

Effect of Polyalthia Longifolia Leaves on Liver Carcinogenesis

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Abstract: This experiment with four groups of rats was designed to evaluate the beneficial properties of polyalthialongifolia consumption on antioxidant enzyme regulation and lipidperoxidation in albino rats in DEN induced stage. For the study, the male albino rats were divided into four groups normal, chemical treated and chemical along with the methanol extract of plants treated groups for initiation phase. The Intraperitoneal injection of DEN with one day and 15 days incubation caused carcinogenesis and the other group had plant treatment up to 15 days. Effects of polyalthia consumption on LPO and antioxidants enzymes changes were also evaluated. The plant treatment had remarkable effects on LPO and antioxidant enzymes level changes in the male albino rats. An improvement in lipidperoxidation and antioxidants enzymes were observed with lower lipidperoxidation and improved antioxidant enzymes changes after 15 days of polyalthialongifoliamethanol extract treatment. Thus the leaf polyalthialongifolia was found to have antioxidant / antidegradative activity.

Keywords: polyalthialongifolia hepatocarcinogenesis; lipidperoxidation; antioxidant enzymes; male albino rats; antioxidant/antidegradative effect.

1. Introduction

Medicinal plants are of great importance to the health of individuals and communities. The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human body (Edeoga *et al.*, 2005). Phytochemicals defined in the strictest sense, as chemicals produced by plants. However, the term is generally used to describe chemicals from plants that may enhance health status of organisms, but are not essential nutrients (Srivastava *et al.*, 2011). There is ample evidence to support the health benefits of the diet in the form of fruits, vegetable, legumes, whole grains and nuts (Mojabet *et al.*, 2003). Because plant based foods are complex mixtures of bioactive compounds, information on the potential health of individual phytochemical is linked to information on the health effects of foods that contain those phytochemicals (Manjula *et al.*, 2009). The most important of these bioactive constituents of plants are alkaloids, tannins, flavanoids and phenolic compounds (Hill, 1952).

Drug induced hepatotoxicity Drug induced liver injury is responsible for 5% of all hospital admissions and 50% of all acute liver failures. Chemicals and drugs produce a wide variety of clinical and pathological hepatic injury. More than 900 drugs have been implicated in causing liver injury and it is the most common reason for a drug to be withdrawn from the market. Hepatotoxicity and drug induced liver injury also account for a substantial number of compound failures, highlighting the need for drug screening assays that are capable of detecting toxicity early in the drug development process. Drug induced liver injury is of frequent occurrence since the role played by the liver to remove the toxic substances from the portal circulation makes it susceptible to the persistent attack by offending foreign compounds resulting in liver dysfunction (Arundel and Lewis, 2007).

2. Medium - term Bioassay

Carcinogenesis is a complex process that has been divided into three stages - Initiation, promotion and progression. These three stages of tumour formation have been characterized in many mammalian tissues, particularly in the liver (Farber *et al.*, 1979). Similar patterns of development and expression of HCC in mice, rats, and human would support the use of rodents as substitute for identifying risk factors of HCC in human. Hepatocellular carcinoma can be induced in the livers of laboratory animals by variety of chemicals (Ahn *et al.*, 1999).

Multistage carcinogenesis studies have extensively used for the analysis of cancer development. In particular, the two-stage initiation-promotion protocol has been widely used in systematic elucidation of the carcinogenesis (Pitot and Dragan 1994). Although the design of these assays varies with respect to the combination and sequence of initiating and promoting events, analysis of putative initiated cells identified by phenotypic markers such as GST-P continues to be a vital part of studies for understanding the mechanism of tumour promotion (Lucier *et al.*, 1991).

