Research Article

Chemoprotective Effect of *Leucas aspera* Plant in Rats: DEN Induced Hepatocarcinogenesis

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ABSTRACT

Cancer is one of the major causes of mortality in humans throughout the world. Scientists all over the world are focusing on herbal medicines to boost immune cells of the body against cancer. The present study is aimed at evaluating the chemoprotective effect of. *Leucas aspera* in DEN induced and CCL_4 promoted hepato-carcinogenesis in wistar rats. Two weeks after the starting of the experimental protocol all the rats except normal control received a single dose of CCl_4 (2 ml/kg i.p.) to stimulate liver cells proliferation and regeneration. At the end of treatment protocol, blood samples were taken. The degree of protection was measured by evaluating antioxidant parameters; liver biochemical parameters were estimated to confirm the effect of toxicants on the liver as well as to check the chemoprotective potential of the extracts. Administration of DEN in animals showed an increased level of GGT which is indicative of hepatic carcinogenesis, an increase in ALP activity was also seen in animals who received DEN which may be due to altered synthesis of enzymes as in other hepatotoxicity conditions. Administration of extracts resulted in normalization of serum GGT level, lowering of AST and an ALT level shows the hepatoprotective effect and inhibition of carcinogenesis. A significant lowering of the activity of ALP indicates the inhibition of pre-cancerous transformation in the liver on hydro-ethanolic and aqueous extract treatment in DEN+CCL_4 animals. The results thus indicate the chemo-preventive efficacy of both the extracts in decreasing cell proliferation and hepatic nodulogenesis. The results clearly indicate a significant chemo-preventive effect of hydro-ethanolic and aqueous extract of *Leucas aspera* plant in rats.

Keywords: *Leucas aspera*, Chemoprotective, Hepatocarcinogenesis, DEN.

INTRODUCTION

Cancer is one of the major causes of mortality in humans throughout the world.[1] Every year, millions of people are diagnosed with cancer which leads to deaths in majority of the cases. The American cancer society has recorded 2-3% of deaths arising from cancer every year worldwide.[2,3] Because of high death rate and serious side effects associated with chemotherapy and radiation therapy many cancer patients seek alternative and or complementary medicine of treatment. A large number of medicinal plants act as anticancer herbs in experimental and/or clinical cancers/tumors of various organs like sarcoma, leukemia, lymphoma and carcinoma.[1,2]

Some herbs reduce toxic side effects of chemotherapy and radiotherapy. Scientists all over the world are focusing on herbal medicines to boost immune cells of the body against cancer. By understanding the complex interactions of various constituents of anticancer herbs, the herbal formulations can be designed to attack the cancerous cells without harming normal cells of the body.[4,5] Medicinal herbs are also significant source of synthetic and herbal drugs.

In recent years pharmaceutical companies have screened more than 25,000 plants for anti-cancer drugs.[4] Medicinal plants possess immunomodulatory and antioxidant activities leading to anticancer activities.[6]

According to Ayurveda (Indian system of traditional medicine) *Leucas aspera* has been proven to possess various pharmacological activities like antifungal, antioxidant, antimicrobial, anti-nociceptive and cytotoxic activity.[7]

The present study is aimed at evaluating the chemoprotective effect of *Leucas aspera* in DEN Induced and CCL_4 promoted hepato-carcinogenesis in wistar rats.

MATERIALS AND METHODS

Collection and Authentication of Plant

Fresh plants of *Leucas aspera* were procured from Mangalore, Karnataka, India. The plant was authenticated by a botanist of St. Agnes College, Mangalore.

Preparation of Plant Extract

The authenticated plant was shade dried and powdered coarsely. Extraction was done according to the standard procedures using analytical grade solvents. Coarse powder (250g) was subjected soxhlet extraction using hydro alcoholic solvent (1:1 water: ethanol).[8] The aqueous extract was prepared using the same marc by the process of maceration.[9] The extract was dissolved in normal saline before oral administration to the rats.[10]

Drugs

Cyclophosphamide tablet (Cadila Healthcare limited).

