Value are expressed as mean ± S.E.M., n = 5, *P < 0.05, ***P < 0.001, [a] Compared to stress (swim) control

4.12 Toxicity

4.12.1 Cage side observation

Normal behavior with no mortality was observed in animals of both the sexes at all dose levels of methanolic extract of *P. longifolia* during the entire period of acute toxicity study. In sub-acute toxicity study also, normal behavior with no mortality was observed in animals of both the sexes at all dose levels of methanolic extract of *P. longifolia* during entire period of sub-acute toxicity study.

4.12.2 Feed and water consumption and body weight measurement

Feed and water consumption pattern during acute toxicity study i.e. 14 days treatment period, did not show dose-dependent and time related decline (Fig. 20a and 20b and Fig. 21a and 21b). The amount of feed and water consumption of dosed groups was not significantly different from that of control group. Food and water consumption was normal during the entire study in animals of both sexes at all dose levels.

Feed and water consumption pattern during sub-acute toxicity study i.e. 21 days treatment period, also did not show dose-dependent and time related decline (Figs. 22a and 22b and Figs. 23a and 23b). The amount of feed and water consumption of dosed groups was not significantly different from that of control group. Food and water consumption was normal during the entire study in animals of both sexes at all dose levels.

There were no significant changes in the body weight of rats of both the sex from day 0 to day 14 in all the groups. Body weight of dosed and control rats did not show any significant change throughout the duration of treatment (Figs. 24a and 24b).

There were no significant changes in the body weight of rats of both the sex from day 0 to day 21 in all the groups. Body weight of dosed and control rats did not show any significant change throughout the duration of treatment (Figs. 25a and 25b).

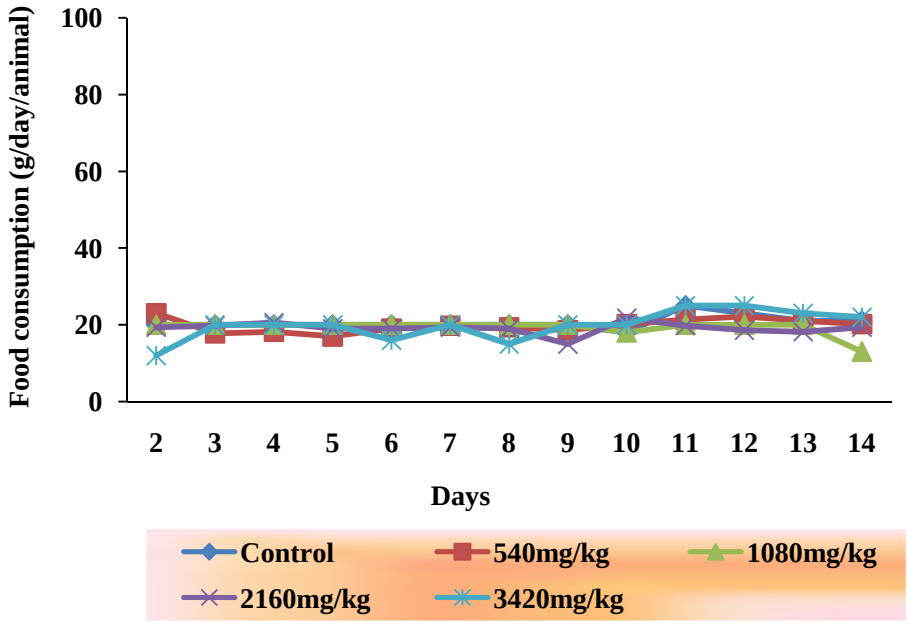


Fig. 20a Changes in feed consumption of male rats during acute toxicity study

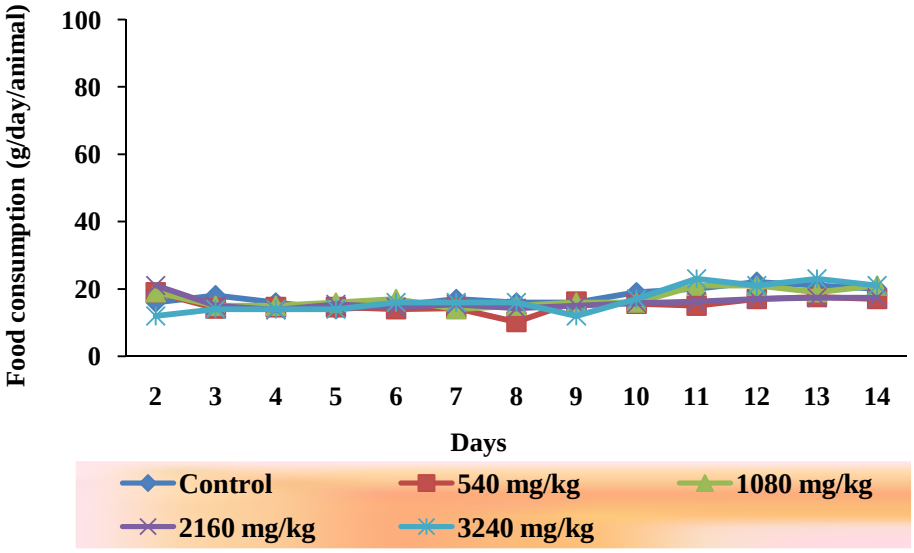


Fig. 20b Changes in feed consumption of female rats during acute toxicity study

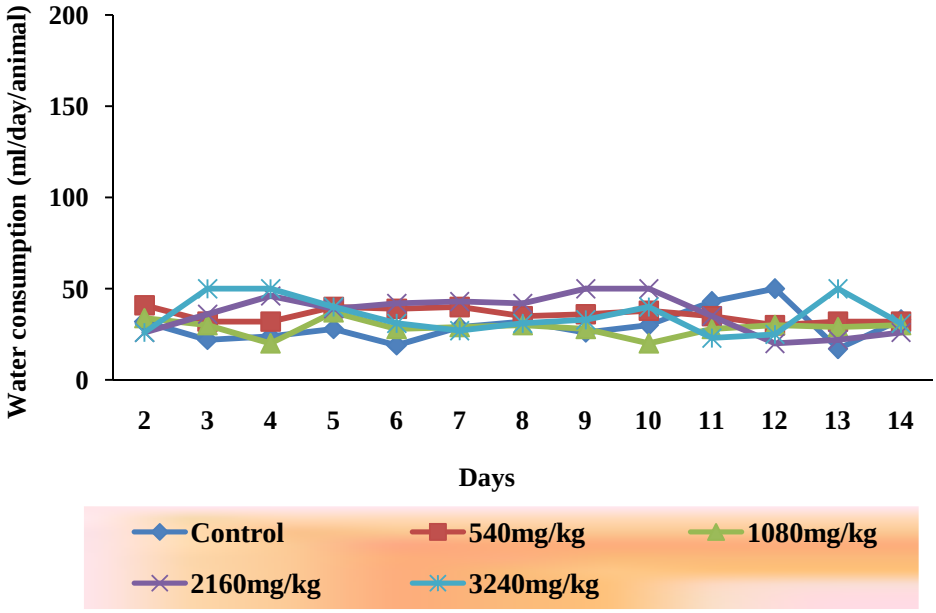


Fig. 21a Changes in water consumption of male rats during acute toxicity study

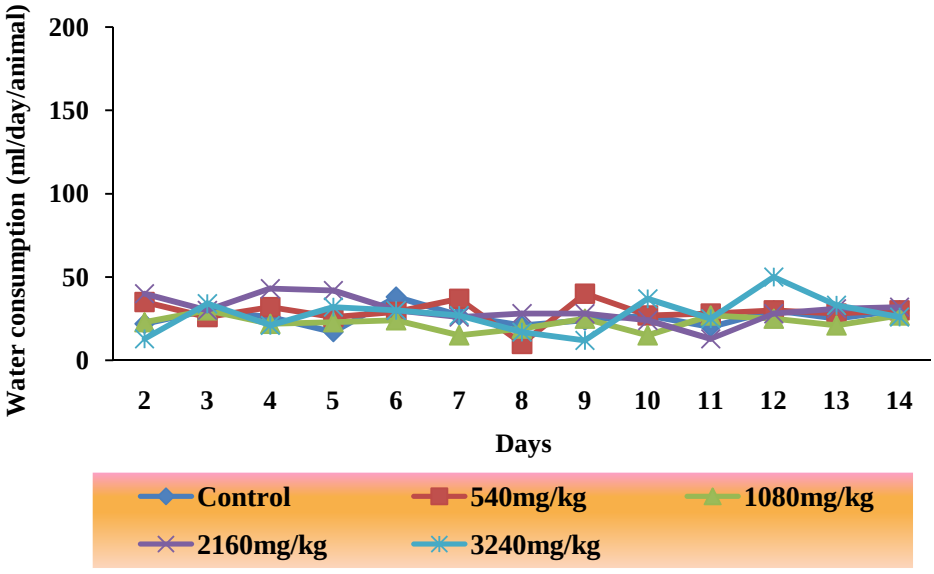


Fig. 21b Changes in water consumption of female rats during acute toxicity study

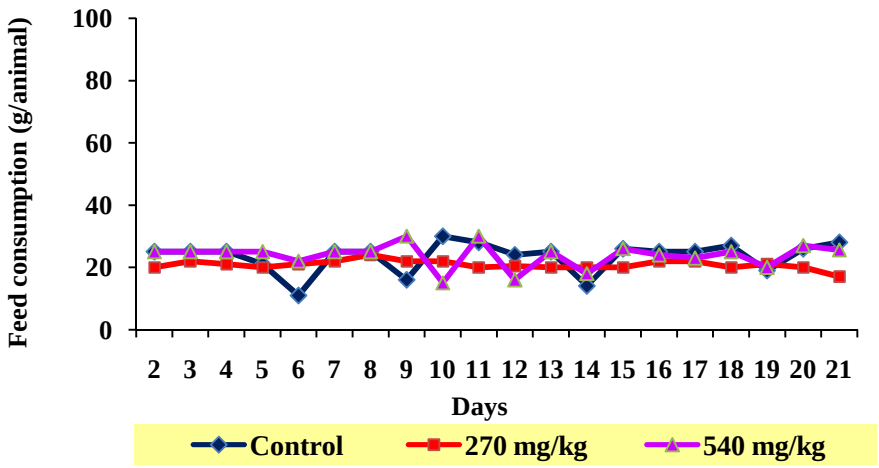


Fig. 22a Changes in the feed consumption of male rats during sub-acute toxicity study

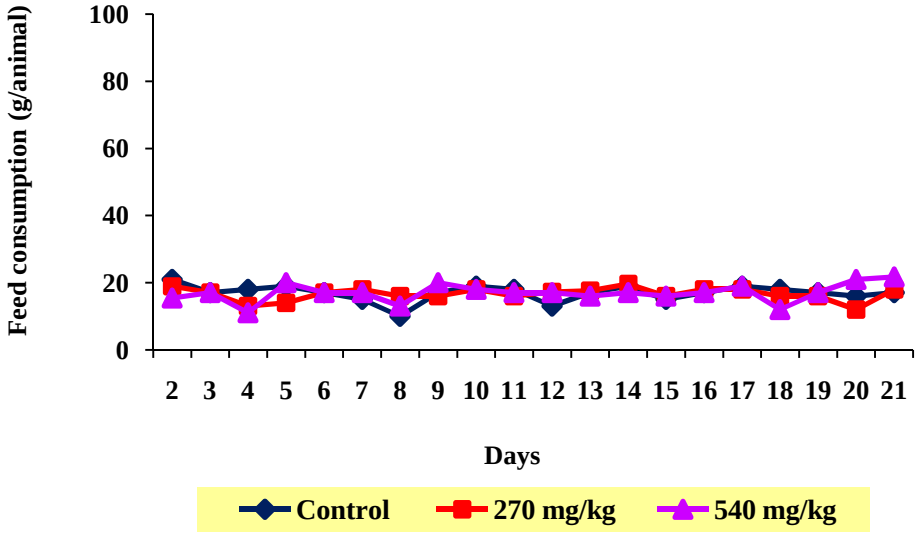


Fig. 22b Changes in the feed consumption of female rats during sub-acute toxicity study

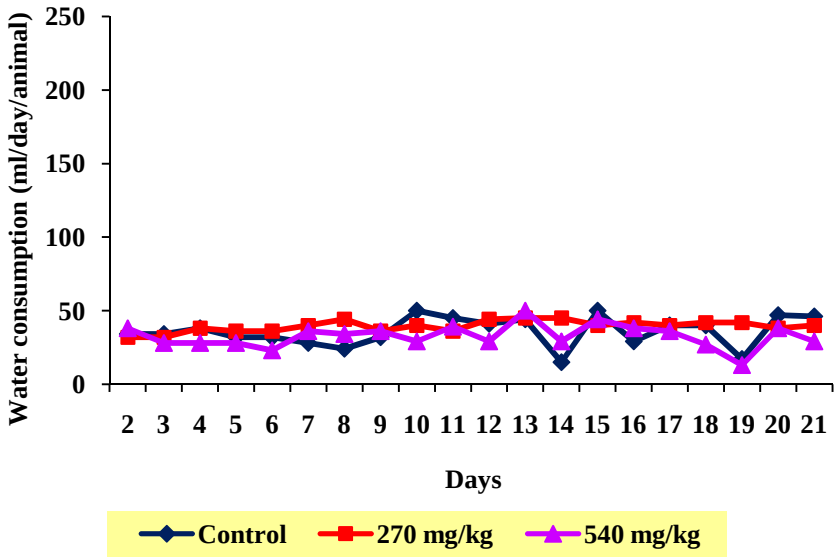


Fig. 23a Changes in the water consumption of male rats during sub-acute toxicity study

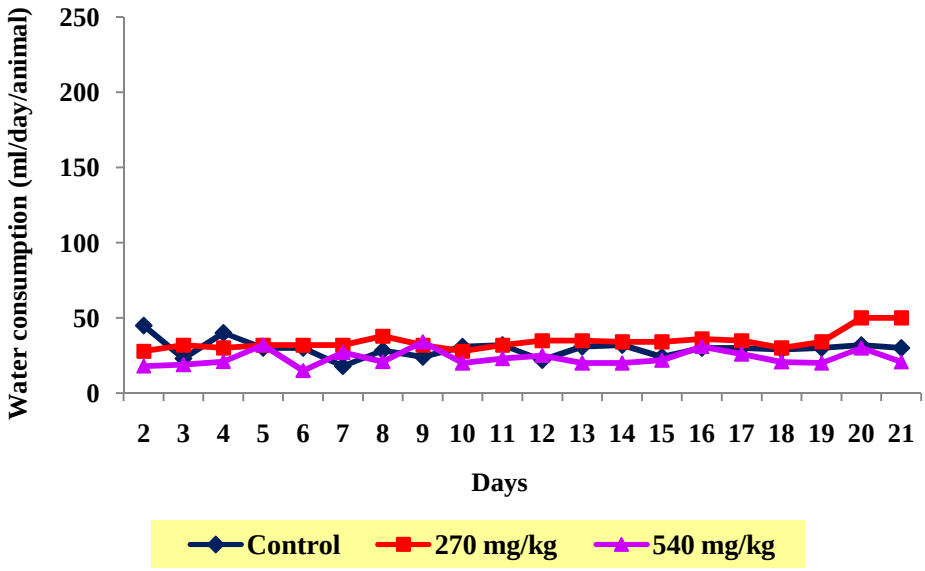


Fig. 23b Changes in the water consumption of female rats during sub-acute toxicity study

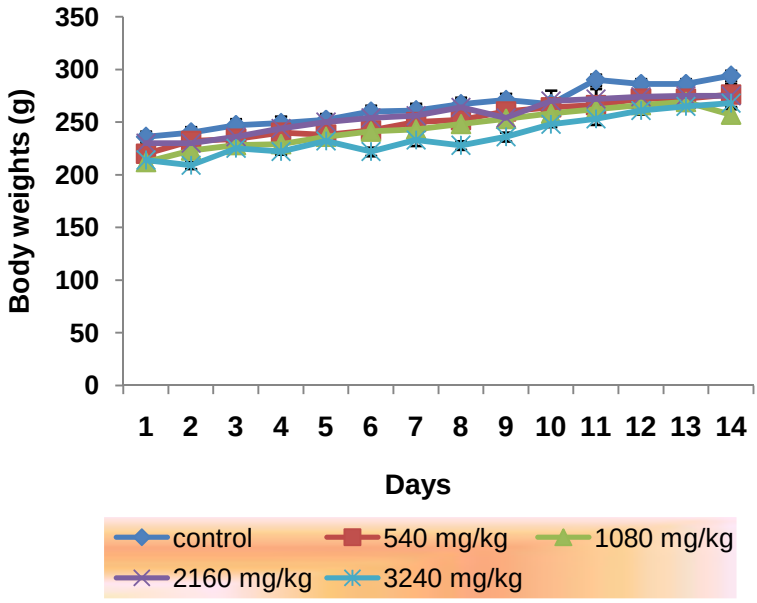


Fig. 24a Changes in body weight of male rats during acute toxicity study

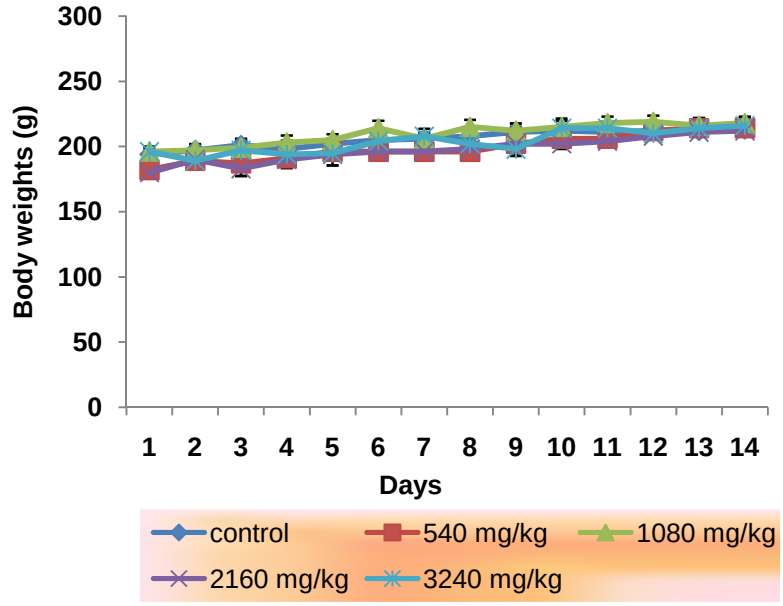


Fig. 24b Changes in body weight of female rats during acute toxicity study

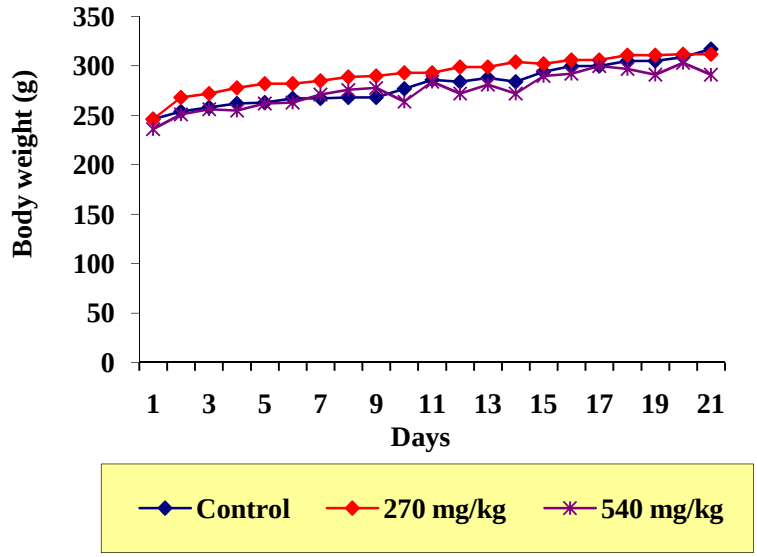


Fig. 25a Changes in body weight of male rats during sub-acute toxicity study

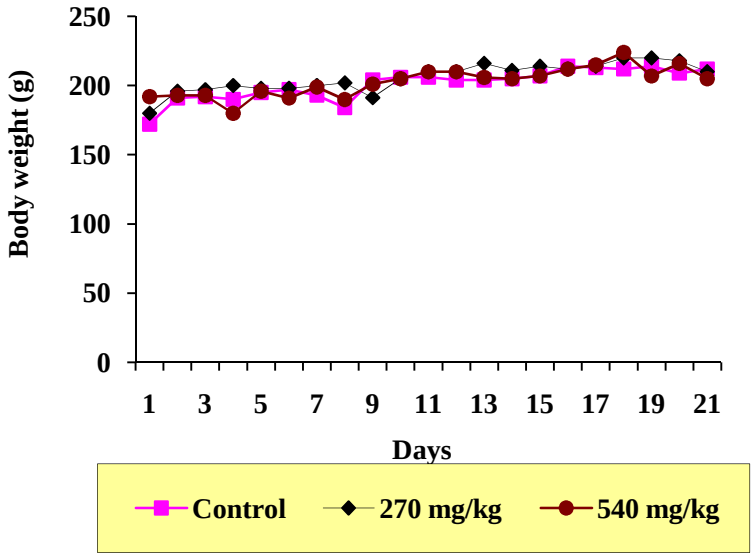


Fig. 25b Changes in body weight of female rats during sub-acute toxicity study

4.12.3 Biochemical analysis of acute toxicity study

The effect of single-dose oral administration of the methanolic extract of *P. longifolia* on the serum biochemical parameters are shown in Tables 12 and 13. The treated male rats showed significant differences from control groups in total protein (1080mg/kg, 2160 mg/kg, $P < 0.05$), albumin (3240 mg/kg, $P < 0.05$), urea (540 mg/kg, 1080 mg/kg, 2160 mg/kg, 3240 mg/kg, $P < 0.05$), ALP (1080 mg/kg, 3240 mg/kg), GOT (1080 mg/kg, 2160 mg/kg, $P < 0.05$), GPT (540 mg/kg, 1080 mg/kg, 2160 mg/kg, 3240 mg/kg, $P < 0.05$). The treated female rats showed significant differences from control in urea (3240 mg/kg, $P<0.05$), cholesterol (540 mg/kg, 2160 mg/kg, $P < 0.05$), GOT (540 mg/kg, $P < 0.05$), GPT (540 mg/kg, 2160 mg/kg, 3240 mg/kg, $P < 0.05$).