The medium term liver foci bioassay developed by Ito *et al* involves the sequential administration of potent initiator, DEN followed by chemical treatment and mitogenic stimulation of hepatocyte growth (Ito *et al.*, 1989). This study is based on Cabral *et al* and smith and cabral Study or initiation / promotion protocol (Cabral *et al.*, 1979; smith and cabral., 1980)

DEN, a genotoxic carcinogen that exclusively resulted in liver cancer. Diethlnitrasamine (DEN) is called complete liver carcinogen, a single dose of which can, under certain condition, induce the formation of HCC. DEN inhibits mitosis in the liver, induces hopomethylation and promotes the development of EAF (Deal *et al.*, 1989). Among these

carcinogens, DEN has been frequently used to study the hepatocarcinogenic process, treatment and drug effects etc. Dunsford *et al* found the carcinogenic effects of DEN and used it opportunistically to improve cancer development in liver cells with enhanced multiplication caused by hepatocyte necrosis (Dunsford *et al.*, 1989). chemical agents including hepatocarcinogenesis have been administered either as DEN alone or in combination with AAF, orotic acid, Phenobarbital and CCl_4 (Kim *et al.*, 1994). In the present study DEN is used as a initiator of hepatocarcinogenesis study in albino rats.

Since the increase in the use of synthetic chemicals in cancer therapy has led to many side effects and undesirable hazards, there is a worldwide trend to go back to natural resources (medical plants) which are therapeutically effective, culturally acceptable and economically within the reach of even the neediest people. Accumulating epidemiological, experimental evidence has revealed the influence of number of naturally occurring and synthetic compounds on drug-detoxification and HCC incidence (Premalatha and Sachdannandam., 2000). Based on this view, this study speculate the effects of the *Polyalthialongifolia* leaves on DEN induced liver carcinogenesis in male albino rats.

Polyalthialongifolia (False Ashoka) is a lofty evergreen tree, native to India, commonly planted due to its effectiveness in alleviating noise pollution. It exhibits symmetrical pyramidal growth with willowy weeping pendulous branches and long narrow lanceolate leaves with undulate margins. The tree is known to grow over 30 ft in height.

Polyalthia is derived from a combination of Greek words meaning „many cures“ with reference to the medicinal properties of the tree while *Longifolia*, in Latin, refers to the length of its leaves (McCann, Charles., 1959) *Polyalthialongifolia* is sometimes incorrectly identified as the Ashoka tree (*Saracaindica*) because of the close resemblance of both trees.

One might mistake it as a tree with effectively no branches, but in fact a *Polyalthia* allowed to grow naturally (without trimming the branches out for decorative reasons) grows into a normal large tree with plenty of shade. In this study, the plant leaves *polyalthialongifolia* was selected for carcinogenesis study.

3. Materials and Methods

Reagents and Chemicals

DEN, HCB, TBA, DNPH, dipyrindyl like all chemicals were of analytical grades and chemicals required for sensitive biochemical assay were obtained from „M/S sigma and Aldrich chemical co., U.S.A.“ Double distilled water was used in all biochemical assays.

Animals

Albino wister rats of male at a age of 15-20 weeks containing 120-150g weight were selected for this study. These animals were purchased from Indian Institute of science, Bangalore, India. Male albino rats were housed in polypropylene cages and maintained in controlled

temperature with standard rat chow. Food and water were provided *ad libitum*.

Preparation of Plant material and extracts

Polyalthialongifolia leaves were collected and shade dried for grinding to get crude powder for treatment. These plants were shade dried and extracted with methanol (70%) by the use of soxhlet extractor. A semisolid extract was obtained after complete elimination of methanol under reduced pressure. The extract was stored in refrigerator until use. The extract was dissolved in normal saline just before oral administration.

Experimental design

The rats were divided into four groups of four animals in each group and the body weight of animals were recorded. Four days fasting and refeeding was continued, before the administration of DEN injection.

Initiation phase

Group – I : Normal control (0.5ml of normal saline / animal / day) up to one week.

Group – II: Rats received only one i.p injection of DEN (20mg in saline/ rat) at 1st day of first week.

Group– III : Received DEN only + *polyalthialongifolia* methanol extract (50mg/kg) upto one week.

Collection of samples

After the completion of experimental regimen, the rats were fasted overnight and blood samples were collected by cervical decapitation with mild ether anesthesia and serum was collected. whole liver was immediately dissected out and washed in ice cold saline. A known weight (1g) of liver was taken and homogenized with (10%) phosphate buffer (pH.7.4). The serum, whole blood with EDTA and liver homogenate were used for various biochemical assays.