Procurement and Housing of Animals

Healthy Wistar albino rats of either sex weighing between 150-180 g were taken for the study. All the animals were procured from Animal house, NIMS University. The

animals were acclimatized by keeping them in the animal house facility of NIMS University for a week.

Five rats were housed per polypropylene (32x24x16 cm) cage and maintained under controlled conditions of temperature (23 ± 2 ^{o}C), and 12h light and 12h dark cycles. The animals were fed with standard pellet diet and water ad libitum. Approval of the Institutional Animals Ethics Committee (IAEC) of NIMS institute of pharmacy, NIMS University, Jaipur was taken (NU/PH/M/COL/12/76).

Preliminary Phytochemical Investigation

Qualitative chemical tests were conducted for hydroethanolic and aqueous extracts of *Leucas aspera* to identify the various phytoconstituents.

Acute Toxicity Studies

The acute oral toxicity study was done by 'Up-and-Down' method in healthy adult female albino rats according to CPCSEA recommended 'OECD' guideline 425. There were no changes from dose level of 175 mg/kg. p.o, to 2000 mg/kg, p.o. Drug extracts did not cause any death up to 2000 mg/kg. The LD_{50} calculated was 2000 mg/kg for both the extracts. So one tenth of the maximum tested dose (i.e. 200 mg/kg, p.o.) was selected as the effective dose.

Methodology

Induction of Hepatocarcinogenesis[11]

Rats were divided into five groups (n=6) as following.

- **Group 1**

Normal control received physiological saline solution, i.p.

- **Group 2**

Hepatocarcinogenic control (DEN 200 mg/kg i.p.) after two weeks.

- **Group 3**

Standard Synthetic Drug

Cyclophosphamide (orally: 50 mg/kg body weight dissolved in sterile water), 2 weeks before DEN injection (200 mg/kg i.p.) and continued throughout the experimental period (i.e. 6 weeks).[12,13]

- **Group 4**

***Leucas aspera* hydro-ethanolic extract** treated group (200 mg/kg orally), 2 weeks before DEN injection (200 mg/kg i.p.) and continued throughout the experimental period (i.e. 6 weeks).[8]

- **Group 5**

***Leucas aspera* Aqueous Extract** treated group (200 mg/kg orally), 2 weeks before DEN injection (200 mg/kg i.p.) and continued throughout the experimental period (i.e. 6 weeks).

Two weeks after the starting of the experiment all the rats except normal control received a single dose of CCl_4 (2ml/kg i.p.) by gavage as 1:1 dilution in corn oil to stimulate liver cells proliferation and regeneration. At the end of 6th week, blood samples were taken by cardiac puncture, under light ether anesthesia and liver were collected. Serum was separated by centrifugation and used for the biochemical assay.

Evaluation of Antioxidant Parameters

The degree of protection was measured by using parameters like Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), and Gamma glutamate transpeptidase (GGT) by kits.

Further, the effects of both extracts on Superoxide Dismutase (SOD) and Catalase (CAT) were also estimated.

Histopathological Examination

Liver pieces were preserved in 10% formaldehyde solution. The pieces of liver processed and embedded in paraffin wax.

Sections of about 4-6 microns were made and stained with hematoxylin and eosin and photographed.

Statistical Analysis

The results were expressed as Mean ± SEM and were analyzed for statistically significant difference using one-way ANOVA.

The difference showing a level of $p < 0.05$ was considered to be statistically significant.

RESULTS

Preliminary Phytochemical Study

The results of preliminary phytochemical study showed that hydro-ethanolic extract contains carbohydrates, saponins, tannins, and flavonoids whereas aqueous extract contains saponins, carbohydrates, glycosides and flavonoids as shown in Table 1.

Efficacy of Both Extracts on Changes in Body and Liver Weight of Rats

In group 2, there was an appreciable loss in the body weight when compared to control rats. The reduction in body weight correlates well with the decreased food intake from week 2 onwards.