4.12.4 Biochemical analysis of sub-acute toxicity study

The effects of repeated-dose oral administration of the methanolic extract of *P. longifolia* on the serum biochemical parameters are shown in Tables 14 and 15. The treated male rats showed significant differences from control groups in total protein (540 mg/kg, $P < 0.05$), albumin (270 mg/kg, $P < 0.05$), Urea (270 mg/kg, $P < 0.05$), cholesterol (270 mg/kg, $P < 0.05$), ALP (270 mg/kg, $P < 0.05$), GOT (270 mg/kg, 540 mg/kg, $P < 0.05$), GPT (270 mg/kg, 540 mg/kg, $P < 0.05$), The treated female rats showed significant differences from control in total protein (270 mg/kg, 540 mg/kg, $P < 0.05$), albumin (270 mg/kg, 540 mg/kg, $P < 0.05$), urea (270 mg/kg, 540 mg/kg, $P < 0.05$), cholesterol (270 mg/kg, 540 mg/kg, $P < 0.05$), GOT (270 mg/kg, 540 mg/kg, $P < 0.05$), GPT (270 mg/kg, $P < 0.05$).

Table 12 Effect of single oral dose (acute toxicity study) of methanolic extract of *P. longifolia* on the biochemical parameters of male rat

Group	Total protein (g/dl)	Albumin (g/dl)	Urea (mg/dl)	Cholesterol (mg/dl)	ALP KA Units	GOT (IU/L)	GPT (IU/L)
Control	6.48 ± 0.04	4.38 ± 0.02	68.22 ± 3.34	89.55 ± 2.73	71.38 ± 7.99	90.20 ± 2.85	21.40 ± 0.60
540mg/kg	6.60 ± 0.06	4.64 ±0.17	46.16 ± 1.07*	78.34 ± 2.95	56.88 ± 7.16	108 ± 9.27	26.20 ± 0.37*
1080mg/kg	6.71 ± 0.05*	4.19 ± 0.08	44.82 ± 2.22*	105.14 ± 3.03	47.65 ± 2.07*	57.40 ± 11.2*	15.60 ± 3.19*
2160mg/kg	6.76 ± 0.07*	4.60 ± 0.10	49.66 ± 0.77*	88.43 ± 2.73	58.42 ± 3.97	108.60 ± 3.84*	37.80 ± 1.66*
3240mg/kg	5.65 ± 0.38	3.87 ± 0.17*	38.86 ± 2.16*	112.29 ± 5.43	44.61 ± 2.14*	85.60 ± 1.03	13.00 ± 1.76*

Values are expressed as mean ± S.E.M., n = 5, *P < 0.05

Table 13 Effect of single oral dose (acute toxicity study) of methanolic extract of *P. longifolia* on the biochemical parameters of female rats

Group	Total protein (g/dl)	Albumin (g/dl)	Urea (mg/dl)	Cholesterol (mg/dl)	ALP KA Units	GOT (IU/L)	GPT (IU/L)
Control	7.05 ± 0.11	4.90 ± 0.10	56.36 ± 2.07	97.04 ± 6.07	27.54 ± 4.39	98.60 ± 3.91	21.80 ± 0.66
540mg/kg	7.03 ± 0.16	4.76 ± 0.26	52.09 ± 1.33	76.68 ± 1.77*	35.69 ± 5.17	128.20 ± 9.32*	34.20 ± 1.59*
1080mg/kg	7.16 ± 0.06	4.84 ± 0.08	54.85 ± 3.77	98.97 ± 4.33	28.27 ± 5.24	99.60 ± 5.55	20.80 ± 0.58
2160mg/kg	6.91 ± 0.10	5.04 ± 0.10	52.17 ± 0.99	70.69 ± 1.86*	31.45 ± 4.95	105.40 ± 6.75	29.80 ± 1.62*
3240mg/kg	6.96 ± 0.10	4.60 ± 0.15	42.96 ± 2.16*	98.24 ± 1.36	18.66 ± 2.49	100.80 ± 10.49	10.80 ± 1.02*

Values are expressed as mean ± S.E.M., n = 5, *P < 0.05

Table 14 Effect of repeated oral dose (sub-acute toxicity study) of methanolic extract of *P. longifolia* on biochemical parameters of male rats

Group	Total protein (g/dl)	Albumin (g/dl)	Urea (mg/dl)	Cholesterol (mg/dl)	ALP KA Units	GOT (IU/L)	GPT (IU/L)
Control	7.06 ± 0.06	3.89 ± 0.13	41.90 ± 1.00	82.17 ± 2.49	21.20 ± 1.83	84.80 ± 2.48	25.80 ± 0.66
270mg/kg	7.08 ± 0.12	4.94 ± 0.07*	52.29± 2.63*	66.67 ± 3.13*	34.56 ± 3.00*	51.60 ± 4.07*	33.60 ± 2.73*
540mg/kg	6.51 ± 0.05*	3.74 ± 0.04	37.54 ± 2.76	88.36 ± 1.94	27.31 ± 2.12	63.20 ± 4.19*	20.80 ± 1.71*

Values are expressed as mean ± S.E.M., n = 5, *P < 0.05

Table 15 Effect of repeated oral dose (sub-acute toxicity study) of methanolic extract of *P. longifolia* on biochemical parameters of female rats

Group	Total protein (g/dl)	Albumin (g/dl)	Urea (mg/dl)	Cholesterol (mg/dl)	ALP KA Units	GOT (IU/L)	GPT (IU/L)
Control	7.12 ± 0.02	4.61 ± 0.14	47.99 ± 1.69	73.06 ± 3.58	14.97 ± 1.66	99.80 ± 6.03	25.60 ± 1.57
270mg/kg	7.46 ± 0.13*	5.19 ± 0.05*	58.71 ± 3.32*	61.71 ± 2.97*	20.11 ± 3.18	56.20 ± 5.77*	32.20 ± 2.06*
540mg/kg	6.64 ± 0.04*	3.92 ± 0.05*	42.72 ± 1.12*	97.88 ± 2.22*	17.52 ± 1.43	68.60 ± 2.48*	22.40 ± 1.60

Values are expressed as mean ± S.E.M., n =5, *P < 0.05

4.12.5 Hematological analysis of acute toxicity study

The effects of single-dose oral administration of the methanolic extract of *P. longifolia* on the hematological parameters are shown in Tables 16 and 17. The treated male rats showed significant differences from control groups in R.B.C. counts (540mg/kg, 2160 mg/kg $P < 0.05$), Packed Cell Volume (540 mg/kg, 2160mg/kg, $P < 0.05$), M.C.H. (540 mg/kg, 1080 mg/kg, 2160 mg/kg, $P < 0.05$), M.C.H.C. (540 mg/kg, 2160 mg/kg, 3240 mg/kg, $P < 0.05$), lymphocytes (540 mg/kg, $P < 0.05$), eosinophils (540 mg/kg, $P < 0.05$). The treated female rats showed significant differences from control groups in hemoglobin (2160 mg/kg, $P < 0.05$), Packed Cell Volume (2160 mg/kg, $P < 0.05$), M.C.H. (540 mg/kg, 2160 mg/kg, $P < 0.05$), M.C.H.C. (540 mg/kg, 2160 mg/kg, $P < 0.05$), total platelet count (540 mg/kg, $P < 0.05$). However, there was no significant change in the levels of total W.B.C. counts, neutrophils, monocytes, and basophiles at all doses.

4.12.6 Hematological analysis of sub-acute toxicity study

The effects of repeated-dose oral administration of the methanolic extracts of *P. longifolia* on the hematological parameters are shown in Tables 18 and 19. The treated male rats showed significant differences from control groups in R.B.C. counts (270 mg/kg, $P < 0.05$), M.C.V. (270 mg/kg, $P < 0.05$), M.C.H. (270 mg/kg, $P < 0.05$), neutrophils (270 mg/kg, $P < 0.05$), lymphocyte (270 mg/kg, $P < 0.05$), monocytes (270 mg/kg, $P < 0.05$). The treated female rats showed significant differences from control in hemoglobin (270 mg/kg, $P < 0.05$), R.B.C. counts (270 mg/kg, $P < 0.05$), M.C.V. (270 mg/kg, $P < 0.05$), M.C.H. (270 mg/kg, $P < 0.05$), total W.B.C. (540mg/kg, $P < 0.05$), eosinophils (270 mg/kg, $P < 0.05$), monocytes (270 mg/kg, $P < 0.05$). However, there was no significant change in the levels of Packed Cell Volume, M.C.H.C., basophiles and total platelet count at any dose levels in both the sexes.

Table 16 Effect of single oral dose (acute toxicity study) of methanolic extract of *P. longifolia* on hematological parameters of male rats

Group	Control	540 mg/kg	1080 mg/kg	2160 mg/kg	3240 mg/kg
Hemoglobin (g/dl)	14.82 ± 0.17	5.48 ± 0.32	14.94 ± 0.47	15.40 ± 0.35	15.22 ± 0.32
Total R.B.C. (ml/cu.mm)	7.91 ± 0.09	8.57 ± 0.19*	7.80 ± 0.28	8.48 ± 0.23*	8.16 ± 0.09
Packed Cell Volume (%)	41.02 ± 0.68	44.48 ± 0.97*	41.02 ± 1.65	45.04 ± 1.00*	41.10 ± 0.61
M.C.V. (cu.micron)	52.08 ± 0.33	51.9 ± 0.43	52.57 ± 0.67	53.12 ± 0.66	50.38 ± 0.78
M.C.H. (pico gram)	18.74 ± 0.08	18.08 ± 0.20*	19.17 ± 0.14*	18.16 ± 0.20*	18.66 ± 0.38
M.C.H.C. (%)	35.98 ± 0.23	34.82 ± 0.28*	36.48 ± 0.43	34.2 ± 0.20*	37.02 ± 0.34*
Total W.B.C. /10^3mm^3	6.8 ± 0.79	7.66 ± 1.06	10.0± 1.9	6.9 ± 1.02	10.8 ± 1.61
Neutrophils (%)	20.6 ± 1.75	12.8 ± 3.77	22.60 ± 1.66	25.6 ± 5.08	31.20 ± 5.30
Lymphocytes (%)	78 ± 1.84	88.4 ± 3.54*	76.80 ± 1.96	73.8 ± 5.34	67.80 ± 5.48
Eosinophils (%)	1.4 ± 0.24	0*	0.60 ± 0.40	0.6 ± 0.40	1 ± 0.32
Monocytes (%)	0	0	0	0	0
Basophiles (%)	0	0	0	0	0
Total platelet count /10^3mm^3	126.8 ± 10.8	123.7 ± 10.0	141.2 ± 7.2	90.5 ± 16.5	120.3 ± 12.2

Values are expressed as mean ± S.E.M., n = 5, *P < 0.05

Table 17 Effect of single oral dose (acute toxicity study) of methanolic extract of *P. longifolia* on hematological parameters of female rats

Group	Control	540 mg/kg	1080 mg/kg	2160 mg/kg	3240 mg/kg
Hemoglobin (g/dl)	15.74 ± 0.17	15.24 ± 0.25	15.38 ± 0.34	15.08 ± 0.09*	15.52 ± 0.24
Total R.B.C. (ml/cu.mm)	7.89 ± 0.05	8.28 ± 0.20	7.73 ± 0.28	8.22 ± 0.13	8.01 ± 0.18
Packed Cell Volume (%)	41.08 ± 0.47	42.66 ± 0.99	38.26 ± 2.74	42.8 ± 0.29*	41.06 ± 1.07
M.C.V. (cu.micron)	52.04 ± 0.41	51.22 ± 0.22	49.35 ± 2.60	52.1 ± 0.61	51.29 ± 0.27
M.C.H. (pico gram)	19.94 ± 0.14	18.4 ± 0.18*	19.94 ± 0.33	18.36 ± 0.28*	19.41 ± 0.29
M.C.H.C. (%)	38.32 ± 0.07	35.74 ± 0.27*	41.04 ± 3.10	35.22 ± 0.20*	37.84 ± 0.44
Total W.B.C. /10^3mm^3	7.60 ± 0.70	8.3 ± 1.44	9.0± 0.98	5.9 ± 0.79	10.0 ± 1.67
Neutrophils (%)	22.8 ± 3.87	24.2 ± 3.65	19.60 ± 2.09	15.6 ± 1.36	26.20 ± 4.07
Lymphocytes (%)	77 ± 3.99	75.8 ± 3.65	80 ± 2.28	84.4 ± 1.36	73.20 ± 4.40
Eosinophils (%)	0.20 ± 0.20	0	0.40 ± 0.40	0	0.60 ± 0.40
Monocytes (%)	0	0	0	0	0
Basophiles (%)	0	0	0	0	0
Total platelet count /10^3mm^3	119.6 ± 9.8	77.7 ± 9.1*	123.0 ± 8.5	151.7 ± 6.7	106.2 ± 18.3

Values are expressed as mean ± S.E.M., n = 5, *P < 0.05

Table 18 Effect of repeated oral dose (sub-acute toxicity study) of methanolic extract of *P. longifolia* on hematological parameters of male rats

Group	Control	270 mg/kg	540 mg/kg
Hemoglobin (g/dl)	15 ± 0.29	16.02 ± 0.32	15.42 ± 0.15
Total R.B.C. (ml/cu.mm)	8 ± 0.19	9.29 ± 0.17*	8.66 ± 0.15
Packed Cell Volume (%)	42 ± 0.94	43.7 ± 0.58	43.98 ± 0.64
M.C.V. (cu.micron)	51 ± 0.34	47.08 ± 0.64*	50.794 ± 0.54
M.C.H. (pico gram)	18 ± 0.21	17.24 ± 0.21*	17.82 ± 0.26
M.C.H.C. (%)	36 ± 0.27	36.64 ± 0.29	35.08 ± 0.27
Total W.B.C. /10^3mm^3	3.02 ± 1.94	1.34 ± 0.07	1.10 ± 0.10
Neutrophils (%)	14 ± 2.43	27.02 ± 3.63*	24 ± 3.59
Lymphocytes (%)	84 ± 2.38	68.4 ± 3.58*	74.8 ± 4.63
Eosinophils (%)	1 ± 0.24	1.56 ± 0.21	1.4 ± 0.75
Monocytes (%)	0	3.02 ± 0.24*	0.2 ± 0.20
Basophiles (%)	0	0	0
Total platelet count /10^3mm^3	113.34 ± 13.79	116.70 ± 11.66	116.72 ± 3.82

Values are expressed as mean ± S.E.M., *P < 0.05, n = 5

Table 19 Effect of repeated oral dose (sub-acute toxicity study) of methanolic extract of *P. longifolia* on hematological parameters of female rats

Group	Control	270 mg/kg	540 mg/kg
Hemoglobin (g/dl)	16.44 ± 0.19	15.86 ± 0.13*	16.36 ± 0.26
Total R.B.C. (ml/cu.mm)	8.51 ± 0.13	8.99± 0.11*	8.51 ± 0.27
Packed Cell Volume (%)	43.92 ± 0.52	42.8 ± 0.25	44.02 ± 0.98
M.C.V. (cu.micron)	51.56 ± 0.28	47.62 ± 0.55*	51.8 ± 0.63
M.C.H. (pico gram)	19.32 ± 0.27	17.64 ± 0.22*	19.26 ± 0.36
M.C.H.C. (%)	37.44 ± 0.33	37.04 ± 0.09	37.2 ± 0.29
Total W.B.C. /10^3mm^3	8.26 ± 0.65	8.97 ± 1.7	11.88 ± 1.28*
Neutrophils (%)	20.2 ± 3.84	16.6 ± 1.16	24.6 ± 2.56
Lymphocytes (%)	77 ± 3.56	76.34 ± 1.98	74 ± 2.92
Eosinophils (%)	1.8 ± 0.37	4.34 ± 0.96*	1 ± 0.55
Monocytes (%)	1 ± 0.32	2.66 ± 0.35*	0.40 ± 0.40
Basophiles (%)	0	0.06 ± 0.06	0
Total platelet count /10^3mm^3	117.14 ± 7.67	123.42 ± 4.82	106.20 ± 9.22

Values are expressed as mean ± S.E.M., *P < 0.05, n = 5

4.12.7 Relative organ weight in acute toxicity study

The effect of single-dose oral administration of the methanolic extract of *P. longifolia* on the relative organ weights are shown in Figs. 26-34. The treated male rats showed significant differences from control groups in liver (2160 mg/kg, $P < 0.05$) (Fig. 29a), spleen (540 mg/kg, 2160 mg/kg $P < 0.05$) (Fig. 30a), kidneys (3240 mg/kg, $P < 0.05$) (Fig. 31a), adrenal glands (3240 mg/kg, $P < 0.05$) (Fig. 32a) and testes (540 mg/kg, 3240 mg/kg, $P < 0.05$) (Fig. 33). However there was no significant change in the relative weights of lungs, heart and thymus glands at any dose levels of treated male rats as compare to control. The treated female rats showed significant differences from control in heart (2160 mg/kg, $P < 0.05$) (Fig. 28b), liver (2160 mg/kg, $P < 0.05$) (Fig. 29b), adrenal glands (2160 mg/kg, $P < 0.05$) (Fig. 32b). However there was no significant change in relative weight of lungs, spleen, kidneys thymus glands and uterus and ovaries at any dose levels of treated female rats as compared to control group.

4.12.8 Relative organ weight of in sub-acute toxicity study

The effect of repeated-dose oral administration of the methanolic extract of *P. longifolia* on the relative organ weights are shown in Figs. 35-43. The treated male rats showed significant differences from control groups in heart (540 mg/kg, $P < 0.05$) (Fig. 37a) and adrenal glands (540 mg/kg, $P < 0.05$) (Fig. 41a). However there was no significant change in the relative weights of lungs, liver, spleen, kidneys, testes and thymus glands at any dose levels in treated male rats as compared to control. The treated female rats showed significant differences from control in thymus glands (540 mg/kg, $P < 0.05$) (Fig. 35b), heart (270 mg/kg, 540 mg/kg, $P < 0.05$) (Fig. 37b), spleen (270 mg/kg, $P < 0.05$) (Fig. 39b), kidney (270 mg/kg, $P < 0.05$) (Fig. 40b), adrenal glands (270 mg/kg $P < 0.05$) (Fig. 41b) and Uterus and ovaries (540 mg/kg, $P < 0.05$) (Fig. 43). However there was no significant change in relative weight of lungs and liver at any dose levels in treated female rats as compared to control group.