Biochemical analysis

The serum and liver were used for the LPO, SOD, CAT, GST, reduced GSH,

Statistical analysis

The data were presented as mean \pm SD. The data were analyzed using students “t” test . A value of $p < 0.05$ was considered statistically significant.

4. Results

Effects of *polyalthialongifolia* leaves on Lipid-Peroxidation and antioxidant.

Table-I depicts the level of serum LPO, reduced glutathione in normal and experimental animals. In chemical control groups, LPO was significantly ($P < 0.05$) increased and the reduced glutathione level was significantly ($P < 0.05$) decreased when compared with the values of the normal control rats. When the plants *polyalthia* was supplemented throughout the study, LPO level was significantly decreased and GSH was nonsignificantly increased in initiation stage. Administration of the plants *polyalthia* normalized the LPO level and increased the GSH value compared with chemical control. Methanol extract of *polyalthia* had more remarkable activity on initiation phase of GSH

Effects of *polyalthialogifolia* leaves on total protein and albumin.

Table-I shows the level of serum protein, albumin in normal and experimental animals. In chemical control groups, protein and albumin were significantly ($P < 0.05$) decreased when compared with the values of the normal control rats. When the plants *polyalthia* was supplemented throughout the study, protein and albumin were significantly increased in initiation stage. Administration of the plants *polyalthian* normalized the protein and albumin compared with chemical control.

Effects of *polyalthialogifolia* leaves on liver marker enzymes

Table-I depicts the level of serum marker enzymes in normal and experimental animals. In chemical control groups liver marker enzymes SGOT, SGPT, GGT and ALP were significantly ($P < 0.05$) increased when compared with the values of the normal control rats. When the plants *polyalthia* was supplemented throughout the study, liver marker enzymes were significantly decreased in initiation stage. Administration of the plants *polyalthian* normalized the protein and albumin compared with chemical control.

Table 1: Effect of *polyalthialongifolia* leaf on LPO and GSH

S.No	Parameter	Normal	DEN treated	<i>Polyalthia</i> treated
1	LPO (□ mole/ml)	10.19±7.6	104.9±7.7*	59.99±13.8*
2	GSH (µg/dl)	464±.130	148±8.0*	268±0.08*

Values are the mean ± SD of 4 animals in each group. Group II was compared with Group I (* $P < 0.05$) Group III was compared with Group IV (** $P < 0.05$).

Table 2: Effect of *polyalthialongifolia* leaf on protein and albumin

S.No	Parameter	Normal	DEN treated	<i>Polyalthia</i> treated
1	Protein (g/dl)	8.18±.03	5.61±0.06*	7.10±.12**
2	Albumin (g/dl)	5.15±0.12	2.52±0.16*	3.62±.20**

Values are the mean ± SD of 4 animals in each group. Group II was compared with Group I (* $P < 0.05$) Group III was compared with Group IV (** $P < 0.05$).

Table 3: Effect of *polyalthialongifolia* leaf on SGOT, SGPT, ALP and GGT.

S.NO	Parameter	Normal	DEN treated	<i>Polyalthia</i> treated
1	SGOT (IU/L)	66.64±7.7	76.16±5.6	54.74±4.7*
2	SGPT (IU/L)	23.80±5.4	47.6±7.7*	33.32±5.4**
3	ALP (IU/L)	27.37±7.4	29.97±2.9*	27.97±2.9**
4	GGT (IU/L)	30.93±10.02	106.5±6.8*	30.71±6.2**

Values are the mean ± SD of 4 animals in each group. Group II was compared with Group I (* $P < 0.05$) Group III was compared with Group IV (** $P < 0.05$).

5. Discussion

Effects of *polyalthialongifolia* leaves on lipid peroxidation

Ito et al reported that there were harmful effects in the liver of DEN (Ito et al., 1989). DEN is the most important environmental carcinogen among nitrosamine in interacting with membrane lipids and consequently inducing free radical formation. They may interact with cellular macromolecules such as DNA, protein, lipid and carbohydrate, to initiate or promote inflammatory, toxic or carcinogenic process.

The table –I represents the level of LPO in experimental animals.