Moreover, a significant increase in the liver weight was observed. The body and liver weights were not affected by treatment with DEN/CCL_4 in animals which received hydro-ethanolic extract.

Animals of group V showed no significant changes in liver weights as compared to control animals (Table 2).

Efficacy of Extracts on Changes in Superoxide Dismutase and Catalase Level in Rats

Our body has an effective defense system against free radical induced damage. It consists of a set of

endogenous antioxidant enzymes, two of the key components of which is catalase (CAT) Superoxide dismutase (SOD). The levels of CAT and SOD were significantly (P<0.001) decreased in DEN/CCL_4 treated rats when compared to control group. Administering hydro-ethanolic and aqueous extracts significantly (P<0.01) increased the decreased levels of SOD and CAT content.

Reduced activities of enzyme (CAT, SOD) antioxidant level of liver homogenate were summarized in Table 3.

Efficacy of Extracts on Changes in Serum Transaminase Levels in Rats

Both serum ALT and AST levels were markedly increased to their maximum value (U/L) at the end of experimental protocol.

Hydro-ethanolic and aqueous extract treatment produced dose-dependent reductions in ALT and AST levels.

Both extracts in group IV and group V reversed these changes to near normal, but it was more effective in group IV than in group V animals (Table 4).

Efficacy of Both the Extracts on Changes in ALP and GGT Level in Rats

Activities of ALP, GGT, had increased after DEN administration as compared with those of the control group. On the other hand, both the extracts reduced enzyme activities in a dose-dependent manner. This enzyme activity was completely restored to the normal level in group IV than V. Reduced activities of ALP and GGT level were summarized in Table 5.

Histopathology

From the histopathology of liver it was found that diseased control group showed severe necrosis and inflammation whereas treatment with hydro-ethanolic showed same effect mild necrosis with inflammation. Aqueous treated show mild effect on the hepatocyte. Cyclophosphamide also showed only the moderate effect on liver subjected to DEN /CCL_4 in short term treatment protocol (Figure 1).

Table 1: Preliminary Phytochemical Screening

Extracts	Carbohydrates	Proteins	Alkaloids	Flavanoids	Glycosides	Tannins	Saponins
Hydro-ethanolic	+	-	-	+	-	+	+
Aqueous	+	-	-	+	+	-	+

(+) indicates the presence of phytoconstituent in the extract and (-) indicates the absence of phytoconstituent in the extract

Table 2: Efficacy of Extracts on the Changes in Body and Liver Weight of Rats

S. No.	Groups	Body Weight		Liver Weight (gms)
		Initial	Final	
1.	Normal	188.0 ± 4.06	225.0 ± 3.53	5.33 ± .21
2.	DEN/CCL_4 treated group	197.0 ± 4.63	167.8 ± 6.65#	6.40 ± .02#
3.	Cyclophosphamide treated group (50 mg/kg)	202.0 ± 4.63	235.0 ± 3.52[a]	5.62 ± .0[a]
4.	Hydro-ethanolic extract treated group (200 mg/kg)	190.0 ± 3.53	205.0 ± 3.53[a]	5.90 ± .02[a]
5.	Aqueous extract treated group (200 mg/kg)	181.0 ± 4.30	215.0 ± 3.50[a]	6.06 ± .03[NS]

Values are expressed as Mean ± S.E.M. (n = 6); a p< 0.001 when compared with DEN/CCl_4 control group, #p<0.001 when compared with vehicle control group, NS: Non-significant

Table 3: Efficacy of Extracts on Changes in Superoxide Dismutase and Catalase Level in Rats Subjected to Experimental Hepatocarcinogenesis