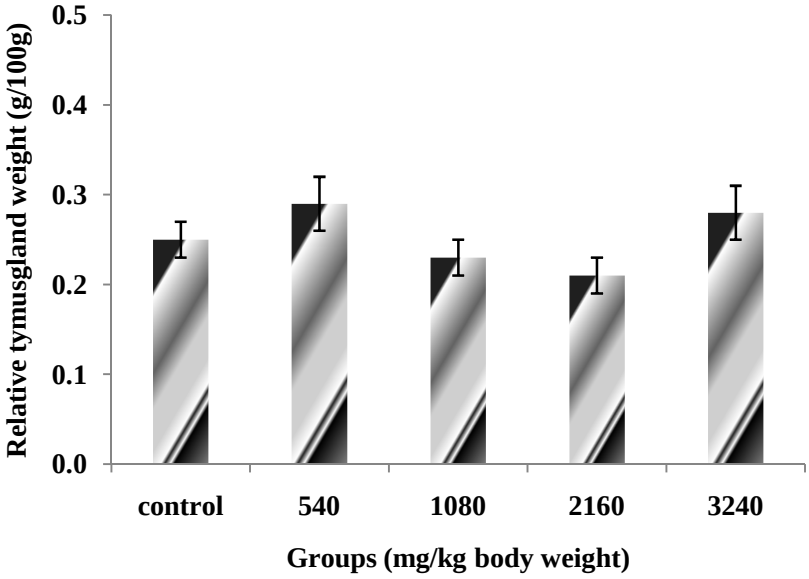


Fig. 26a Changes in relative weight of thymus gland of male rats in **acute** toxicity study

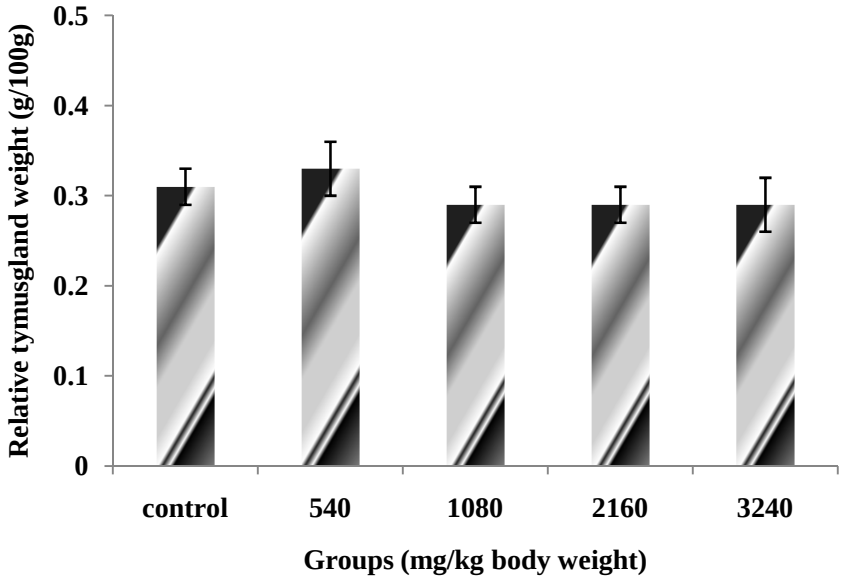


Fig. 26b Changes in relative weight of thymus gland of female rats in **acute** toxicity study

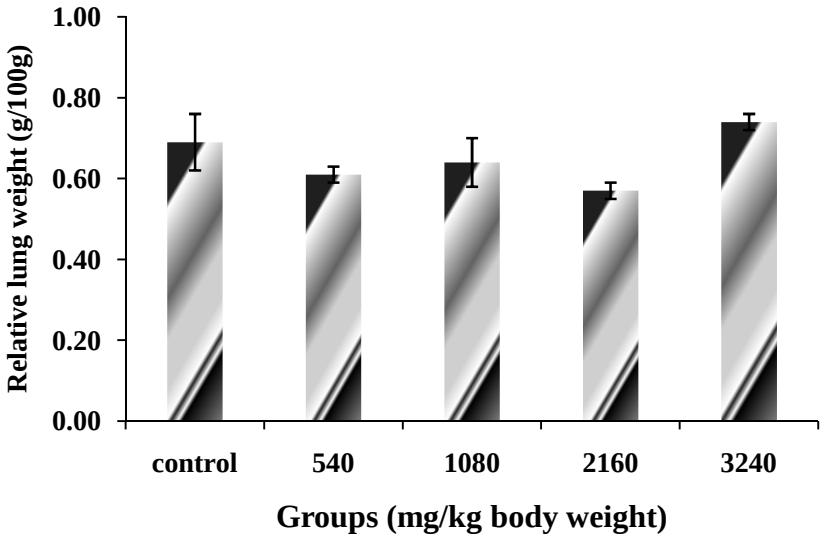


Fig. 27a Changes in relative weight of lung of male rats in **acute** toxicity study

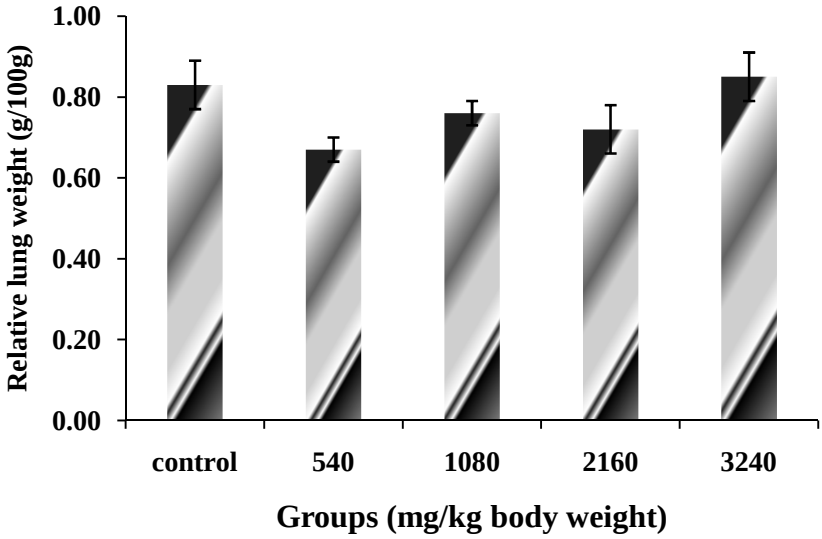


Fig. 27b Changes in relative weight of lung of female rats in **acute** toxicity study

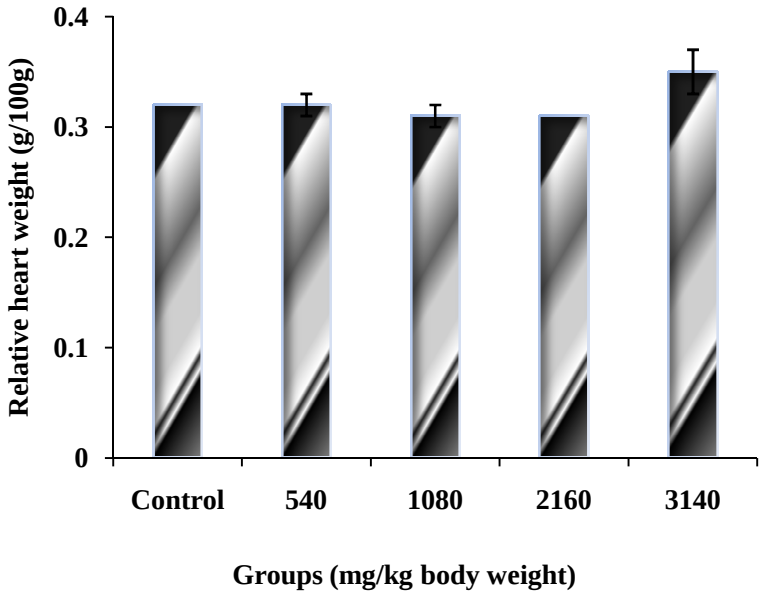


Fig. 28a Changes in relative weight of heart of male rats in **acute** toxicity study

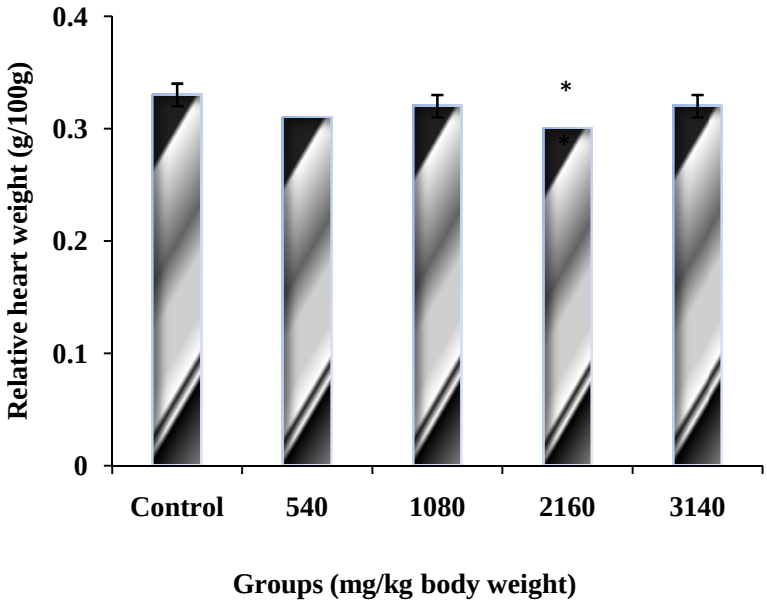


Fig. 28b Changes in relative weight of heart of female rats in **acute** toxicity study

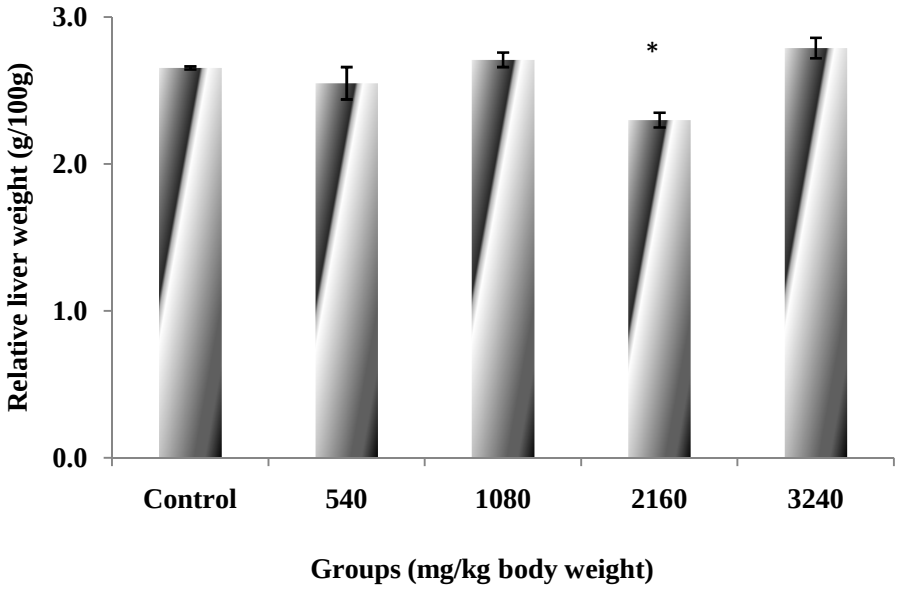


Fig. 29a Changes in relative weight of liver of male rats in **acute** toxicity study

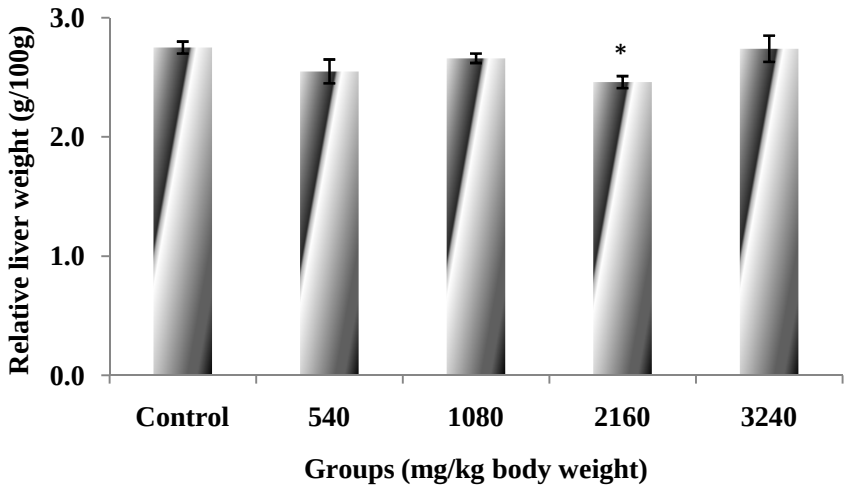


Fig. 29b Changes in relative weight of liver of female rats in **acute** toxicity study

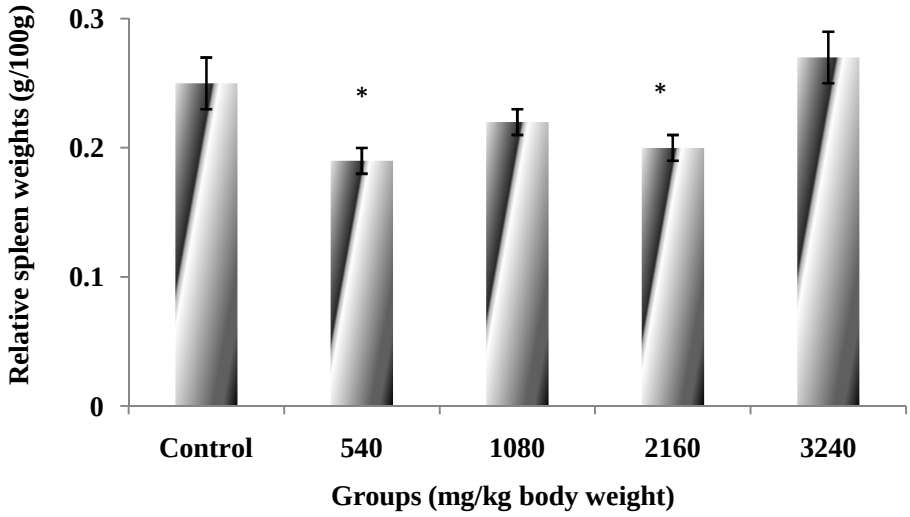


Fig. 30a Changes in relative weight of spleen of male rats in **acute** toxicity study

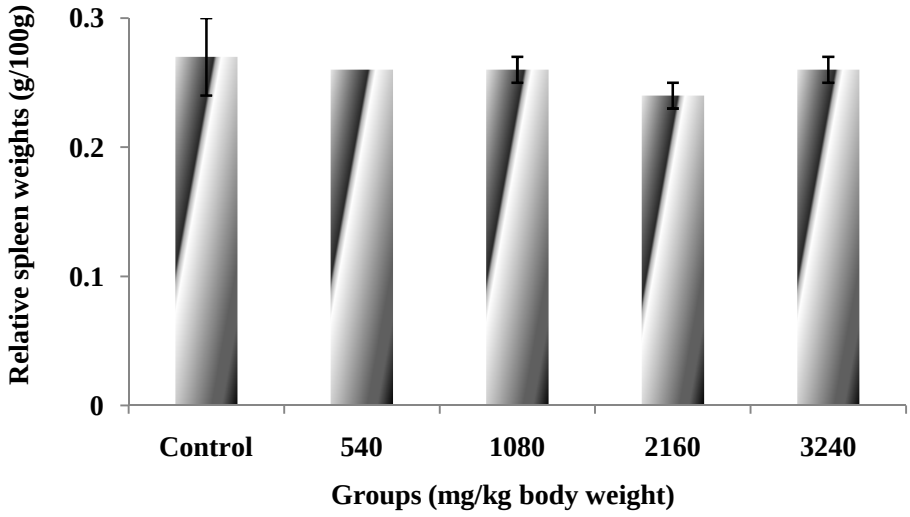


Fig. 30b Changes in relative weight of spleen of female rats in **acute** toxicity study

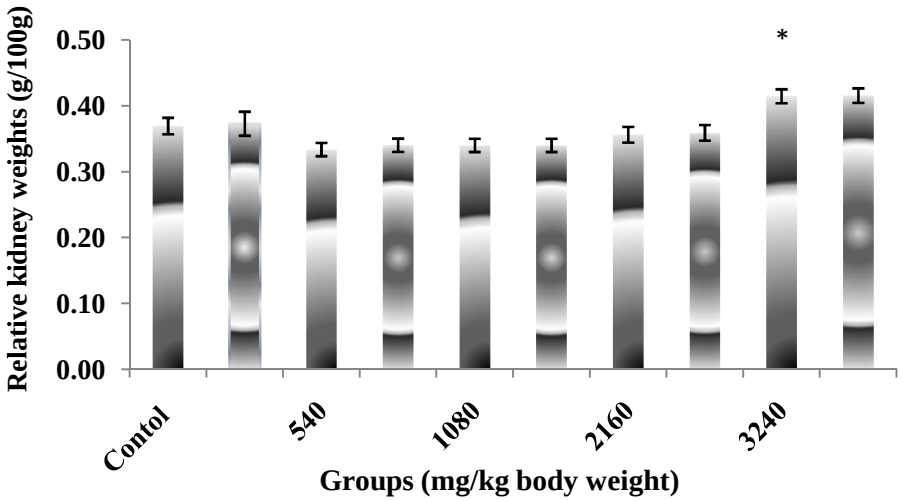


Fig. 31a Changes in relative weight of kidney of male rats in **acute** toxicity study

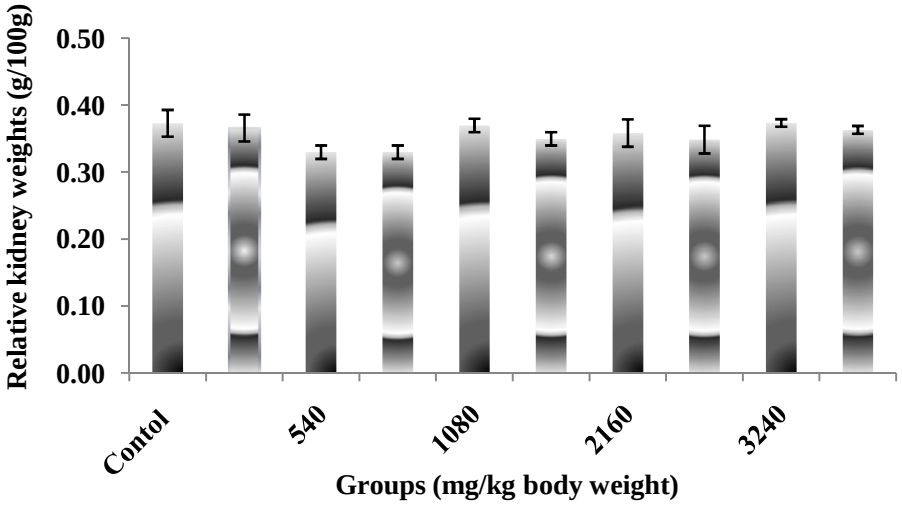


Fig. 31b Changes in relative weight of kidney of female rats in **acute** toxicity study

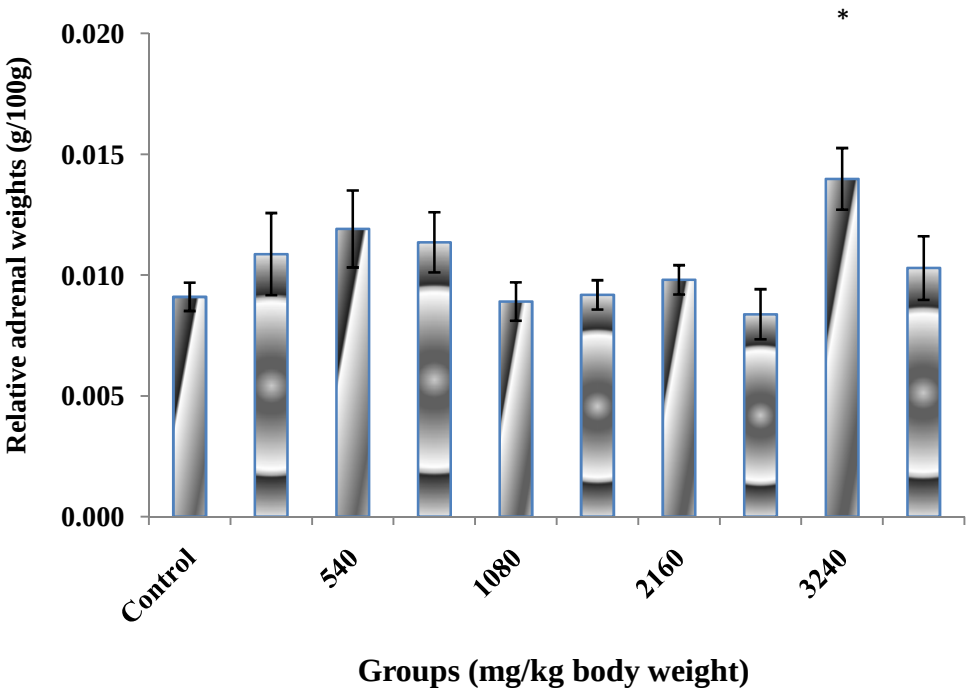


Fig. 32a Changes in relative weight of adrenal gland of male rats in **acute** toxicity study

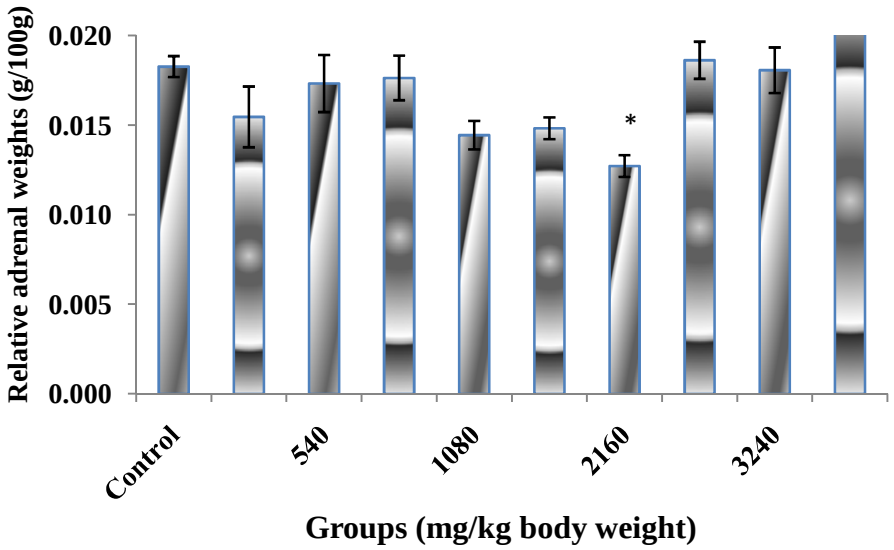


Fig. 32b Changes in relative weight of adrenal gland of female rats in **acute** toxicity study