The level of lipid peroxidation was significantly higher of initiation phase than other groups, although all the control groups had higher value. But in treated groups the serum LPO was significantly lower in *polyalthia* treated group than control group. LPO is regarded as one of the basic mechanisms of cellular damage, caused by free radicals. Free radicals react with lipid causing peroxidation, resulting in the release of products such as malondialdehyde, hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^\cdot). Lipid peroxidation is one among these and it is a process, that is formed by means of the oxidation of polyunsaturated fatty acids. MDA is one of the final products of lipid peroxidation (Bast et al., 1991). An increase in lipid peroxides and hydroxyl radical indicates serious damage to cell membranes, inhibition of several important enzymes, reduced cellular function and cell death.

The role of oxidative damage in carcinogenesis was increasingly being speculated. Given a long term evolutionary development of cancer, these conditions are not normally expected to cause cancer, unless they are the source of a primary mutagenic event. Some in bronchogenic carcinoma, ultraviolet light in skin cancer and ethanol for hepatocellular cancer (Dreher and Junod., 1996). In the study, DEN (Diethyl nitrosamine) was used for induction agent for lipid peroxidation and hepatocarcinogenesis. This oxidative stress may be a reason for the elevated LPO level in the liver of DEN treated animals. LPO may lead to the formation of several toxic by-products such as 4-hydroxynonenal and malondialdehyde which can attack cellular targets including DNA, inducing mutagenicity and carcinogenicity (Ramaiah et al., 1986).

In the study, the level of LPO was increased in DEN, DEN+HCB administered group's serum and liver than normal group. The increase in LPO level in the liver induced by DEN alone, DEN+HCB suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanism to prevent formation of excessive free radicals. Therefore the levels of antioxidant were decreased in chemical control groups than the normal. These results were modified after administration of the methanol extract of *polyalthia*. These results indicated that these plants had an antioxidative effect in carcinogenesis. These plants were rich in phenols and flavanoids. Generally phenols and flavanoids are strong antioxidants (Tiwari., 2001). Therefore these plants act like antioxidants and minimized the lipid peroxidation levels.

Effects of Tz and Gc on nonenzymatic antioxidants

Non-enzymatic antioxidant such as reduced glutathione, vitamin-C and vitamin-E play an excellent role in protecting the cells from oxidative damage (Anuradha and Selvam., 1993). GSH status is a highly sensitive indicator of cell functionality and viability. Excessive production of free radical resulted in the oxidative stress, which leads to the damage of biomolecules and can induce lipidperoxidation *in vivo*. In the present study, decreased level of GSH was associated with enhanced lipidperoxidation in DEN alone, treated rats than the normal group. The values of GSH in these groups were provided in the table – I

Glutathione is one of the abundant tripeptide, (nonprotein thiol), nonenzymatic biological antioxidants present in the liver (Anderson, 1998). It acts as a substrate for the H₂O₂ removing enzymes glutathione peroxidase and for dehydroascorbate reductase. In addition GSH plays a critical role in cellular function which includes the maintenance of membrane protein, the removal of free oxygen radicals, such as peroxy radical, superoxide radical, alkoxy radical, translocation of aminoacids across cell membranes, the detoxification of foreign compounds and biotransformation of drugs (Comporti et al., 1991). GSH plays a central role in coordinating the body's antioxidant defense processes. perturbation of GSH status of a biological system has been reported to lead to serious consequences (Uday Bandyopadhyay., 1999). GSH can conjugate with NO, resulting in the formation of a S-nitroso-glutathione adduct, which was cleaved by the thioredoxin system and to release GSH and NO. GSH interact with glutaredoxin system and thioredoxin, which play as an important role in the regulation of cellular redox homeostasis.

The reduced form of GSH becomes readily oxidized to GSSG on interacting with free radicals. GSH was involved in the protection of normal cell structure and function by maintaining redox homeostasis, quenching of free radicals by participation in detoxification reaction and keeps up the cellular levels of the active form of vitamin-C and vitamin-E by neutralizing the free radicals. When there is a reduction in GSH, the cellular levels of vitamin-C and E were found closely interlinked to each other (Pari and Amadi., 2005). Due to the reason of oxidative stress by DEN, it decreased the level of GSH in initiation stage and also it lead to the lower level of antioxidants. But the plants supplementation increased this GSH level and indicates its antioxidant effect.