S. No.	Treated Group	SOD (U/mg)	CAT (µM of H_2O_2 decomposed/min/mg)
1.	Normal	18.42 ± 0.11	132.3 ± 3.520
2.	DEN/CCL_4 treated group	6.944 ± 0.13	71.28 ± 1.083[#]
3.	Cyclophosphamide treated group (50 mg/kg)	15.15 ± 0.08[a]	122.9 ± 0.86[a]
4.	Hydro-ethanolic extract treated group(200 mg/kg)	11.98 ± 0.12[a]	98.28 ± 0.69[a]
5.	Aqueous extract treated group (200 mg/kg)	10.25 ± 0.10[a]	87.10 ± 0.69[a]

Values are expressed as Mean ± S.E.M. (n = 6); a p< 0.001when compared with DEN/CCl_4 control group, #p<0.001 when compared with vehicle control group, NS: Non-significant

Table 4: Efficacy of Extracts on Changes in Serum Transaminase Level in Rats Subjected to Experimental Hepatocarcinogenesis

S. No.	Treated Group	ALT IU/L	AST IU/L
1.	Normal	62.06 ± 3.65	92.63 ± 5.03
2.	DEN/CCL_4 treated group	166.5 ± 4.45	302.1 ± 7.33[#]
3.	Cyclophosphamide treated group (50 mg/kg)	84.82 ± 2.63[a]	135.4 ± 5.03[a]
4.	Hydro-ethanolic extract treated group(200 mg/kg)	119.0 ± 3.65[a]	183.8 ± 6.12[a]
5.	Aqueous extract treated group (200 mg/kg)	133.4 ± 4.44[a]	213.8 ± 5.03[a]

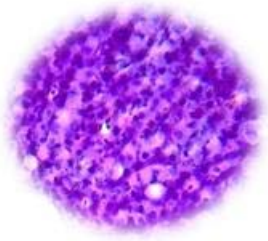
Values are expressed as Mean ± S.E.M. n = 6;a p < 0.001when compared with DEN/CCl4 control group, #p<0.001when compared with vehicle control group, NS: Non-significant

Table 5: Efficacy of Extracts on the Changes in ALP and GGT Level in Rats

S. No.	Treated Group	ALP KA	GGT IU/L
1.	Normal	7.56 ± 1.07	51.06 ± 2.54
2.	DEN/CCL_4 treated group	54.89 ± 1.74#	118.4 ± 2.81#
3.	Cyclophosphamide treated group (50 mg/kg)	18.89 ± 1.11a	69.55 ± 2.26a
4.	Hydro-ethanolic extract treated group(200 mg/kg)	37.78 ± 1.11a	87.60 ± 2.35a
5.	Aqueous extract treated group (200 mg/kg)	45.55 ± 1.36a	94.64 ± 2.69a

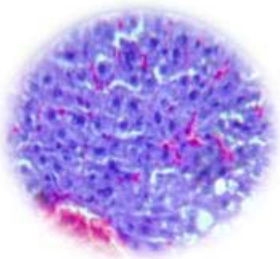
Values are expressed as mean ± S.E.M. n = 6; a p< 0.001 when compared with DEN/CCl_4 control group, #p<0.001 when compared with vehicle control group, NS: Non-significant

Figure 1: Histopathology of Liver of Rats



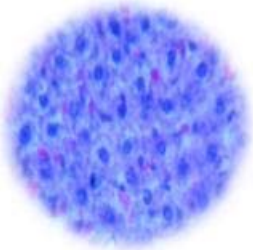
Normal Liver

Liver of the rat treated with vehicle show normal liver histology having a normal portal triad, sinusoids, and arrangement of hepatocytes.



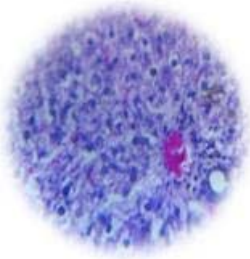
Diseased Control Liver

Liver of the rat treated with DEN/CCL_4 showed necrotic hepatocytes and the adjacent portal tracts were infiltrated by numerous inflammatory cells.