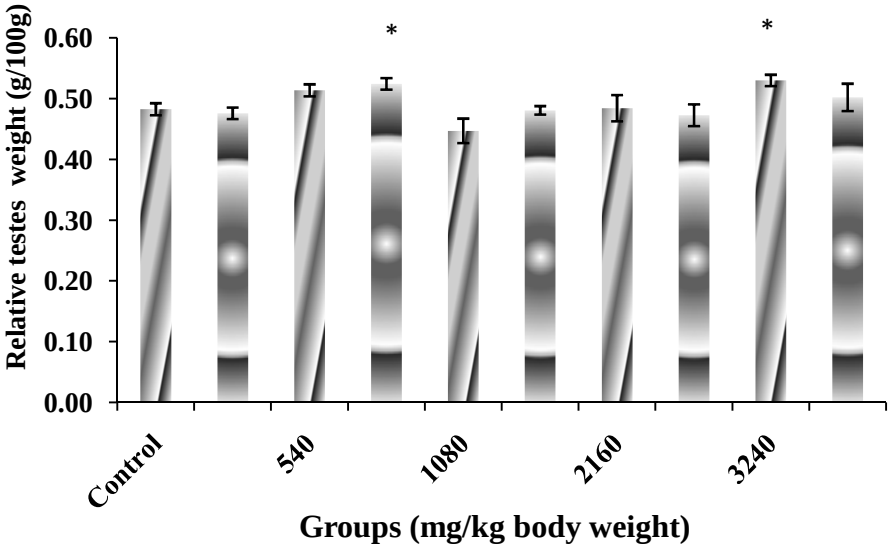


Fig. 33 Changes in relative weight of testes of male rats in **acute** toxicity study

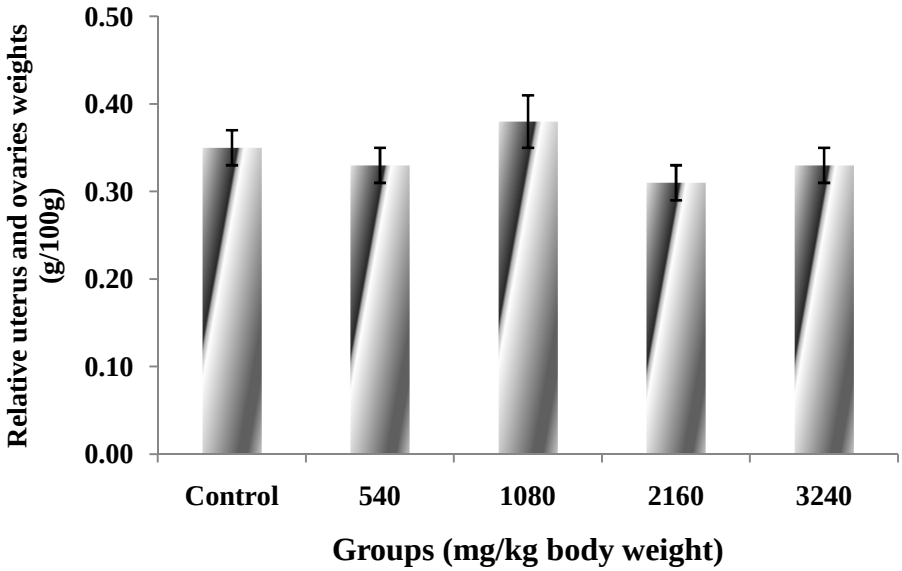


Fig. 34 Changes in relative weight of uterus and ovaries of female rats in **acute** toxicity study

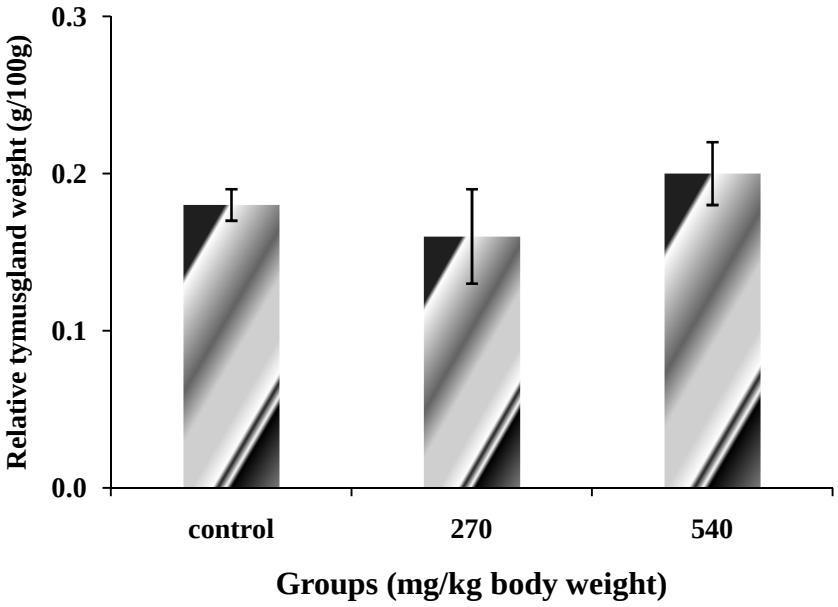


Fig. 35a Changes in relative weight of thymus gland of male rats in **sub-acute** toxicity study

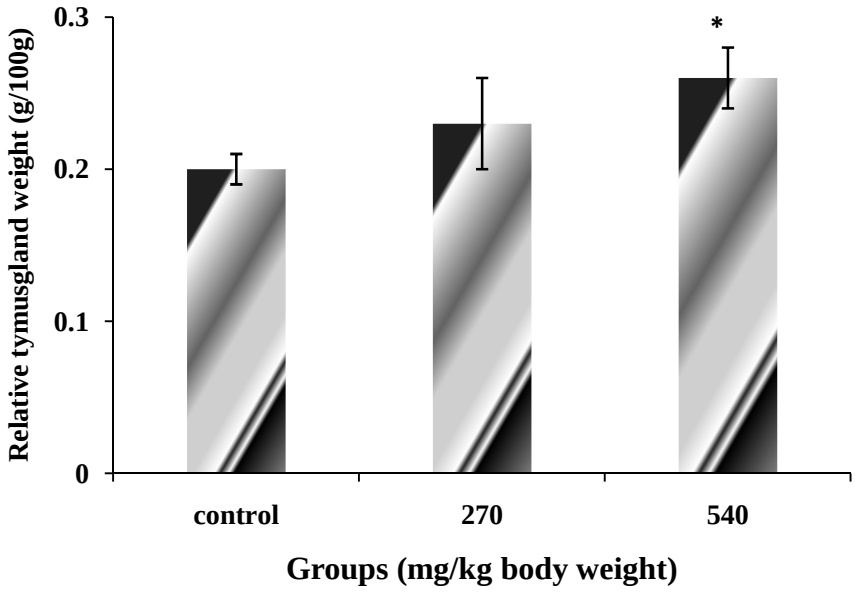


Fig. 35b Changes in relative weight of thymus gland of female rats in **sub-acute** toxicity study

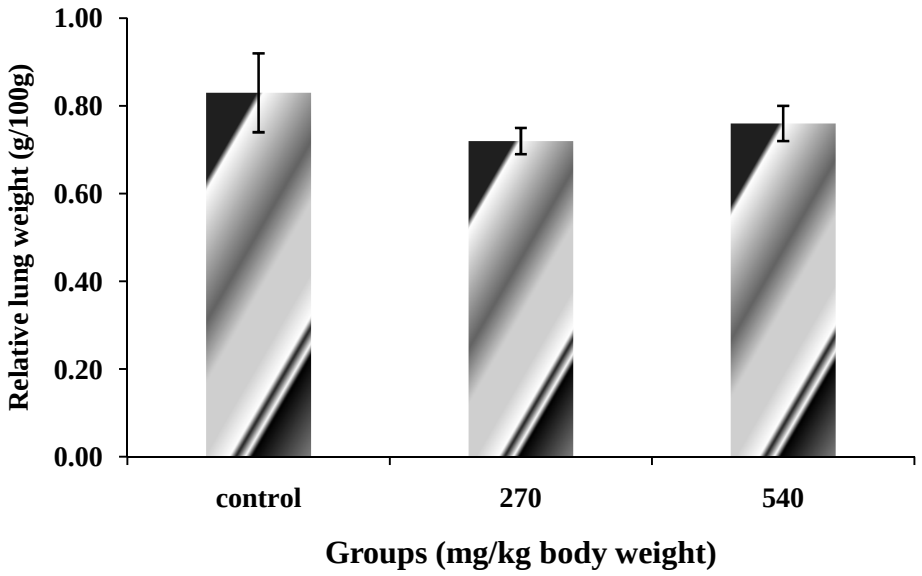


Fig. 36a Changes in relative weight of lung of male rats in **sub-acute** toxicity study

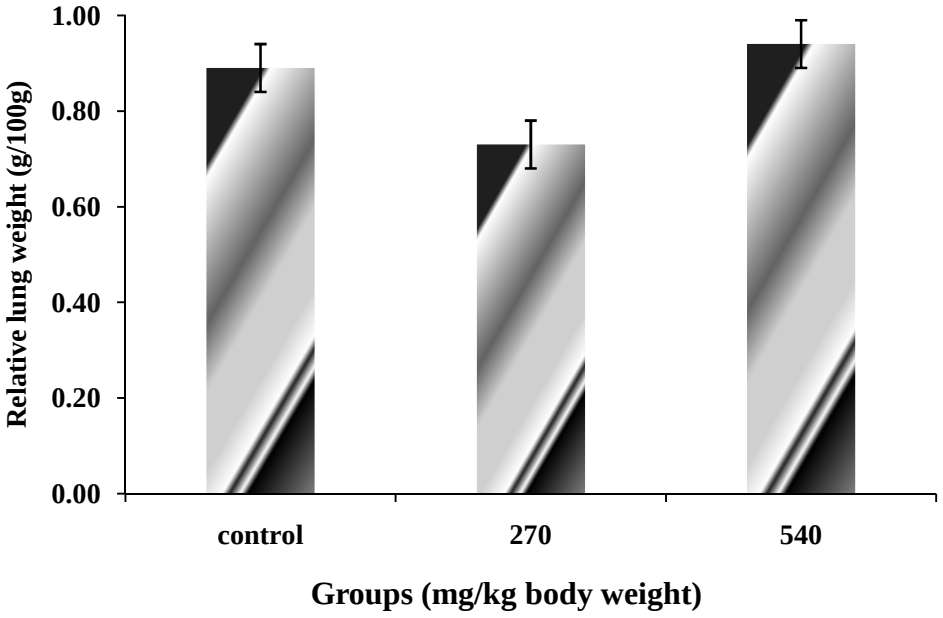


Fig. 36b Changes in relative weight of lung of female rats in **sub-acute** toxicity study

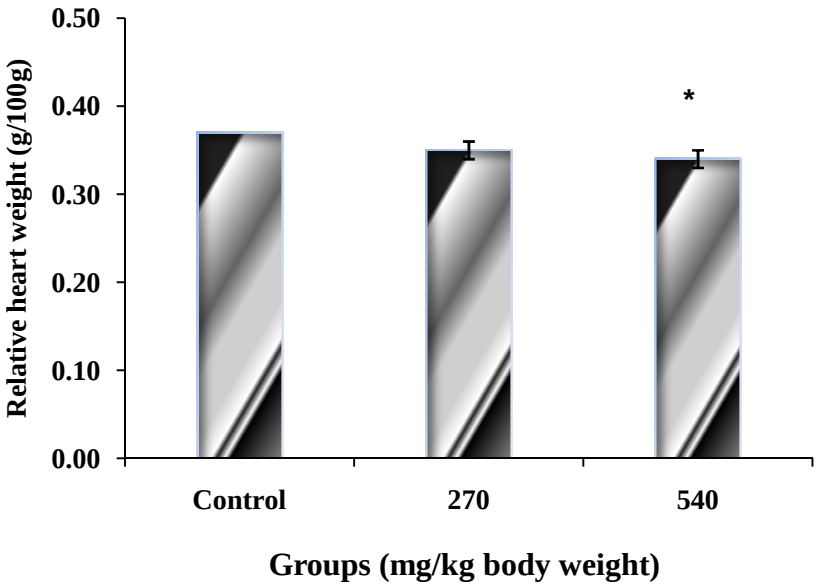


Fig. 37a Changes in relative weight of heart of male rats in **sub-acute** toxicity study

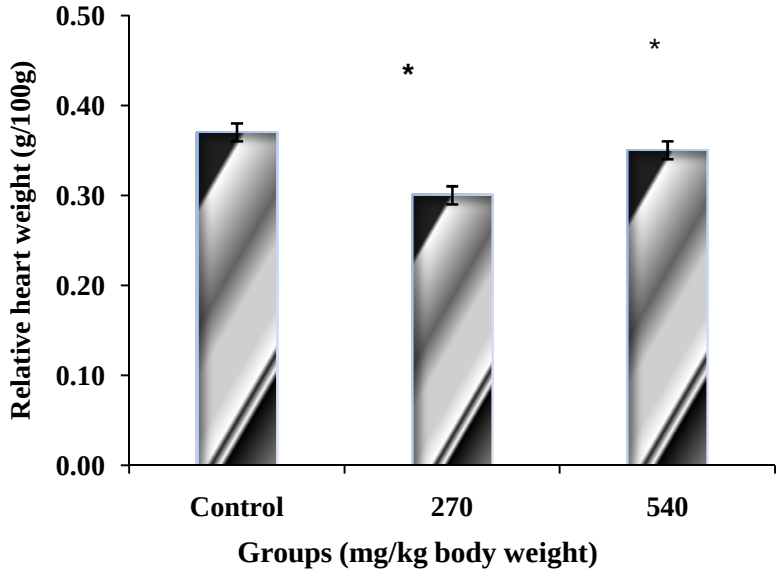


Fig. 37b Changes in relative weight of heart of female rats in **sub-acute** toxicity study

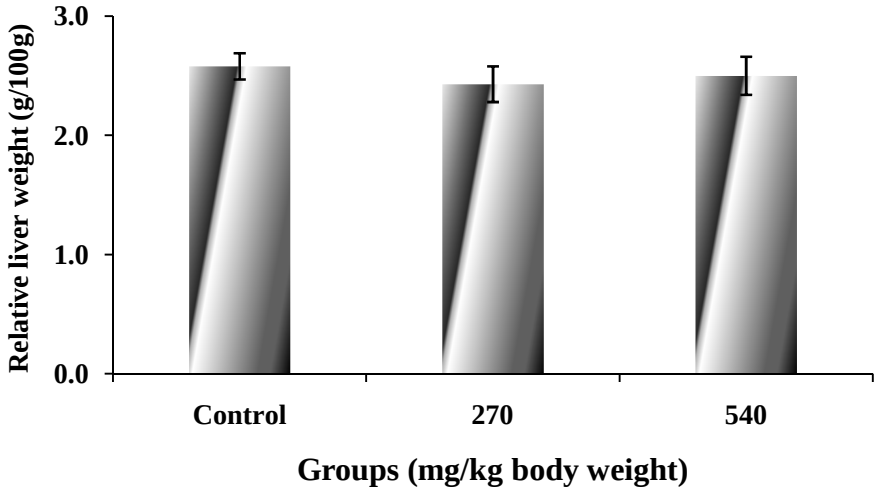


Fig. 38a Changes in relative weight of liver of male rats in **sub-acute** toxicity study

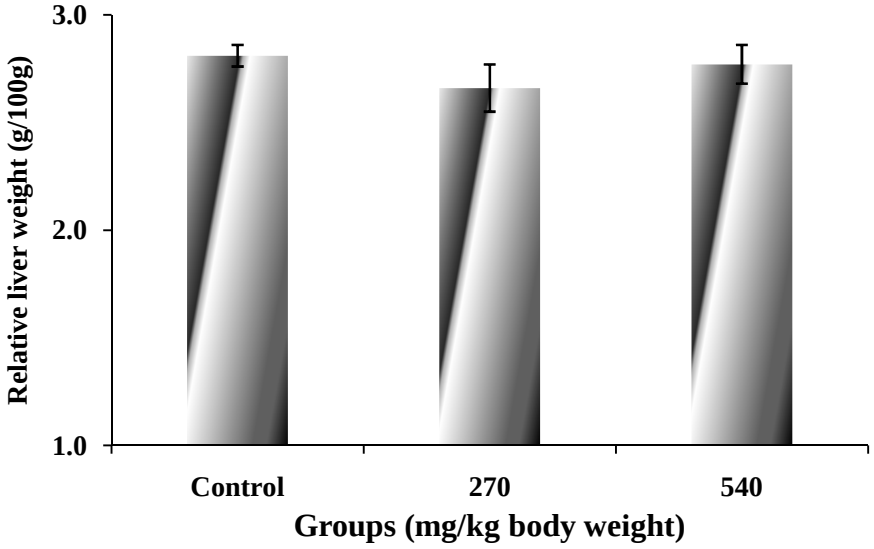


Fig. 38b Changes in relative weight of liver of female rats in **sub-acute** toxicity study

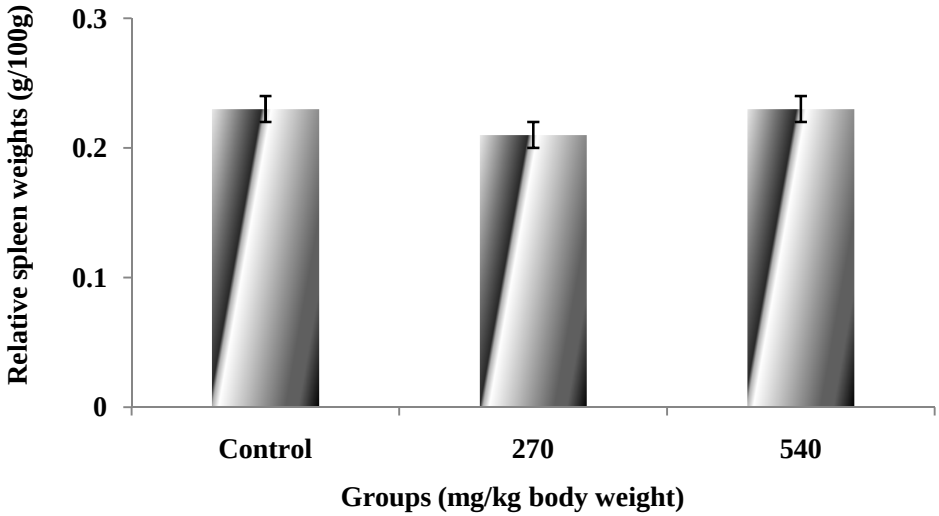


Fig. 39a Changes in relative weight of spleen of male rats in **sub-acute** toxicity study

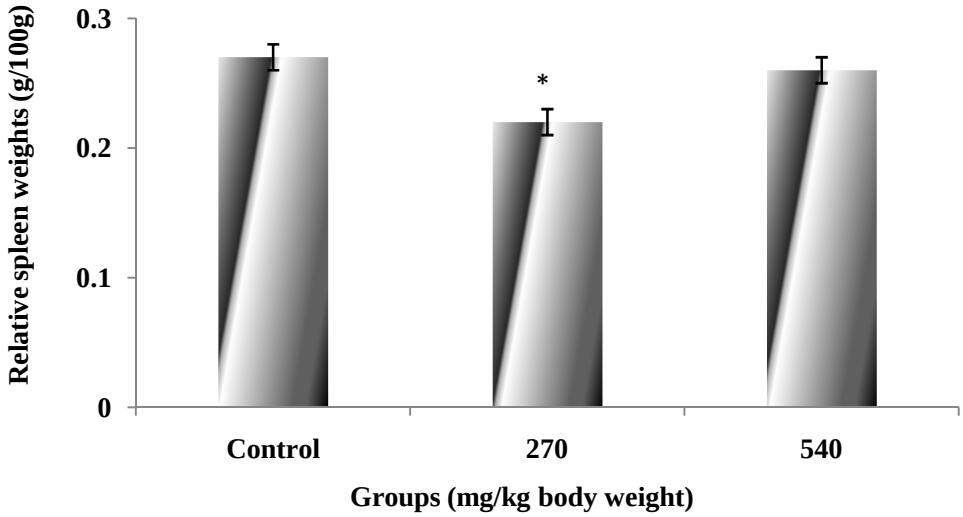


Fig. 39b Changes in relative weight of spleen of female rats in **sub-acute** toxicity study

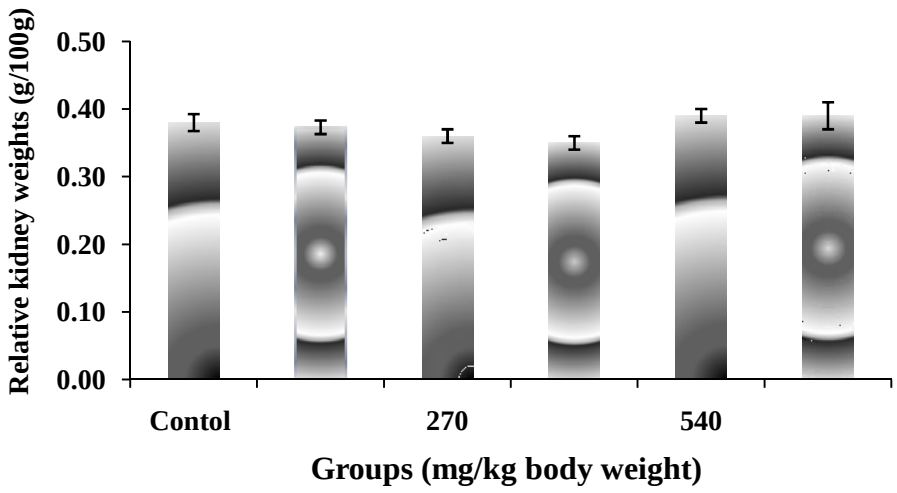


Fig. 40a Changes in relative weight of kidney of male rats in **sub-acute** toxicity study