Effects of *polyalthialongifolia* on proteins.

Proteins were easily attacked by ROS directly or indirectly through lipid peroxidation. Protein radicals can be rapidly transferred to other sites within the protein infrastructure. This may result in further modification of enzyme activity (Stimulation or inhibition). In particular, it seems to be that, membrane bound enzymes, may play an important role in a variety of physiological functions of the membranes. In addition to the enzymes, damage to the membrane transport proteins may produce cellular ionic homeostasis and lead to alterations in intracellular Ca and K that will trigger a series of changes in cells. Thus protein oxidative damage can result in the modification of structure, enzyme activity and signal pathways (Bellomo et al., 1983).

Initiation of carcinogenesis involves direct interaction of ultimate carcinogens to DNA of target cells, leading to irreversible alterations of cellular genome. The reaction of chemical adduct with protein could result in the induction (or) repression of proteins controlling cell replication, growth or differentiation. The reaction of carcinogen with DNA or RNA involves strand breakage and carcinogen base adduct formation. Unless these alterations are repaired effectively, they become propagated from cell to cell during normal tissue growth and development (Feig *et al.*, 1994). Due to the free radical damage on DNA, RNA and protein of carcinogenic group in initiation, these contents were decreased than the normal group of rats in the investigation. similarly the requirement of GSH and thiols to DNA synthesis might be related to the proliferation of cells. In this study during oxidative damage GSH level was found decreased. Therefore DNA, RNA synthesis also declined. The SOD plays major role in the protection of cells against oxidative damage. The Mn-SOD protects mitochondrial proteins, membranes and DNA from O₂ generated, as a result of the respiratory activity (Verhoeven *et al.*, 1985). Vitamin-C and E decrease the some target organ/cell specific induction of oxidative DNA damage (Meneghini and Martins., 1993).

The significant decreased level of total protein in the DEN and intoxicated rats were observed in this research. The decrease of total protein could be attributed to an increase in aminoacid deamination. Likewise the significant decreased levels of protein were observed in the DBNA intoxicated rats in study made by Thirunavukkarasu and Sakthisekaran (Thirunavukkarasu and Sakthisekaran., 2003). Likewise the albumin levels were decreased in initiation phase of chemicals groups. Similarly albumin also decreased due to the degradation activity of chemicals.

Oxidative damage in proteins might be a critical pathological event because enzyme inactivation can result rapid effects. By nature of their catalytic functions. Protein oxidation is currently considered to be an important factor in a variety of diseases such as hypertension, cardiovascular disease, cancer, etc., (Butterfield and Kanski., 2001). The adduct formed by the covalent binding of genotoxic chemicals with proteins were useful biomarkers for assessing the risk in molecular epidemiology. Unlike DNA adducts, protein adducts were not repaired. Therefore, protein adduct form a stable repository of accumulated exposure to carcinogens over the lifetime of the protein (Sharma and Farmer., 2004).

Albumin is the most abundant serum protein produced by the liver. The synthesis and excretion of albumin is one of the major functions of the differentiated hepatocytes in mammalian liver. In the adult animal, it is produced at high and constant levels (Sala-Trepat *et al.*, 1979). Level of albumin in the serum continues to serve as an important marker for the presence, progress or improvement of many diseases, even though it is the complex end result of synthesis, degradation or loss and distribution between intra and extravascular space.

The level of Albumin synthesis and excretion are frequently reduced in chemically induced primary hepatoma and

transplantable hepatoma cells *in vivo* and *in vitro* (Cassio et al., 1958). Decreased level of albumin among subjects with high level of carbonyl might be, the result of oxidative modification of proteins, leading to accelerated catabolism (Grant et al., 1992) or suppression of albumin synthesis in the liver due to oxidative stress or both. Likewise in the study, the albumin level decrement was observed in the chemicals groups

The decrement to total protein and albumin in DEN treated group of rat, may be due to oxidative damage of protein in serum by free radical formation from DEN. In the present study, DEN induction, declined the level of antioxidant system; therefore it may cause free radical mediated damage of proteins, decreased the level of these contents. But the administration of the plant *polyalthialongifolia* extracts prevents this decrement by its antilipidperoxidative or antistress effects.