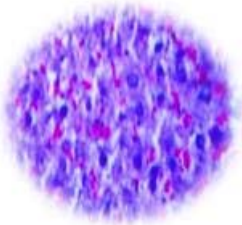
Cyclophosphamide Treated Liver

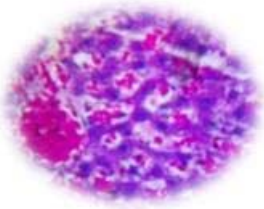
DEN/CCL_4+Cyclophosphamide treated group reduced the carcinogenic potential DEN/CCL_4 by showing the normal appearance of hepatocytes and mild presence of necrosis.



Hydro-ethanolic Treated Group

Pre treatment of rat with hydro-ethanolic extract disarrangement of hepatocytes and presence of necrosis can also be seen in some area.





Aqueous Extract Treated Liver

Liver exposed to aqueous extract showed a moderate degree of necrosis and very little arrangement of hepatocytes

DISCUSSION

This study demonstrates a potential role of hydroethanolic and aqueous extract of *Leucas aspera* in limiting preneoplastic changes in DEN-initiated and CCl_4-promoted experimental hepatocarcinogenesis in rats. Both extract supplementations has also been found to abate the development of preneoplastic lesions in carcinogen-challenged rat hepatocytes. The results thus indicate the chemo-preventive efficacy of hydro-ethanolic extract and aqueous extract in decreasing cell proliferation and hepatic nodulogenesis. The data presented demonstrated that treatment of rats with

carcinogen DEN-CCl_4 increased significantly serum GGT level. The increased level of GGT is an indicative of hepatic carcinogenesis. Long-term extract administration 2 weeks before induction of hepatocarcinogenesis and throughout the experimental period resulted in normalization of serum GGT level. The observed reduction in the levels of GGT in extract treated animals was presumably due to inhibition of stress and glutathione and therefore decreases in the tumors production rates. An increase in AST, ALT activities in DEN induced animals correlate to the hepatotoxicity and carcinogenesis with the development of preneoplastic changes, increased severity and advanced stage of liver carcinoma. The lowering in the activities of AST and ALT on extract treatment shows the hepatoprotective effect and inhibition of carcinogenesis. ALP is used as a specific tumor marker during diagnosis in the early detection of cancer.[14] An increase in ALP activity on DEN administration may be due to altered synthesis of enzymes as in other hepatotoxic conditions.[15]

Activities of ALP are increased in precancerous lesion in primary liver cell carcinoma and carcinoma of bile duct. The lowering of these enzyme activities significantly ($P<0.001$) indicates the inhibition of pre- cancerous transformation in the liver on hydroethanolic and aqueous extract treatment in DEN+CCL_4 animals.

Catalytic dismutation of highly reactive and potentially toxic superoxide radicals to H_2O_2.[16] Activities of the enzymic antioxidants are reverted to near normal in extract treated animals. This indicates the antioxidant potency of the drug and so preventing the inactivity of these enzymes from ROS.

Phytochemical studies have shown the presence of flavonoids in both the extracts. Flavonoids are known to possess antimutagenic and antimalignant effects.[17] Moreover, flavonoids have a chemopreventive role in cancer through the induction of enzymes affecting carcinogen metabolism and inhibit various activities of tumor promoters, which are involved in the process of carcinogenesis.[18] Chemopreventive effect of the both the extracts may be due to the presence of these compounds.

A more detailed study is needed to examine the molecular mechanisms by which both extract exhibited anti-cancer effects in the DEN-induced CCL_4 promoted hepatocarcinogenesis model.

CONCLUSION

The results clearly indicate a significant chemopreventive effect of hydroethanolic and aqueous extract of *Leucas aspera* plant in rats. The extracts inhibited the levels of AST, ALT, ALP and GGT and significantly increased the enzymic antioxidant defense mechanisms in DEN induced and CCL_4 promoted experimental Hepatocellular carcinogenesis. All these observations clearly indicate a chemopreventive function of the plant *Leucas aspera* extracts.

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