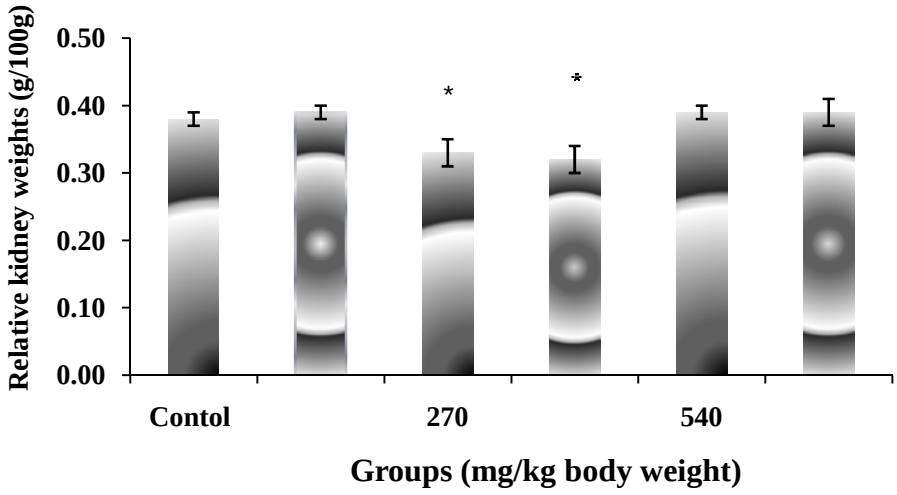


Fig. 40b Changes in relative weight of kidney of female rats in **sub-acute** toxicity study

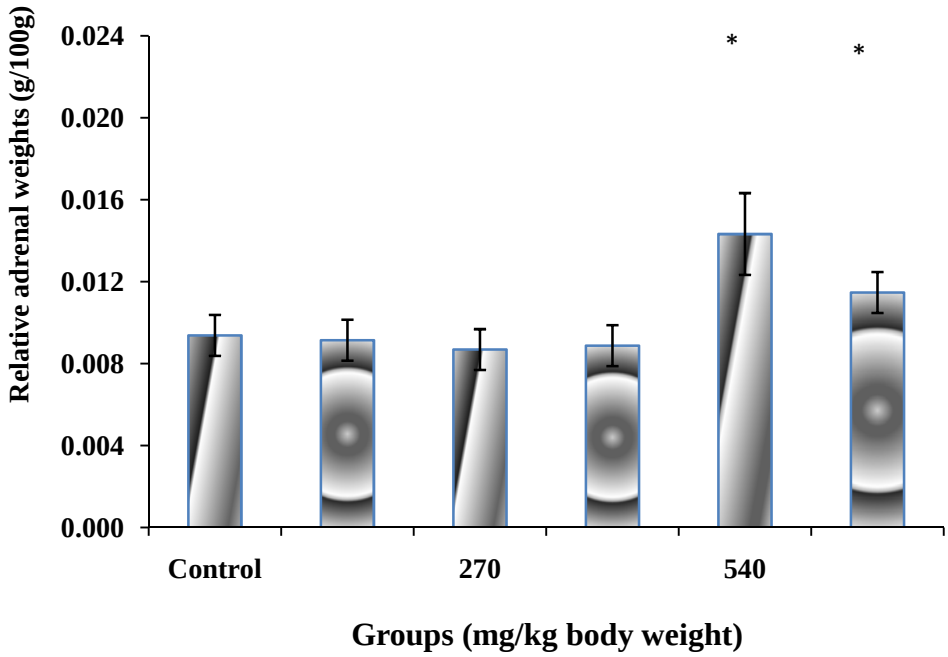


Fig. 41a Changes in relative weight of adrenal gland of male rats in **sub-acute** toxicity study

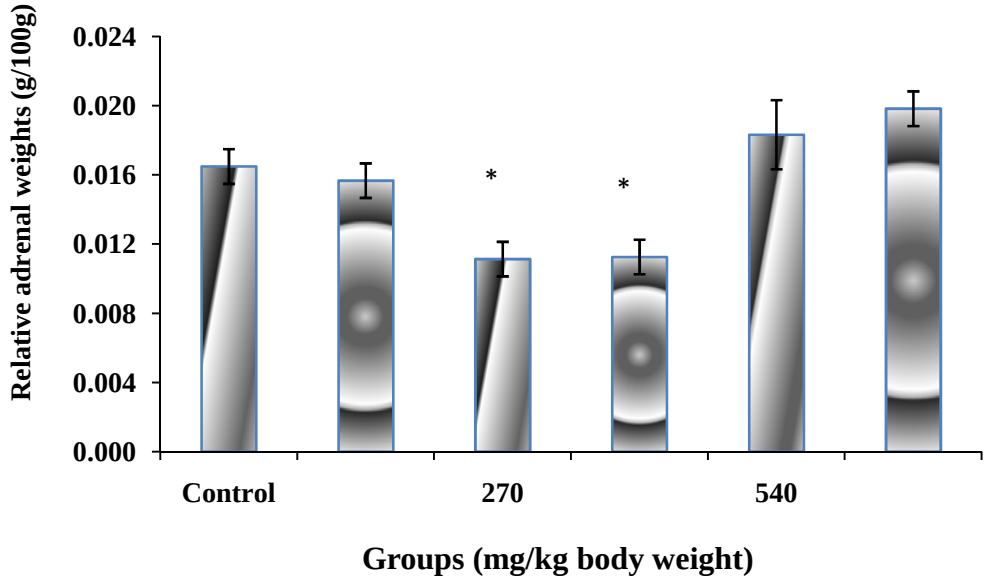


Fig. 41b Changes in relative weight of adrenal gland of female rats in **sub-acute** toxicity study

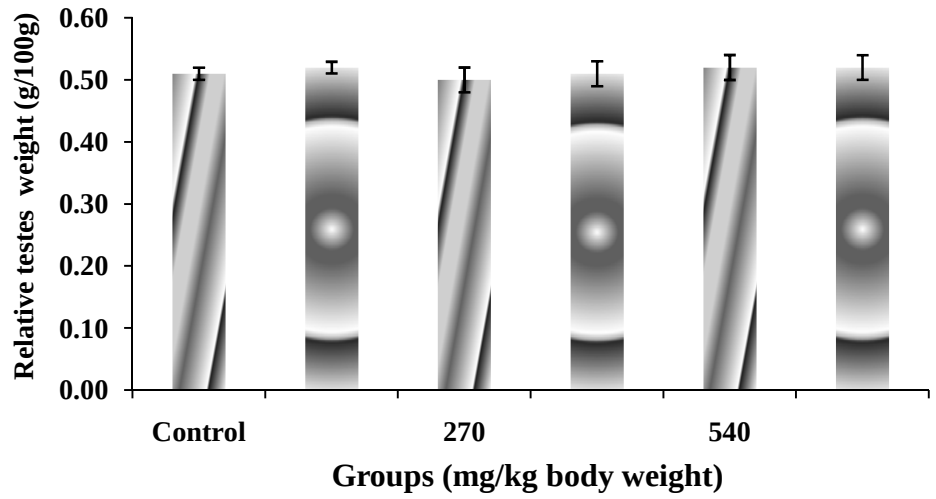


Fig. 42 Changes in relative weight of testes of male rats in **sub-acute** toxicity study

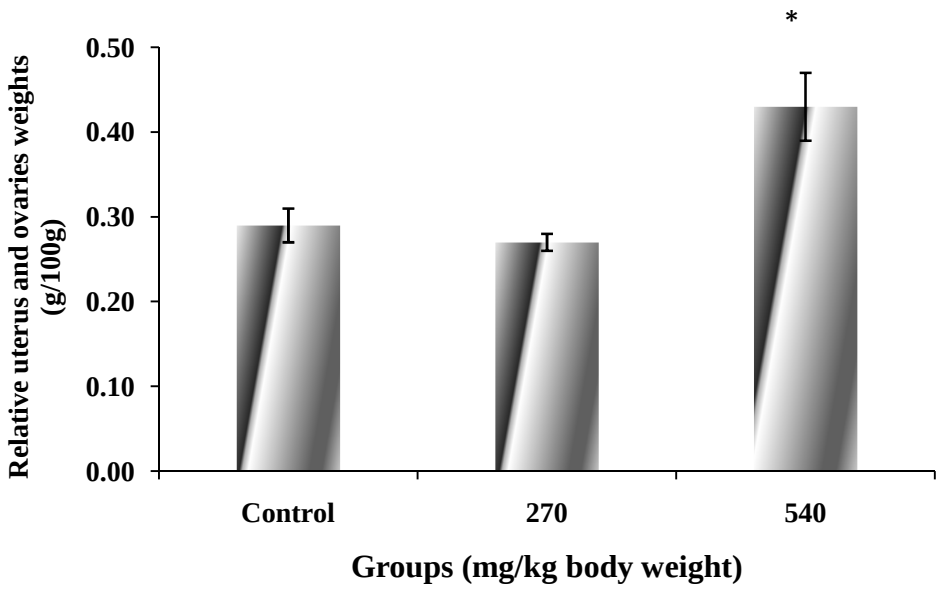


Fig. 43 Changes in relative weight of uterus and ovaries of female rats in **sub-acute** toxicity study

4.12.9 Histopathology of studied organs

The histological analyses of liver, heart, kidney, spleen and thymus gland revealed no morphological change in treated animals (Figs. 44-48). The histological analyses of liver revealed no morphological change in treated animals, as shown by a normal lobular architecture and portal-space containing arterioles, venule and bile ducts. No fatty changes were observed in treated groups (Fig. 44). The histological analyses of heart revealed no morphological change in treated animals. The methanolic extract of *P. longifolia* did not produce any gross changes in cell density and pattern in heart at any dose levels (Fig. 45). The histological analyses of kidney revealed no morphological change in treated animals. In all groups, renal cortex and renal corpuscles were preserved, as well as all types of tubules (Fig. 46). The methanolic extract of *P. longifolia* increased white pulp in spleen at the dose of 540 mg/kg. However, there was no change at the dose of 270 mg/kg. In all groups, red pulp and capsule were preserved (Fig. 47). The histological analyses of thymus gland revealed no morphological change in treated animals. In all groups, medulla, lymphocytes and capsule were preserved (Fig. 48).

PLATE - 1

Fig. 44a Photomicrographs of T.S. of Liver (Control)

The liver cross-section illustrates sinusoids (S) and hepatocytes (H) at magnification (400×). All these structures were found to be totally conserved. Normal cytoarchitecture was observed.

Fig. 44b Photomicrographs of T.S. of Liver (270 mg/kg b.w.)

The liver cross-section illustrates sinusoids (S) and hepatocytes (H) at magnification (400×). All these structures were found to be totally conserved. The methanolic extract of *P. longifolia* did not produce any histological changes in hepatic cells at 270 mg/kg b.w.

Fig. 44c Photomicrographs of T.S. of Liver (540 mg/kg b.w.)

The liver cross-section illustrates sinusoids (S) and hepatocytes (H) at magnification (400×). All these structures were found to be totally conserved. The methanolic extract of *P. longifolia* did not produce any histological changes in hepatic cells at 540 mg/kg b.w.

Plate - 1 Photomicrographs of T.S. of Liver

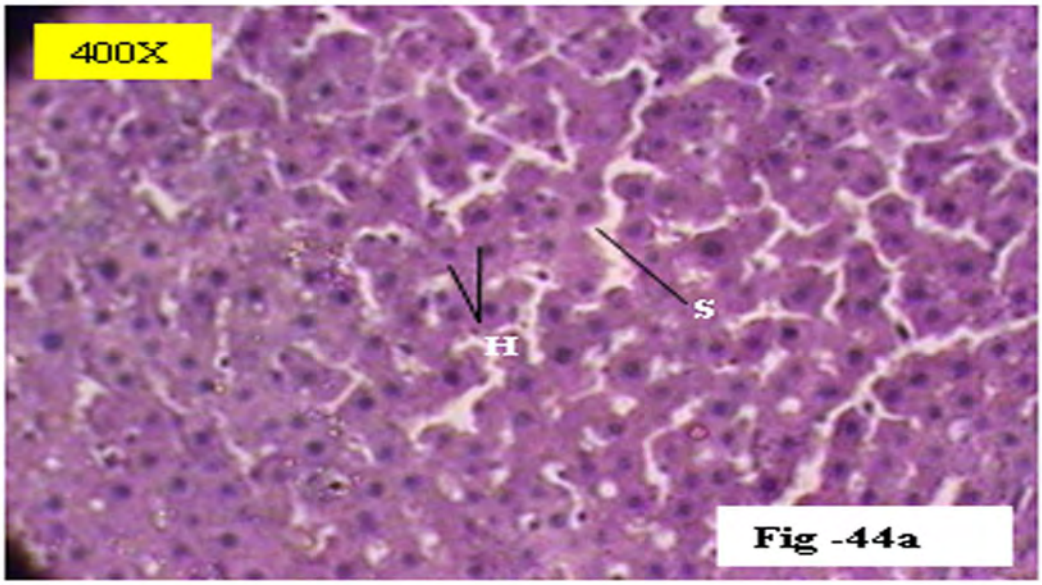


Fig -44a

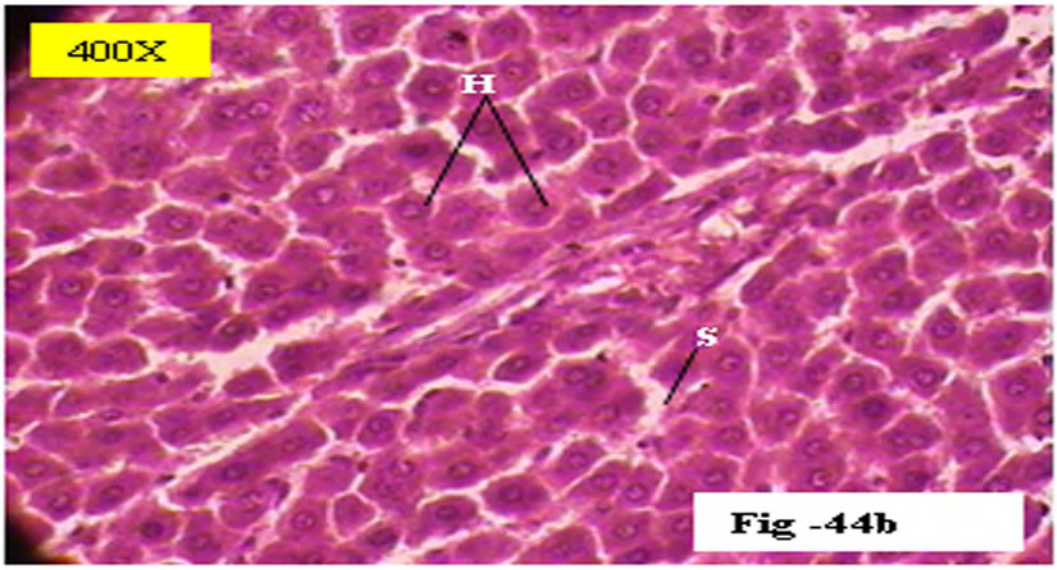


Fig -44b

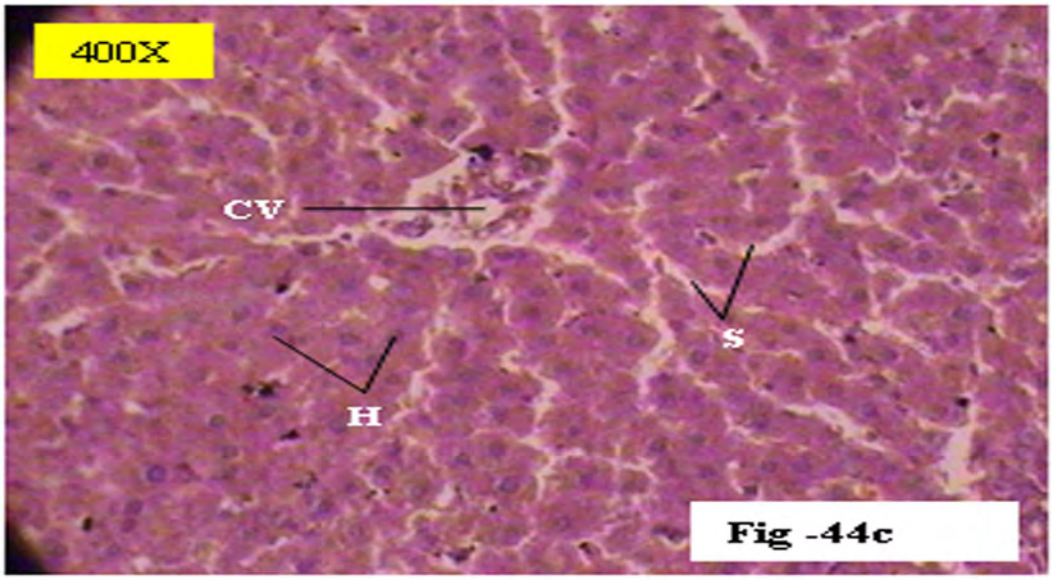


Fig -44c

PLATE - 2

Fig. 45a Photomicrographs of T.S. of Heart (Control)

The heart cross-section illustrates connective tissue (CT) and myocardium (MC) at magnification (400×). All these structures were found to be totally conserved. Normal cytoarchitecture was observed.

Fig. 45b Photomicrographs of T.S. of Heart (270 mg/kg b.w.)

The heart cross-section illustrates connective tissue (CT) and myocardium (MC) at magnification (400×). All these structures were found to be totally conserved. The methanolic extract of *P. longifolia* did not produce any gross changes in cell density and pattern in heart at 270 mg/kg b.w.

Fig. 45c Photomicrographs of T.S. of Heart (540 mg/kg b.w.)

The heart cross-section illustrates connective tissue (CT) and myocardium (MC) at magnification (400×). All these structures were found to be totally conserved. The methanolic extract of *P. longifolia* did not produce any gross changes in cell density and pattern in heart at 540 mg/kg b.w.

Plate - 2 Photomicrographs of T.S. of Heart

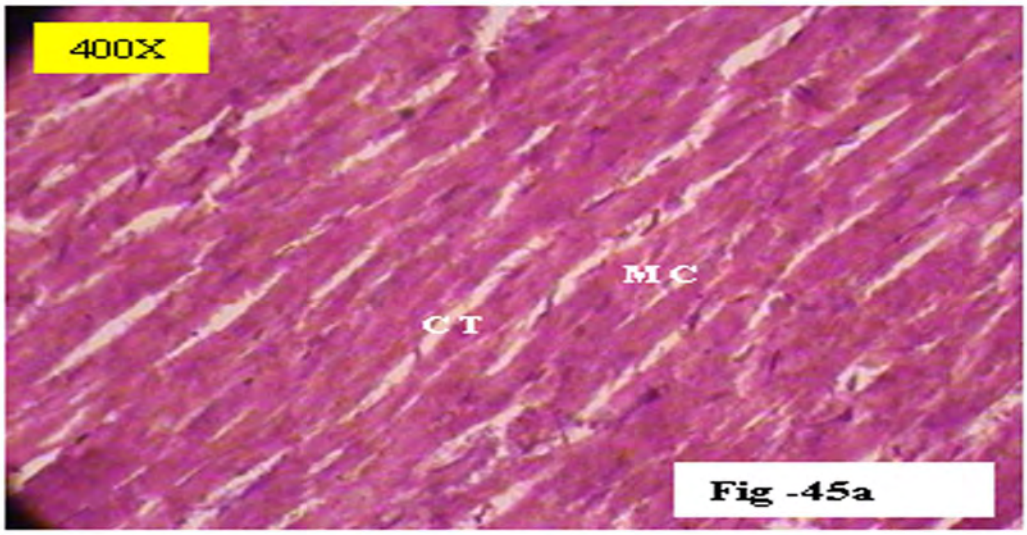


Fig -45a

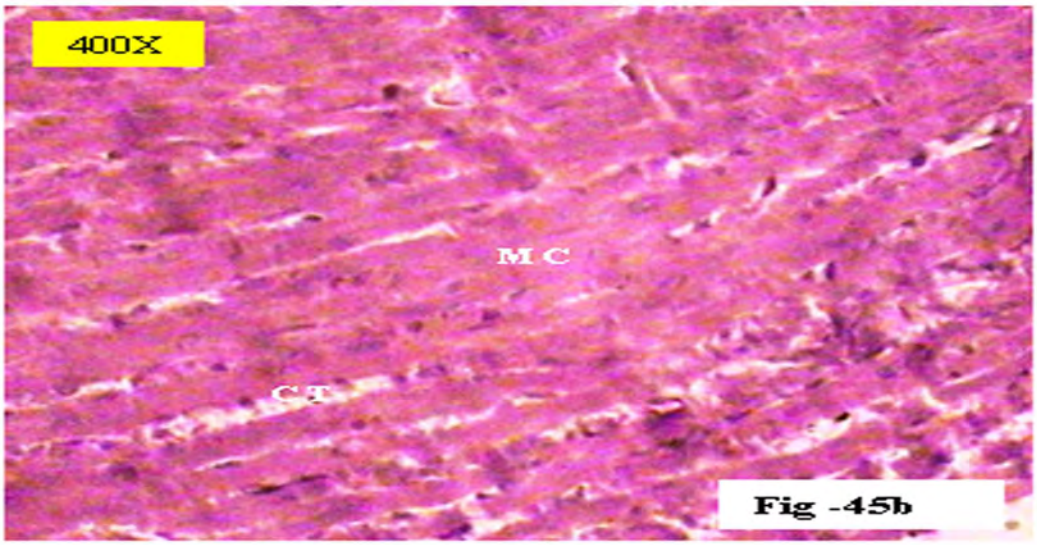


Fig -45b

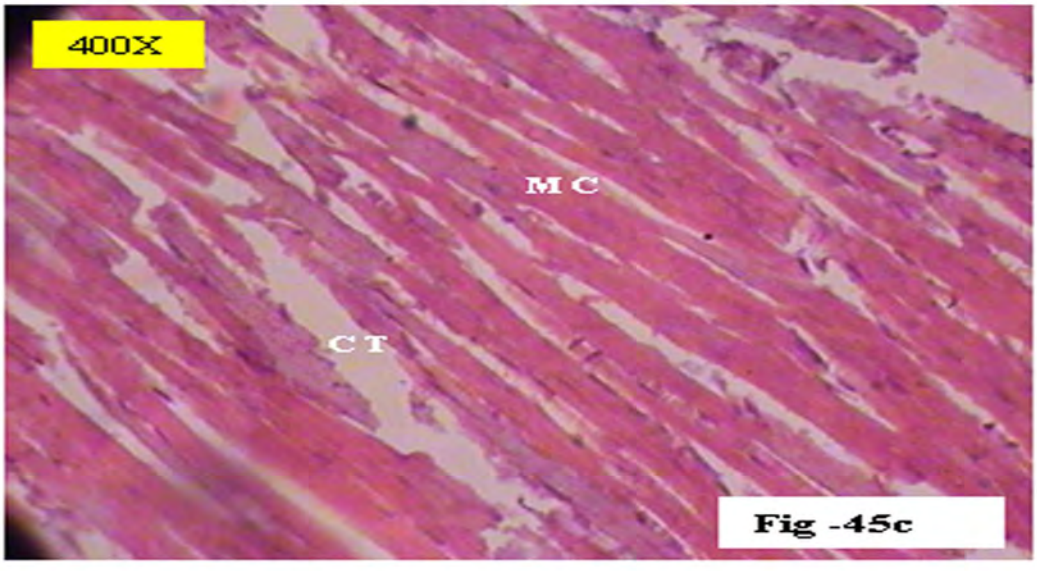


Fig -45c

PLATE - 3

Fig. 46a Photomicrographs of T.S. of Kidney (Control)

The kidney cross-section illustrates bowman's space (BS), tubules (T) renal corpuscles (RC) at magnification (400×). All these structures were found to be totally conserved. Normal cytoarchitecture was observed.