Effects of *polyalthialongifolia* on Hepatic marker enzymes

ALT, AST, ALP and GGT which were known altogether as cholestatic liver enzymes. Elevation of these enzyme can indicate the presence of liver disease. AST and ALT (SGOT/SGPT) were jointly known as transaminases. They are associated with inflammation or injury to liver cells, a condition which is known as hepatocellular liver injury. Damage to the liver typically result in a leak of AST and ALT into the bloodstream. High levels of GGT and ALP hint at a blockage of the bile ducts (or) possible injury (or) inflammation of bile ducts. It characterized by an impairment of bile flow, which were known as cholestasis. When a blockage or inflammation of the bile ducts occurs, the GGT and ALT can overflow into the bloodstream (Melissa Palmer., 2004).

Hepatospecific enzymes were increased when hepatocellular damage and these enzymes were activated in hepatoma (Ha et al., 2001). AST, ALT, GGT, ALP and ACP exhibit high levels in the abnormally functioning of liver. The administration of carcinogenic substances may bring changes in enzyme levels arising from cellular proliferation. So, it is of some importance to analyse the enzyme activity variation quantitatively, in order to understand the process involved. DEN had considerable effects on the serum and tissue specific enzymes. Some researches had indicated, that, it increases the activities of ALT, AST and GGT (Chakraborty and Selvaraj., 2000). In the research of Guiliani and Zaki, i.p DEN were given to rats for 5 days in dose of 2mg/kg body weight. It was determined that serum ALT and AST enzymes activity increased (Guiliani and Zaki., 1983). In this study, the liver marker enzymes levels were given in the table -V. The marker enzymes ALT, AST, ALP, ACP and GGT were significantly increased in DEN and DEN+ HCB groups as compared with normal as well as treatment groups.

Proteins were also easily attacked by ROS directly or indirectly through lipidperoxidation modifying their enzyme activity (Clayson et al., 1994). DEN is not only carcinogenic as a potent alkylating agent, but also inhibits protein synthesis. After this inhibition, changes in the activity of enzyme may occur. It is observed that DEN can

cause an increase in some enzyme activities and decrease in some other enzyme activities. Bansal et al (2005) found that, activities of AST, ALT and ALP were increased significantly following N-nitroso compounds treatment to rats (Bansal et al., 2005). The liver enzymes were normally found in circulation in small amounts because of hepatic growth and repair.

The increased level of SGOT, SGPT, ALT and serum bilirubin were indicative of cellular leakage and loss of functional integrity of liver cell membrane (Drotman and Lowhorn., 1978). In the study, elevated serum level of SGOT, SGPT and ALT and total bilirubin were indicative of poor hepatic function in DEN treated animals. All these indicate an induction of hepatocellular carcinogenesis by DEN. consequently, elevated activities of ALT and AST were observed in the current study in response to DEN administration could be a common sign of impaired liver function. On the otherhand Alkaline phosphatase belongs to a group of enzymes catalyze the hydrolysis of phosphomonoesters at alkaline P^H. ALP is found present in cell surface in most human tissues. The highest concentration were found in the intestine, liver, bone, spleen and kidney (Moss and Handeson., 1999). Acute cell necrosis liberate ALP in the circulation and serum enzyme level were elevated. GGT was found predominantly in liver. Elevated levels of ALP indicate, that something was wrong with the liver only if, the amount of GGT was raised as well. GGT can be elevated without ALP being elevated, as GGT was a sensitive marker of certain hepatotoxic drugs. In the study, the increment in the liver marker enzymes were nearnormalized by the administration of the plant *polyalthialongifolia* leaves.

6. Conclusion

This present study proved that the plant *polyalthialongifolia* had the properties of antioxidant and antidegradative effect on liver cell of DEN induced carcinogenesis stage of male albino rats. This effect may due to the presence of phytoconstituents in these leaves

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