Fig. 46b Photomicrographs of T.S. of Kidney (270 mg/kg b.w.)

The kidney cross-section illustrates bowman's space (BS), tubules (T) renal corpuscles (RC) at magnification (400×). All these structures were found to be totally conserved. The methanolic extract of *P. longifolia* did not produce any histological changes in kidney at 270 mg/kg b.w.

Fig. 46c Photomicrographs of T.S. of kidney (540 mg/kg b.w.)

The kidney cross-section illustrates bowman's space (BS), tubules (T) renal corpuscles (RC) at magnification (400×). All these structures were found to be totally conserved. The methanolic extract of *P. longifolia* did not produce any histological changes in kidney at 540 mg/kg b.w.

Plate - 3 Photomicrographs of T.S. of Kidney

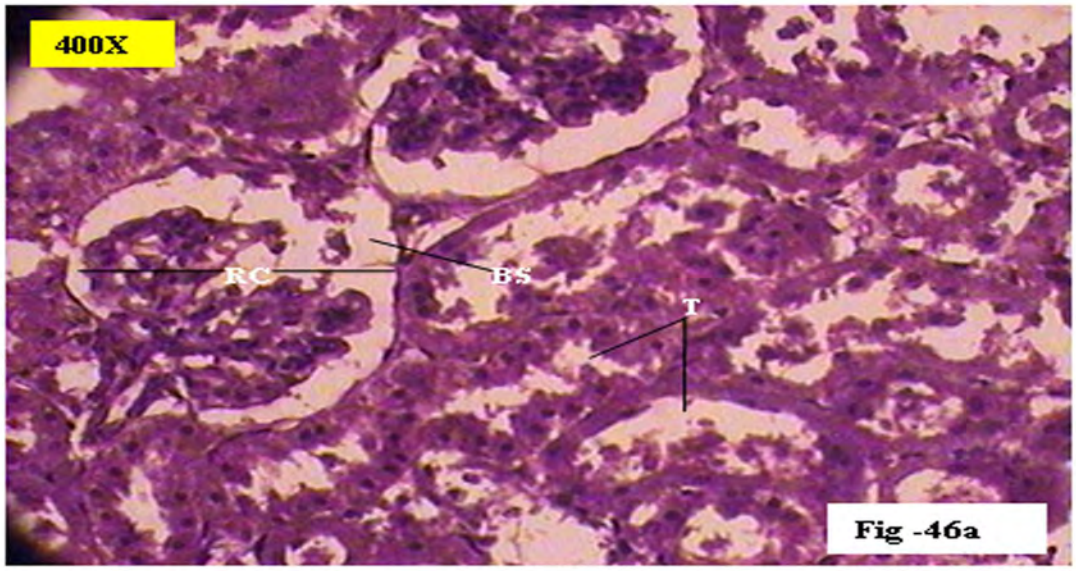


Fig -46a

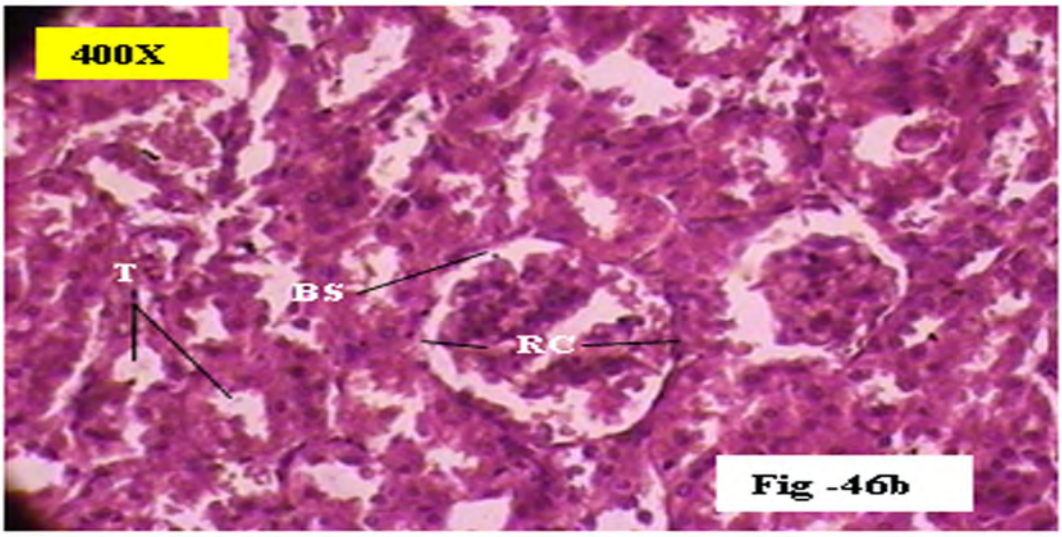


Fig -46b

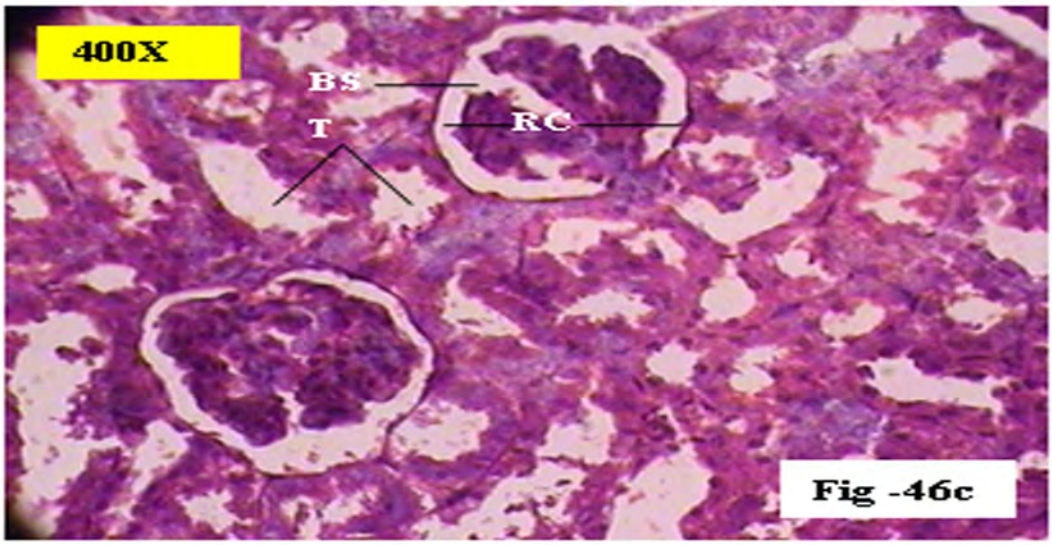


Fig -46c

PLATE - 4

Fig. 47a Photomicrographs of T.S. of Spleen (Control)

The spleen cross-section illustrates red pulp (RP), white pulp (WP) and capsule (C) at magnification (400×). All these structures were found to be totally conserved. Normal cytoarchitecture was observed.

Fig. 47b Photomicrographs of T.S. of Spleen (270 mg/kg b.w.)

The spleen cross-section illustrates red pulp (RP), white pulp (WP) and capsule (C) at magnification (400×). All these structures were found to be totally conserved. The methanolic extract of *P. longifolia* did not produce any histological changes in spleen at 270 mg/kg b.w.

Fig. 47c Photomicrographs of T.S. of Spleen (540 mg/kg b.w.)

The spleen cross-section illustrates red pulp (RP), white pulp (WP) and capsule (C) at magnification (400×). All these structures were found to be totally conserved. The methanolic extract of *P. longifolia* increased white pulp in spleen at 540 mg/kg b.w.

Plate - 4 Photomicrographs of T.S. of Spleen

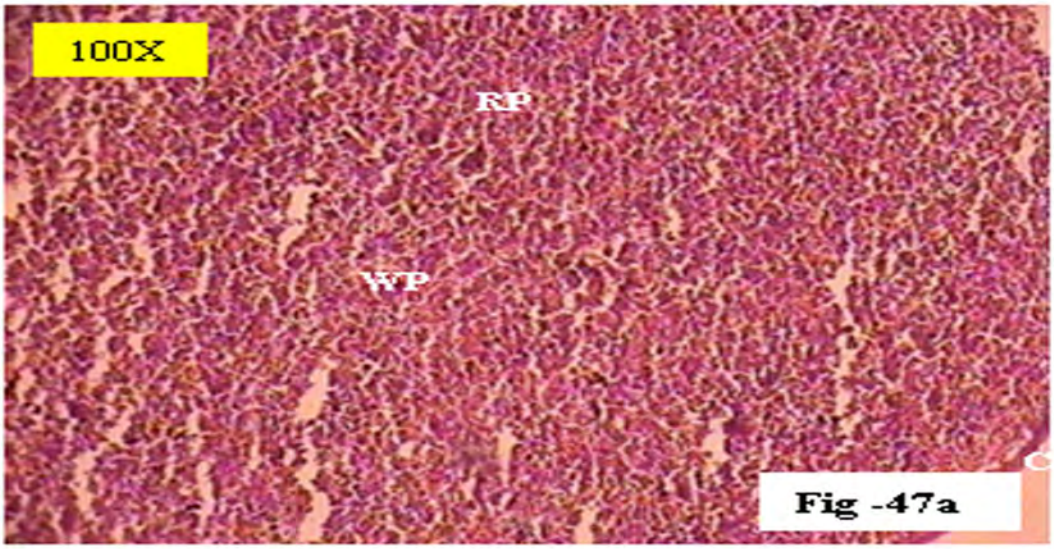


Fig -47a

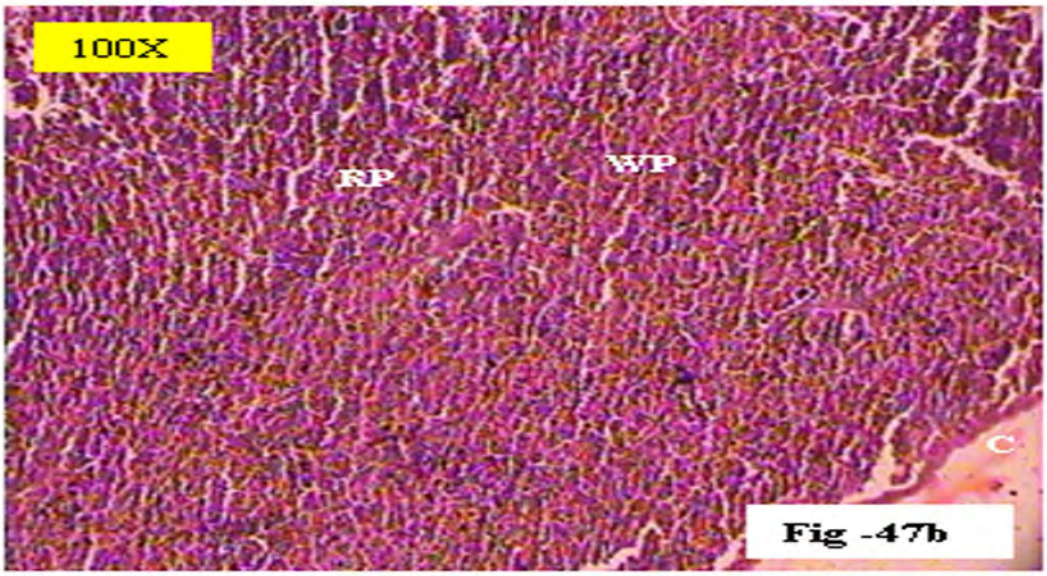


Fig -47b

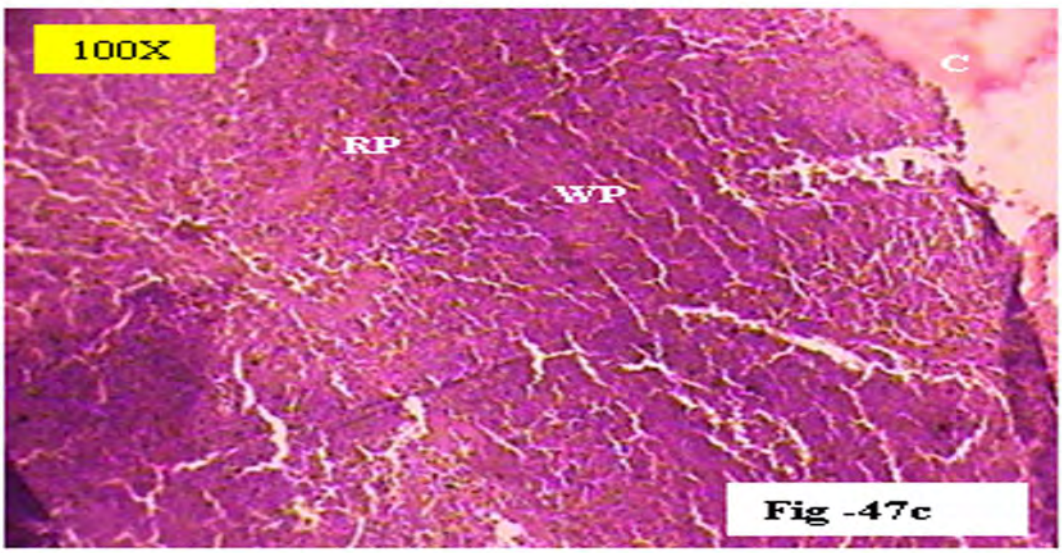


Fig -47c

PLATE - 5

Fig. 48a Photomicrographs of T.S. of Thymus gland (Control)

The thymus gland cross-section illustrates medulla (M), lymphocytes (Lc) and capsule (C) at magnification (400×). All these structures were found to be totally conserved. Normal cytoarchitecture was observed.

Fig. 48b Photomicrographs of T.S. of Thymus gland (270 mg/kg b.w.)

The thymus gland cross-section illustrates medulla (M), lymphocytes (Lc) and capsule (C) at magnification (400×). All these structures were found to be totally conserved. The methanolic extract of *P. longifolia* did not produce any histological changes in thymus gland at 270 mg/kg b.w.

Fig. 48c Photomicrographs of T.S. of Thymus gland (540 mg/kg b.w.)

The thymus gland cross-section illustrates medulla (M), lymphocytes (Lc) and capsule (C) at magnification (400×). All these structures were found to be totally conserved. The methanolic extract of *P. longifolia* did not produce any histological changes in thymus gland at 540 mg/kg b.w.

Plate - 5 Photomicrographs of T.S. Thymus gland

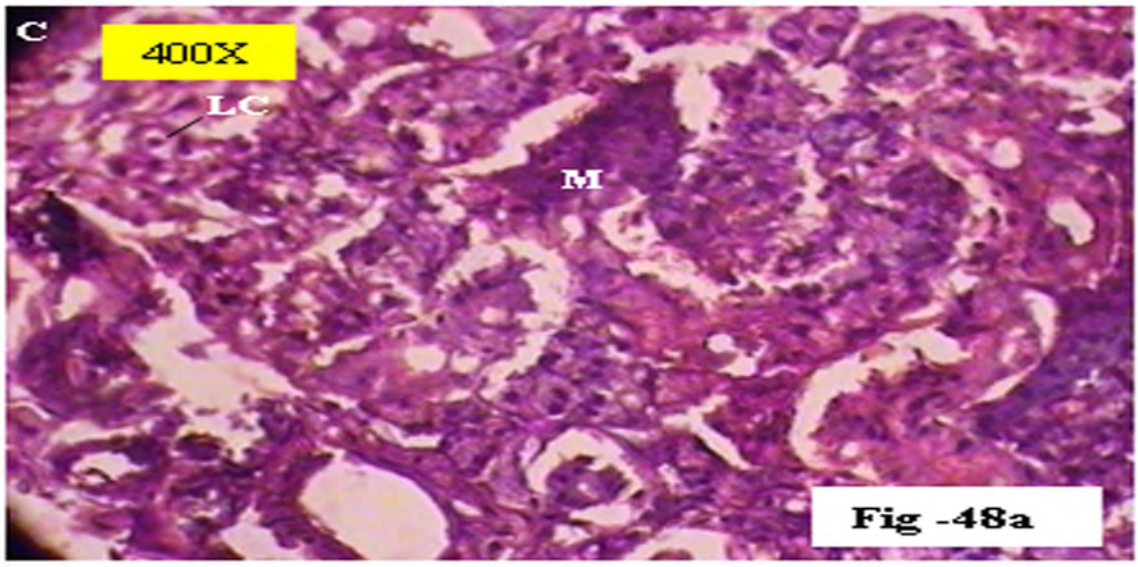


Fig -48a

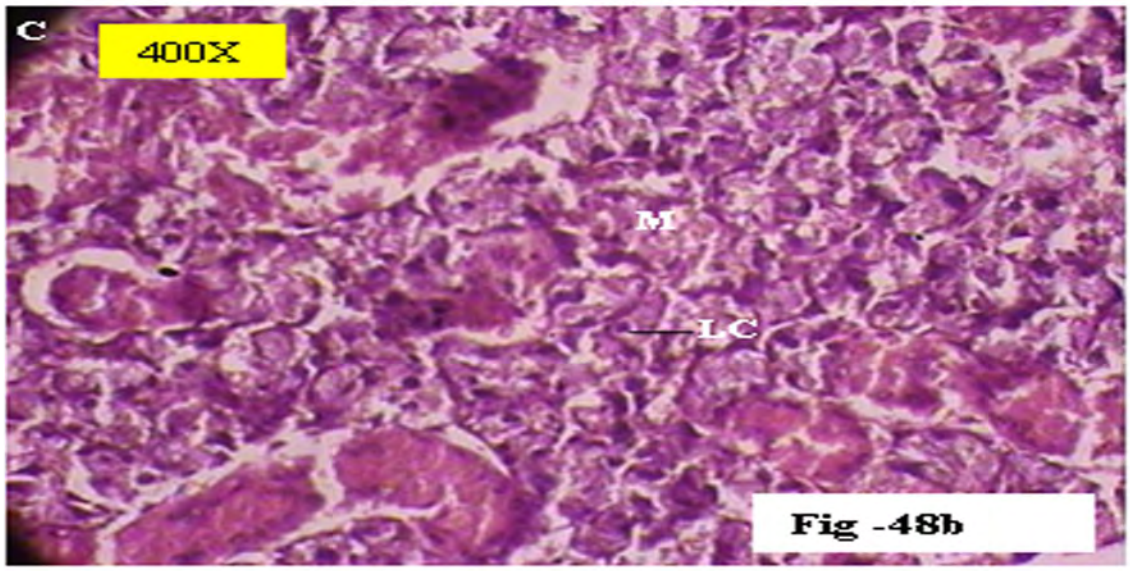


Fig -48b

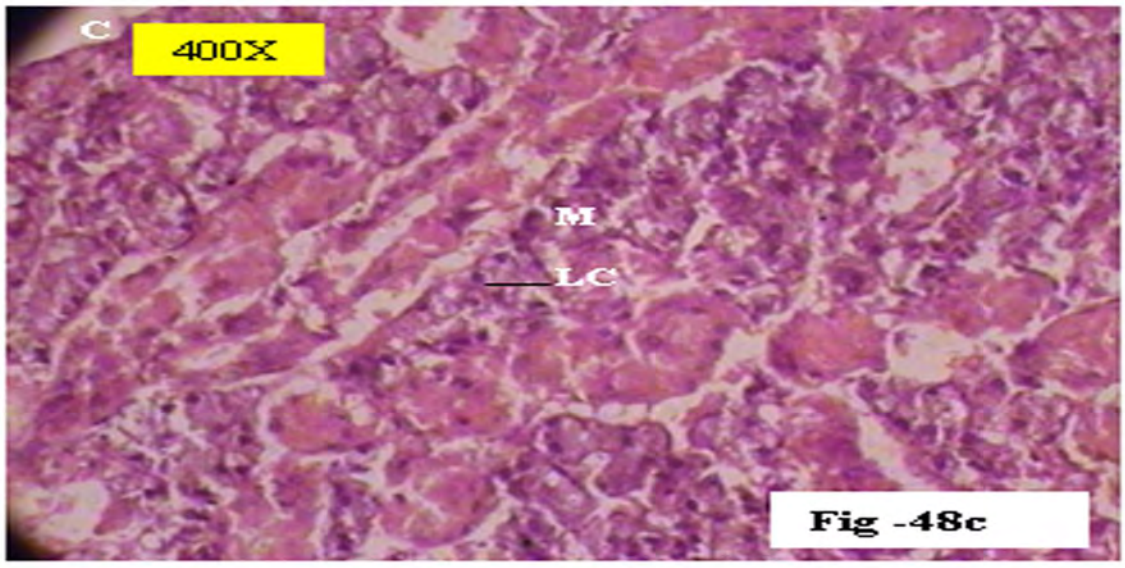


Fig -48c

CHAPTER 5: DISCUSSION

5.1 Pharmacognostic study

Natural products provide a significant source of potential drugs from which humankind has identified not only phytomedicines and herbal remedies, but also most of our current anticancer and antibiotics drugs. The evaluation of a crude drug is an integral part of establishing its correct identity. Before any crude drug can be included in herbal pharmacopoeia, pharmacognostical parameters and standards must be established. Therefore some diagnostic features have been evaluated to identify and to differentiate *P. longifolia* leaf from other crude drugs and adulterants. The leaf primordial in *P. longifolia* was in an alternate pattern, the initiation of the appendage taking place in the second tunica layer (Ramji, 1960). The venation pattern of the leaf was pinnate, the secondary veins traversing obliquely from the midrib and anastomosing with adjacent ones by tertiary veins near the margin (Ramji, 1961). Flowers were in axils of leaves. The leaves of *P. longifolia* were bright green in color and polished above, paler beneath. The transverse section of the leaf showed single layered epidermis. Oil glands were found in palisade tissue and in lower midrib. Prismatic crystals of calcium oxalate were found. These were some typical characteristics which may be used for its identification and prevention of drug adulteration. Pharmacognostic studies on different plants has been done by various workers (Khatoon et al., 2006; Abere et al., 2007; Chanda et al., 2010a; Dave et al., 2010; Essiett et al., 2010; Sandhya et al., 2010).

5.2 Physicochemical analysis

The physical constant evaluation of the drugs is an important parameter in detecting adulteration or improper handling of drugs. The moisture content of dry powder of leaves of *P. longifolia* was 12 % which is not very high, hence it would discourage bacteria fungi or yeast growth. The residue remaining after incineration of plant material is the ash content or ash value, which simply represents inorganic salts, naturally occurring in crude drug or adhering to it or deliberately added to it, as a form of adulteration. The ash value was determined by three different methods *viz.* total ash, acid-insoluble ash and water-soluble ash. The total ash method is employed to measure the total amount of material remaining after ignition. This includes both ‘physiological ash’ which is derived from the plant tissue itself, and ‘non-

physiological ash', which is the residue of the extraneous matter adhering to the plant surface. Acid-insoluble ash is a part of total ash and measures the amount of silica present, especially as sand and siliceous earth. Water soluble ash is the water soluble portion of the total ash (Evans, 2002; Kokate et al., 2006). These ash values are important quantitative standards. Low amount of total ash, acid insoluble ash and water soluble ash indicates that the inorganic matter and non-physiological matter such as silica is less in leaves of *P. longifolia*. The variation in extractable matter in various solvents is suggestive of the fact that the formation of the bioactive principle of the medicinal plant is influenced by number of intrinsic and extrinsic factors. High alcohol soluble and water soluble extractive value reveal the presence of polar substance like phenols, tannins and glycosides, as also reported by Sharma et al. (2009).

The pH is an important parameter for formulations (Soares et al., 2005). The pH of powder of leaves was acidic which implies that it may be irritating to the gastrointestinal tract. It might also not be useful in the formulation of acidic drugs. The solubility of the methanolic extract of *P. longifolia* was more in methanol followed by dimethylsulphoxide and less in acetone. There was minor presence of some heavy metals in the methanolic extract. The permissible limit of lead, cadmium, arsenic and mercury in the dietary contents as per the WHO (1998) is 10 ppm, 0.3 ppm, 10 ppm, 1 ppm. Although WHO (1998) has not yet decided the permissible limits of chromium, zinc and other metals because they are considered as micronutrients (Meena et al., 2010; Swamy and Ravikumar, 2010). All these parameters are useful for the compilation of a suitable monograph for it proper identification.

5.3 Phytochemical analysis

P. longifolia leaves were rich in alkaloids, flavonoids, tannins, triterpenes, saponins and cardiac glycosides while steroids and phlobatannins were absent. It is possible that these secondary metabolites might be responsible for the bioactivity of the plant extract (Nino et al., 2006). The presence of alkaloids and saponins in the plant indicates that the plant extract could be used for antifungal activity (Rani and Murty, 2006). Secondary metabolites may be used for the preparation of drug in a systematic

way which may lead to the cure of many ailments in the future (Shanthi and Amudha, 2010). The extract had high total phenol and crude alkaloid content. Plant phenolics are one of the major groups of compounds acting as primary antioxidant free radical terminators. These compounds possess a wide spectrum of chemical and biological activities including radical scavenging properties. Several studies have described the antioxidant properties of medicinal plants, foods and beverages which are rich in phenolic compounds (Wang et al., 2008b; Kaneria et al., 2009; Chanda et al., 2010b). In spectral analysis, comparison of the data with the reported spectral values of the IR, NMR and GC-MS spectra; it was found that the methanolic extract might contain some compounds like clerodane diterpenoids, allantoin and ent-halimane diterpenes (Phadnis et al., 1988; Hara et al., 1995).

5.4 Antioxidant study

5.4.1 *In vitro* antioxidant activity

The antioxidant reactions involve multiple steps including the initiation, propagation, branching and termination of free radicals. The antioxidants which inhibit the formation of free radicals from their unstable precursors are called preventive antioxidants, and those which interrupt the radical chain reaction (propagation and branching) are the 'chain-breaking' antioxidants (Ou et al., 2001).

Generally, there are various methods for determination of antioxidant activities. The measurement of radical scavenging activity of any antioxidant is commonly associated with the using of DPPH method because it is quick, reliable and reproducible method. It is widely used to test the ability of compounds as free radical scavengers or hydrogen donors and to evaluate the antioxidative activity of plant extracts. In the DPPH assay, the antioxidants reduce the stable radical DPPH to the yellow coloured diphenyl-picrylhydrazine which has maximum absorption at 517 nm. The concentration of antioxidant needed to decrease the initial DPPH concentration by 50% (IC_{50}) is a parameter widely used to measure the antioxidant activity. A lower IC_{50} value indicates a higher antioxidant power. In the present study, the IC_{50} value of methanolic extract was >1000 µg/ml. It indicates that this extract was not a good scavenger of DPPH radical. Similar results i.e. low DPPH activity was reported in

methanolic extract of seeds and fruit rinds of some plants belonging to the family Fabaceae (Chanda et al., 2010b).

In hydroxyl radical scavenging assay, free radicals were generated by Fentons reaction (Nordberg and Arner, 2001), which react with deoxyribose and produce MDA. MDA on being incubated with TBA produces a pink chromogen (Paya et al., 1992). When methanolic extract was added to the reaction mixture, it removed the hydroxyl radicals from the sugar and prevented the reaction. The IC_{50} value of methanolic extract was 610 µg/ml which indicates that methanolic extract had capacity to inhibit hydroxyl radical-mediated deoxyribose degradation in Fe^{3+}-EDTA -ascorbic acid and H_2O_2 reaction mixture, to a certain level.

Superoxide anion radical is a weak oxidant but it gives rise to the generation of powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress (Dahal and Richardson, 1978). The IC_{50} value of methanolic extract was 810 µg/ml which again indicated a moderate superoxide anion scavenging activity.

ABTS radical cation is a blue chromophore produced by the reaction between ABTS and potassium persulfate. Addition of the plant extract to this pre-formed radical cation reduced it to ABTS in a concentration-dependent manner. The methanolic extract of *P. longifolia* has good hydrogen-donating capacity, as evidenced by its IC_{50} value (75 µg/ml).

Compounds with reducing power indicate that they are electron donors, and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Yen and Chen, 1995). Many reports have revealed that there is a direct correlation between antioxidant activities and reducing power of certain plant extracts (Yildirim et al., 2001b; Oktay et al., 2003). The methanolic extract showed reducing capacity though it was much less than that of standard ascorbic acid which suggests that methanolic extract had a potency to donate electron to reactive free radicals, converting them into more stable non-reactive species and terminating the free radical chain reaction but to a limited extent.

The scavenging capacity of the ABTS radical by the extract was found to be much higher than that of hydroxyl radical scavenging activity and superoxide radical scavenging activity as well as reducing power. Factors like stereoselectivity of the radicals or the solubility of the extract in different testing systems have been reported to affect the capacity of extract to react and quench different radicals (Yu et al., 2002). Wang et al. (1998) found that some compounds which have ABTS radical scavenging activity did not show DPPH radical scavenging activity. In this study also, the extract showed strong ABTS radical scavenging activity but did not show DPPH radical scavenging activity. This suggests that the methanolic extract of *P. longifolia* had the capability to scavenge different free radicals differently in different systems, indicating that it may be useful as a therapeutic agent for treating some radical-related pathological damage. This study also proves our earlier conclusion that it is very essential to evaluate more than one antioxidant assay when natural plant extracts are being evaluated for their antioxidant potential (Chanda and Dave, 2009); and one plant may show good activity in one assay but poor in another assay (Chanda and Nagani, 2010).

5.4.2 *In vivo* antioxidant activity

The mechanism of hepatic damage by CCl_4 is well documented. CCl_4 is metabolized cytochrome P_{450} in the liver endoplasmic reticulum to active trichloromethyl radical ($CCl_3^{\bullet}$). This is turn reacts with molecular oxygen and gets converted to trichloromethyl peroxyl radical ($CCl_3O_2^{\bullet}$). Trichloromethyl peroxyl bonds covalently to cellular macromolecules and causes peroxidative degradation of lipids membrane of the adipose tissue. This leads to the formation of lipid peroxides, which in turn yield products like MDA, which causes loss of integrity of cell membranes and damage to hepatic tissue (Thabrew et al., 1987).

Mammalian cells contain wide antioxidant defense system. They contain endogenous antioxidant enzymes such as catalase, glutathione peroxidase and superoxide dismutase. The levels of antioxidant enzymes are strongly regulated within all cells to ensure the maintenance of the body's redox balance. These enzymes are able to detoxify free radicals by converting them back to more stable molecules within the cell, to be used or disposed accordingly (Saeed et al., 2005**).** Superoxide dismutases

are a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide, which is subsequently detoxified by the combined activities of catalase, glutathione peroxidase, and glutathione (reduced), resulting in the reduction of hydrogen peroxide and finally the production of water. The activity of catalase results in the conversion of hydrogen peroxide to water and oxygen while glutathione peroxidase facilitate the conjugation of hydrogen peroxide to glutathione reduced leading to the generation of water and oxidized glutathione (Akindele et al., 2010).

The lipid peroxidation is induced when free radicals are formed as the results of losing a hydrogen atom from the double bond in the structure of unsaturated fatty acids. Antioxidants work to protect lipids from peroxidation by radicals (Jain et al., 2008). Lipid peroxidation is found to be an important pathophysiological event in a variety of diseases such as aging, cancer, diabetes, cardiovascular disorders and rheumatoid arthritis (Ajitha and Rajnarayana, 2001). MDA is a useful index of lipid peroxidation being a major breakdown product of lipid peroxides. The engagement and overwhelming of antioxidant enzymes by free radicals result in the depletion of the antioxidant defenses and induction of lipid peroxidation evident in elevation of MDA level (Akindele et al., 2010). The results obtained in this study showed that CCl_4 significantly reduced the activity of hepatic *in vivo* antioxidant enzymes; GPx, CAT and non-enzyme GSH level and consequently increased the level of MDA. Methanolic extract of *P. longifolia* (270 mg/kg) significantly increased CCl_4-diminshed hepatic GSH level and GPx and CAT activities and reduced the CCl_4-elevated MDA level. Low dose of methanolic extract decreased protein content non-significantly, while high dose (540 mg/kg) of methanolic extract non-significantly increased total protein content. The high dose of extract significantly increased CCl_4-diminshed hepatic GPx activity and GSH level and non-significantly increased CCl_4-diminshed hepatic CAT and reduced the CCl_4-elevated MDA level.

The administration of CCl_4 resulted in elevated activities of enzymes in serum, an indicator of cellular leakage and loss of activities of cell membranes in liver (Naziroglu et al., 1999). The elevation of liver enzymes especially GPT has more importance as a specific marker of liver injury due to toxic drugs, alcohol and virus (Sherlock and Dooley, 2002). ALP activity on the other hand is related to the functioning of hepatocytes, increase in its activity being due to increased synthesis in

the presence of increased biliary pressure (Moss and Butterworth, 1974). Reduction in the levels of GPT and GOT towards the respective normal value is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damages caused by CCl_4 (Mukherjee, 2002). The total protein level including albumin level will be depressed in hepatotoxic conditions due to defective protein biosynthesis in liver. The CCl_4 intoxication cause disruption and disassociation of polyribosomes on endoplasmic reticulum and thereby reducing the biosynthesis of protein (Clawson, 1989). In this study, CCl_4 significantly decreased serum total protein and albumin content which is a further indication of hepatocellular damage (Navarro and Senior, 2006). The methanolic extract of *P. longifolia* showed hepatoprotective activity by reducing CCl_4-induced elevated levels of GPT, GOT, urea and BUN and increased CCl_4-induced reduction of serum albumin and total protein. These results suggest that the methanolic extract of *P. longifolia* possibly protect the structural integrity of the cell membrane of hepatocytes or enhance regeneration of damaged liver cells.

It is well known that free radicals are involved in the progression of ulcer and in water immersion stress model; increase in oxidative and decrease in antioxidative biomarkers have been reported by Singh et al., (2008b); Sood et al., (2010). The levels of various endogenous antioxidants decreased by chronic stress after immune activation (Gupta et al., 2009). In water immersion model, stress causes both sympathetic and parasympathetic stimulation of stomach leading to local hypoxia. The ischemic condition caused an increase in the levels H_2O_2 which in conjugation with O_2 generates $^{\bullet}OH$ ions which oxidized various cellular constituents such as proteins, membrane lipids (Tandon et al., 2004). GSH in tissues has been proposed to be a potential chemopreventive agent due to its antioxidant and detoxification properties. Reduced GSH plays pleiotropic role, including maintaining cells in a reduced state and serving as an electron donor for certain free radical scavenging enzymes (Alican et al., 1994). The GSH level was reduced in rats subjected to increased stress level as also reported by Sahin and Gumuslu (2007).

Total protein content significantly decreased at the low dose of extract but significantly increased at the high dose of extract. Various antioxidant enzymes like GPx, CAT and SOD prevent the accumulation of ROS but stress results in an imbalance in the activity of these enzymes which lead to faulty disposal of free

radicals and their accumulation (Halliwell, 1981). Treatment with methanolic extract of *P. longifolia* remarkably restored the levels of GSH content and GPx, CAT and SOD activities as compared to stress group. The results suggested that *P. longifolia* had potent *in vivo* antioxidant activity.

Arakawa et al., (1997) has shown in rats exposed to water immersion restrain stress for 6 h that this stress causes increases in plasma GPT, GOT, and LDH activities and urea nitrogen, creatinine, and glucose levels. The results obtained in this study showed water immersion stress caused increase in ALP, GPT, GOT activities and also increase in urea, BUN and cholesterol levels while total protein and albumin levels decreased. Treatment with methanolic extract reduced the level of this elevated enzymes activities and increased the level of total protein. However, albumin and GPT were unaffected by the methanolic extract.

5.5 Toxicity study

Herbal medicines are worldwide used for the treatment and prevention of various acute and chronic diseases and are gaining popularity in developing countries. Herbal medicines are often believed to be harmless because they are “natural,” easily available, and are commonly used for self-medication without supervision. Despite this widespread use of herbal medicine worldwide, few scientific studies have been undertaken to ascertain their safety and efficacy. This has raised concern regarding toxicity and adverse effects of these medicines (Saad et al., 2006). These medicines contain bioactive constituents with the potential to cause adverse effects (Bent and Ko, 2004). Toxicity profile of any bioactive compounds is a key feature in its further development to turn it into a successful molecule.

The results of the study suggested that *P. longifolia* is a relatively non toxic plant. According to Nair et al. (2009) there were no gross behavioral changes in mice fed with various solvent extracts of *P. longifolia* and suggested that this plant is safe in acute study. The determination of food and water consumption are important in the study of safety of a natural product, as proper intake of food and water are necessary to the physiological status of the animals and to the achievement of the proper response to the drug tested instead of a “false” response due to improper nutritional

conditions (Stevens and Mylecraine, 1994). During acute toxicity evaluation, Wistar rats were not associated with any mortalities and abnormalities in general conditions, behavior, growth, and food and water consumption of animals. Body weight changes are indicators of adverse side effects, as the animals that survive cannot lose more than 10% of the initial body weight (Teo et al., 2002). Body weight gain and food consumption levels were similar in both control and treated animals. In sub-acute studies, methanolic extract of *P. longifolia* at the doses used did not produce any marked changes in both male and female rats, as evidenced by the toxic symptoms, no changes in food and water consumption and the normal body weight. All animals survived until the scheduled euthanasia. The body weight and food consumption was not affected by administration of the drug in a single dose or repeated doses suggesting that methanolic extract of *P. longifolia* did not induce appetite suppression and had no deleterious effect on health status, growth or development of animals.

Total protein measurement is used in the diagnosis and treatment of a variety of diseases involving the liver or kidney as well as other metabolic disorders. A decrease in albumin level has been attributed to several causes, such as massive necrosis of the liver, deterioration of liver function, hepatic resistance to insulin and glycogen impairment of oxidative phosphorylation (Rao, 1995). In acute toxicity study, the total protein level increased in treated animals which demonstrate that the methanolic extract of *P. longifolia* had no toxic effect on liver and kidney function. Albumin levels showed a similar trend but the increased levels were not significant. Urea and creatinine are compounds derived from proteins which are eliminated by the kidney. In the present work, accumulation of urea, a relevant indicator for renal impairment (Vidal et al., 2003) was not observed. In fact, the urea level significantly decreased at all dose levels in male rats while in female rats the levels decreased but were significant only at highest dose. In sub-acute toxicity study, protein levels decreased in higher dose and increased in low dose groups. Albumin levels showed a similar trend, in fact, albumin levels showed significant increase in low dose; therefore; it suggests non toxic effect on liver and kidney function of the extract. Also the decrease in the urea concentration at higher dose directly suggests no kidney damage. The decrease in cholesterol levels in the treated animals in both types of toxicity (acute and sub-acute) studies indicated that the methanolic extract of *P. longifolia* had hypolipidemic activity. This is similar to the results obtained by Zhou et al. (2004) in

Ginkgo biloba. Also this suggests that the methanolic extract has some beneficial effects by reducing cardiovascular risk factors, which contributes to the death of diabetic patients (Barnett and O'Gara, 2003), and establishes the use of the formulation as a hypoglycemic agent.

ALP, GOT, GPT in tissue and blood are important marker enzymes which are used to assess the integrity of the cell membrane, cytosolic activity and cell death (Akanji et al., 1993). The extent of hepatocellular injury is assessed by the increased serum levels of ALP, GOT and GPT. These damages could be acute or chronic, reversible or irreversible (Janbaz et al., 2002). In the present acute toxicity study, ALP levels decreased significantly by administration of methanolic extract of *P. longifolia* which suggest that no possible cholestasis occurred at the dose levels tested since a rise in ALP level is usually a characteristic finding in cholestatic liver disease (Aniagu et al., 2005). In sub-acute toxicity study, there was no significant change in ALP levels except in low dose of male rats when compared to control group. The differences were not dose dependent therefore it can be stated that the extract had no alteration on liver function.

In acute toxicity study, in male rats, GOT level increased significantly only at dose level 2160 mg/kg b.w. and in female rats at dose level 540 mg/kg b.w. In both sexes, at other dose levels, GOT level decreased or slightly increased but they were insignificant. GPT level in both sexes, at some dose level showed increased activity though the extract showed significant decrease at highest dose in both male and female rats. All the three serum marker enzymes showed decreased levels except in some which were not dose dependent hence it can be stated that methanolic extract of *P. longifolia* was hepatoprotective in nature. While in sub-acute toxicity study, GOT level showed significant decrease in treated animals at all the dose levels. The GPT level showed increase in low dose of treated animals though the methanolic extract of *P. longifolia* showed decrease at higher dose in both sexes of animals. Repeated dose administration decreased GOT and GPT levels and could therefore serve to protect the liver especially at 540 mg/kg b.w.

The fall in hemoglobin content, RBC count and PCV can be correlated with induction of anemia, defective haematopoiesis, weakness and morbidity in experimental rats

(Criswell et al., 2002). In acute toxicity study, there was increase in hemoglobin content and RBC count, also increase in PCV levels was observed which suggests that the extract had no allergic response and there was no risk of such diseases up to the dose of 3240 mg/kg b.w. In sub-acute toxicity study, the observed increase in haemoglobin levels could be due to the increased absorption of iron in treated male rats (Ogbonnia et al., 2009). The hemoglobin levels decreased in female rats but it did not show dose dependent manner. The RBC levels showed significant increase in treated animals. Also it showed increase in PCV levels suggests that the beneficial effect of the extract on the animals. The methanolic extract has some polar constituents which may alter the amount of hemoglobin and RBCs in the blood. This is similar to the results obtained with some other plants (Sanchez-Elsner et al., 2004; Mbaka et al., 2010).

The increase in MCV and decrease in MCHC indicate macrocytic and hypochromic anemia (Barger, 2003). The acute toxicity study showed that there was no significant difference in MCV at any dose levels of animals of both sexes. The MCHC significantly decreased at two intermediate doses except at high dose in male rats but it was unaffected in female rats. In the sub-acute toxicity study, the MCV and MCH levels showed significant decrease in treated animals at the dose level 270 mg/kg b.w. The MCV and MCH levels showed non-significant decrease in treated animals at the dose level 540 mg/kg b.w. The MCHC was unaffected in both sexes of animals. The results of MCV, MCH and MCHC suggest that the methanolic extract is safe and there was no risk of hypochromic anemia.

WBC and its subpopulations relating to it such as lymphocytes usually show increase in activity in response to toxic environment (Robins, 1974). In the acute toxicity study, WBC was not significantly altered while lymphocytes, the main effectors cells of the immune system (Mc Knight et al., 1999) showed significant increase at low dose level of male rats. However, at other doses, male rats showed decreased levels of lymphocytes thus suggesting that the methanolic extract of *P. longifolia* has no risk on the immune system of the animals. In the sub-acute toxicity study, the total WBC counts increased significantly in female rats at high dose. This means that the extract may activate the defense mechanism. Total WBC count was unaffected in male rats. The reduction in lymphocyte count and increased neutrophils count suggested that the

methanolic extract of *P. longifolia* may possess some anti-lymphocytic activity (Garg et al., 1997).

Eosinophils normally constitute up to 7% of total circulating leukocytes. Eosinophils are important in the phagocytosis of foreign bodies. Eosinophils are also involved in allergic reactions. (Oyesanmi et al., 1999). Eosinophils level usually show increase in allergic reaction, but in acute toxicity study showed that the eosinophils decreased at all the dose levels in male rats and at two intermediate doses in female rats. So it can be stated that animals had no allergic reaction up to dose of 3240 mg/kg b.w. In sub-acute toxicity study, the eosinophils were unaffected except in the low dose in female rats. It did not show dose dependent effect, therefore it can be stated that the extract is safe and animals had no allergic reaction when methanolic extract of *P. longifolia* was administered orally for 21 days.

In the acute toxicity study, level of platelets decreased significantly in female rats at low dose. The treated male rats showed non-significant decrease in the levels of platelets. Platelets also known as thrombocytes, help to mediate blood clotting, which is a meshwork of fibrin fibres. The fibres also adhere to damaged blood vessels; therefore, the blood clot becomes adherent to any vascular opening and thus prevents further blood clot (Andrews et al., 1997). The extract could thus precipitate thrombocytopaenia which is the presence of low level platelets in the circulatory system. If someone suffers from thrombocytopaenia, there is a tendency to bleed (Adedapo et al., 2008b). This observation of decreased platelet level in the circulatory system by the methanolic extract of *P. longifolia* also means that it had anticoagulant property. In sub-acute toxicity study, the basophiles and platelets had no significant change in treated animals.

Organ weight is an index of swelling, atrophy or hypertrophy (Amresh et al., 2008). The relative organ weight is fundamental to diagnose whether the organ was exposed to the injury or not. The heart, liver, kidneys, spleen and lungs are the primary organs affected by metabolic reactions caused by toxicants. The liver is the major site of foreign compounds metabolism in the body (Dybing et al., 2002). In acute toxicity study, the relative weight of the organs such as liver, heart, spleen, kidneys, adrenal glands and testes did not show a dose-dependence effect (increase or decrease) thus,

it can only represent a normal variation and not as a sign of toxicity (Avancini et al., 2007). In sub-acute toxicity study, the absence of an effect on the weight of the liver and the lungs of the rats treated with the extract suggest that *P. longifolia* leaf extract at the doses investigated for 21 days did not cause swelling, atrophy or hypertrophy of these organs. The increase in the weight of the thymus glands, adrenal glands, and uterus and ovaries in treated female rats following extract administration for 21days may either imply hypertrophy or that growth of the organs is proportional to the growth of the animals. The reduction in the weight of the heart, spleen and kidney of the animals could possibly be due to cellular constriction; it also did not show a dose-dependent effect. This is an indication that the extract had no toxic effect on the weight of the studied organs. The histological analysis also did not suggest any alterations in any of the organs examined in this study.

CHAPTER 6: CONCLUSIONS

In pharmacognostic study of *Polyalthia longifolia* leaf, standards like macroscopic and microscopic characteristics, ash values, extractive values, heavy metals analysis, pH, solubility and qualitative phytochemical analysis were done which could be useful for the compilation of a suitable monograph for its proper identification. Pharmacognostic investigation revealed presence of oil glands in large number. Prismatic crystals of calcium oxalate were found. The stomata were surrounded by small subsidiary cells, whereas the guard cells were comparatively larger in size. The inorganic matter and non-physiological matter such as silica was less in leaves of *P. longifolia*. The pH of methanolic extract was acidic. The extract was free from heavy metal contamination. The solubility of extract was more in polar solvents than non polar.

Qualitative phytochemical analysis revealed that *P. longifolia* leaves were rich in alkaloids, cardiac glycosides, tannins, triterpenes and saponins. Quantitative phytochemical analysis revealed that *P. longifolia* leaves had more total phenol content than flavonoids; crude alkaloid content was more than saponin content.

In *in vitro* antioxidant studies, the methanolic extract of *P. longifolia* showed best ABTS radical scavenging activity followed by reducing capacity assessment; hydroxyl radical scavenging activity and superoxide anion radical scavenging activity was moderate while the methanolic extract could not scavenge DPPH free radical. It is generally believed that plants which are having more phenolic content show good antioxidant activity i.e. there is a direct correlation between total phenol content and antioxidant activity but not always; sometimes it is vice versa. In this study, the phenolic content of methanolic extract of *P. longifolia* showed correlation with ABTS radical scavenging activity but was not correlated with other antioxidant assays. The study once again confirmed that it is essential to do more than one antioxidant assay to evaluate antioxidant property of any medicinal plants.

In vivo antioxidant activity revealed that methanolic extract protects oxidative injury induced by CCl_4 and water immersion stress. Treatment with methanolic extract for 5 days in CCl_4 induced stress model revealed that the methanolic extract of *P. longifolia* significantly decreased the level of serum biochemical enzymes activities such as ALP, GOT and GPT as well as urea and BUN content while significantly increased

the level of total protein and albumin content. The extract significantly increased the level of liver antioxidant enzymes activities such GPx and CAT as well as total protein and GSH content while significantly decreased the level of lipid peroxidation.

Treatment with methanolic extract for 7 days in water immersion stress model revealed that the methanolic extract of *P. longifolia* significantly decreased the level of the serum biochemical enzymes activities such as ALP and GOT as well as urea, BUN and cholesterol content while significantly increased the level of total protein content. The extract significantly increased the level of hepatic enzymes activities such as GPx, CAT and SOD as well as total protein and GSH content. Thus, it can be stated that *P. longifolia* is a good source of natural antioxidants.

The methanolic extract of *P. longifolia* was well tolerated, lack of mortality and neither produced overt signs of clinical toxicity (diarrhea, loss of hair, behavioural changes, impairments in food intake and body weight gain), nor any signs of hepato-, nephro-, or hemato-toxicity, also was well supported by biochemical data. Acute toxicity study suggests that the methanolic extract of *P. longifolia* is safe up to the dose of 3240 mg/kg b.w. especially when consumed by oral route. Sub-acute toxicity findings provided more information on therapeutic safety of methanolic extract of *P. longifolia*. The significant decrease in platelet levels in treated animals demonstrated the anti-coagulate properties of the methanolic extract.

Further work should be carried out on the characterization of specific antioxidant components of *P. longifolia* and evaluation of their therapeutic significance in prevention of diseases induced by oxidative stress.

CHAPTER 7: REFERENCES

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CHAPTER 8: SUMMARY

Herbal medicines are a wonderful and precious gift of the nature and have been playing a significant role in the prevention and treatment of various health ailments. The major population of the southeastern Asian countries relies heavily on the efficacy of herbal remedies.

Herbs are staging a comeback and herbal renaissance is happening all over the globe. The herbal products today symbolize safety in contrast to the synthetic drugs that are regarded as unsafe to human and environment. Thus, the blind dependence on synthetic drugs is over and people again have started looking at the ancient healing systems like Ayurveda, Siddha and Unani. An estimate of the WHO states that around 85–90% of the world's population consumes traditional herbal medicines. Therapeutic plants have always been valued as a mode of treatment for a variety of ailments in folk cultures and have played a very important role in discovering the modern day medicines. In the past, infectious diseases killed our ancestors early, so they did not display the current epidemic of chronic diseases that arise in older age. Now medical status has been improved. Nowadays, people live longer and therefore express symptoms of chronic diseases such as atherosclerosis, cancer, diabetes, hypertension, coronary heart disease, etc.

A majority of the present day diseases are reported to be due to the shift in the balance of the pro-oxidant and the antioxidant homeostatic phenomenon in the body. Oxygen centered free radicals and other reactive oxygen species are by product of numerous physiological and biochemical processes in human body. Over production of such free radicals causes oxidative damage to biomolecules such as lipid, protein and DNA, eventually leading to many chronic diseases in human. The dire need of the hour is to discover or identify medicinal plants, rich in antioxidants.

The use of plants for healing purpose is getting increasingly popular as they are believed to be beneficial and free of side effects. However, the rationale for the utilization of medicinal plants has rested largely on long-term clinical experience with little or no scientific data on their efficacy and safety. Toxicological studies help to decide whether a new drug should be adopted for clinical use or not. Toxicity data are required to predict the safety associated before the use of medical products.

Polyalthia longifolia (Sonn.) Thw. var. pendula (Annonaceae) was selected in our laboratory and was evaluated for antimicrobial, anti-inflammatory, analgesic, antidiabetic and hepatoprotective properties and it showed very promising results. In continuation of this work, in the present study, *Polyalthia longifolia* (Sonn.) Thw. var. pendula leaf was selected for its antioxidant and toxicological assessment.

8.1 Pharmacognostic analysis

Herbal medicine is a triumph of popular therapeutic diversity. Almost in all the traditional medicine, the medicinal plants play a major role and constitute the backbone for the same. In order to make sure the safe use of these medicines, a necessary first step is the establishment of standards of quality, safety and efficacy. Keeping this into consideration, attempts were made to establish pharmacognostic, physicochemical and phytochemical study of the *P. longifolia* leaf. According to WHO the macroscopic and microscopic description of a medicinal plant is the first step towards establishing its identity and purity and should be carried out before any tests are undertaken.

Fresh leaves of *P. longifolia* were collected in the year 2007, Rajkot, Gujarat, India. The plant was compared with voucher specimen (No. PSN 4) deposited at Department of Biosciences, Saurashtra University, Rajkot, Gujarat, India. The fresh and mature leaves were used for pharmacognostic study.

The leaves of *P. longifolia* were ovate-oblong to narrowly lance-shaped, 11-22 cm X 2-5 cm size, wedge-shaped to round at base, wavy at margins and 4-8 mm long petiole. Leaf was dorsiventral. Transverse section of the leaf showed that the upper and lower epidermises were single layered. The palisade tissue was two layered. Prismatic crystals of calcium oxalate were found. Oil glands were found in palisade tissue and in lower midrib region. The anomocytic stomata were present in lower epidermis.

The crude powder of *P. longifolia* leaf was light green in color with characteristic odour. The characteristics determined from the powder study were epidermis, mesophyll, multicellular blunt tip trichome, phloem and spiral xylem vessel.

The dried powder of leaves was defatted with hexane and extracted in methanol for 24 h on a rotary shaker by cold percolation method. The methanolic extract was concentrated to get the solid mass. The yield obtained was 8.62%. The methanolic extract was used for the entire study.

8.2 Physicochemical analysis

The physicochemical parameters carried out in dried leaf powder and or methanolic extract of *P. longifolia* were loss on drying, total ash , acid insoluble ash, water soluble ash, petroleum ether soluble extractive, alcohol soluble extractive, methanol soluble extractive, water soluble extractive, solubility test, pH, heavy metal analysis, melting and boiling point. The physical constant evaluation of the drugs is an important parameter in detecting adulteration or improper handling of drugs. The moisture content of dry powder of leaves of *P. longifolia* was 12 % which is not very high, hence it would discourage bacteria fungi or yeast growth. Low amount of total ash, acid insoluble ash and water soluble ash indicates that the inorganic matter and non-physiological matter such as silica is less in leaves of *P. longifolia*. High alcohol soluble and water soluble extractive value reveal the presence of polar substance like phenols, tannins and glycosides. The pH is an important parameter for formulations. The pH of powder of leaves was acidic in nature. The solubility of the methanolic extract of *P. longifolia* was more in methanol followed by dimethylsulphoxide and less in acetone. There was minor presence of some heavy metals in the methanolic extract but it did not exceed permissible limit. All these parameters are useful for the compilation of a suitable monograph for it proper identification.

8.3 Phytochemical analysis

Qualitative analysis of alkaloids, flavonoids, tannins, phlobatannins, triterpenes, steroids, saponins and cardiac glycosides were done in dried leaf powder and in methanolic extract of *P. longifolia*. In methanolic extract, total phenol and flavonoid

content was estimated while in dried crude powder alkaloid and saponin content were estimated.

Qualitative analysis indicated that *P. longifolia* leaves were rich in alkaloids, flavonoids, tannins, triterpenes, saponins and cardiac glycosides while steroids and phlobatannins were absent.

Quantitative phytochemical analysis revealed that *P. longifolia* leaves had more total phenol content than flavonoids; crude alkaloid content was more than saponin content. In spectral analysis, comparison of the data with the earlier reported spectral values of the IR, NMR and GCMS spectra, it was found that the methanolic extract might contain some compounds like clerodane diterpenoids, allantoin and ent-halimane diterpenes.

8.4 *In vitro* antioxidant activity

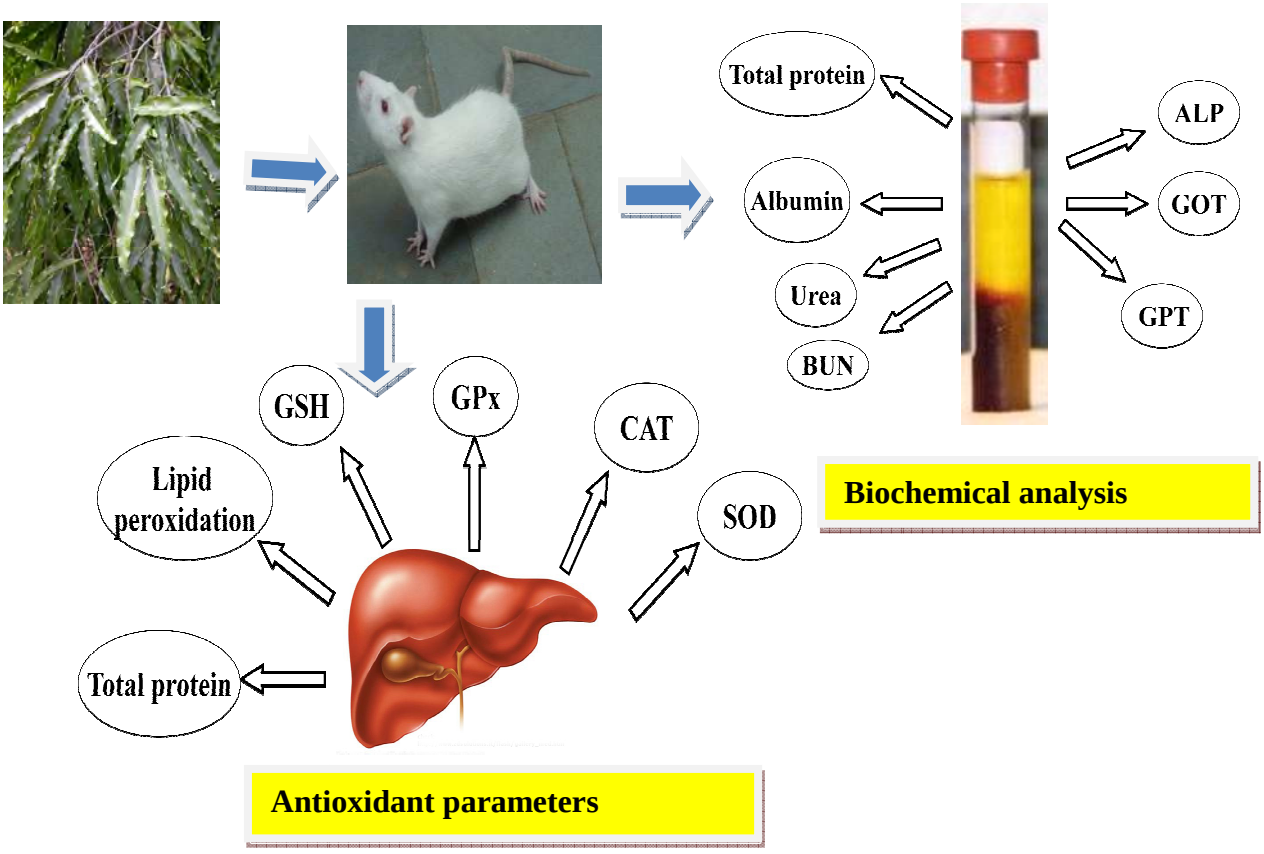
The present study was carried out to evaluate *in vitro* antioxidant activity of the methanolic extract of *P. longifolia* by DPPH radical scavenging activity, hydroxyl radical scavenging activity, superoxide anion radical scavenging activity, ABTS radical cation scavenging activity and reducing capacity assessment.

The result of *in vitro* antioxidant activity revealed that the methanolic extract had capacity to scavenge free radicals. The IC_{50} value of methanolic extract was 75, 610 and 810 µg/ml in ABTS radical scavenging activity, hydroxyl radical scavenging activity and superoxide anion radical scavenging activity respectively. The scavenging capacity of the ABTS radical by the extract was found to be much higher than that of hydroxyl radical scavenging activity and superoxide radical scavenging activity as well as reducing power. The extract did not show DPPH radical scavenging activity. There are reports that some compounds which have ABTS radical scavenging activity did not show DPPH radical scavenging activity. It showed the capability of the extract to scavenge different free radicals in different systems, indicating that they may be useful therapeutic agents for treating radical-related pathological damage.

8.5 *In vivo* antioxidant activity

Wistar albino rats were used for the study. The study was approved by CPCSEA approved local ethical committee. *In vivo* antioxidant activity was studied by 2 models, *viz.* CCl_4 induced stress model and water immersion stress model. Treatment rats were dosed by oral gavage with aqueous suspensions of methanolic extract of *P. longifolia* with dosages of 270 and 540 mg/kg body weight in both the models. In both the models various biochemical parameters were evaluated in serum and antioxidant parameters from liver homogenate. Treatment with methanolic extract for 5 days in CCl_4 induced stress model and 7 days in water immersion stress model revealed that the methanolic extract of *P. longifolia* significantly decreased the serum biochemical enzymes such as ALP, GOT and GPT and significantly increased hepatic enzymes activities such as GPx, CAT, SOD. The results suggest that methanolic extract of *P. longifolia* protect the structural integrity of the cell membrane of hepatocytes or enhance regeneration of damaged liver cells.

***In vivo* antioxidant study by graphical presentation**



8.6 Toxicity study

The objective of this study was to elucidate acute (14 days) and sub-acute (21 days) toxicity of *P. longifolia* in Wistar albino rats. Treatment rats were dosed by oral gavage with aqueous suspensions of methanolic extract of *P. longifolia* with single dosages of 540, 1080, 2160 and 3240 mg/kg b.w. for acute toxicity study and 270 and 540 mg/kg b.w. for sub-acute toxicity study. In both the studies, biochemical parameters were estimated from serum, organ weight and haematological analysis was done. Histopathology of liver, heart, kidney, spleen and thymus gland was done in sub-acute toxicity study.

The result of toxicity study revealed that the methanolic extract of *P. longifolia* was well tolerated, lack of mortality and neither produced overt signs of clinical toxicity nor any signs of hepato-, nephro-, or hemato-toxicity, also well supported by biochemical data. Acute toxicity study suggests that the methanolic extract of *P. longifolia* is safe up to the dose of 3240 mg/kg b.w. especially when consumed by oral route. Sub-acute toxicity findings provided more information on therapeutic safety of methanolic extract of *P. longifolia*.

Toxicity study provides valuable data on toxicity profile of *P. longifolia* that should be useful for the planning of future preclinical and clinical studies of the formulation. Methanolic extract was non-toxic. The methanolic extract demonstrated effective *in vitro* and *in vivo* antioxidant activity. Further research needs to be carried out to identify the active antioxidant molecules and evaluation of their therapeutic significance in prevention of diseases induced by oxidative stress. .

Toxicity study by graphical presentation