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Hepato protective activity of ethanolic and ethyl acetate extract of *Ruellia Tuberosa* L. (whole plant) against N-Nitrosodiethylamine induced liver cancer in wistar albino rats

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ABSTRACT

In Indian traditional system of medicine, herbal remedies are prescribed for the treatment of various diseases including liver diseases. The present study aims to carry out a systematic investigation of the protective influences of the ethyl acetate and ethanolic extract of *R.tuberosa* against N-Nitrosodiethylamine-induced hepato-carcinogenesis in wistar albino rats. Liver function was assessed by the determination of AST, ALT and ALP levels along with histopathological examinations. The serum biochemical analysis suggests that the use of ethanolic extract of *R.tuberosa* exhibited significant protective effect from hepatic damage in N-Nitrosodiethylamine induced hepato-toxicity model. Hepatic enzyme induction is generally an adaptive response associated with increases in liver weight, induction of gene expression, and morphological changes in hepatocytes. Histopathological studies revealed further evidence for its hepatoprotective activity.

Keywords: *Ruellia tuberosa;* N-Nitrosodiethylamine; Hepato carcinogenesis; Hepato toxicity.

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INTRODUCTION

Hepatocellular carcinoma is said to be stimulated by toxic industrial chemicals, air and water pollutants, food additives and fungal toxins.[1] N-Nitrosodiethylamine (DEN), a hepato-carcinogen, is known to cause perturbations in the nuclear enzymes involved in DNA repair/replication and is normally used as a carcinogen to induce liver cancer in animal models.[2] Liver is more susceptible to carcinogenic insult, because of its metabolizing efficacy. Liver, the key organ of metabolism and excretion has an immense task of detoxification of xenobiotics, environmental pollutants and chemotherapeutic agents. Hence, this organ is subjected to variety of diseases including hepatocellular carcinoma and other disorders. Several hundred plants have been examined for use in a wide variety of liver disorders. Antioxidants play an important role in inhibiting and scavenging free radicals and thus providing protection against infections and degenerative diseases.[3] Hepatic enzyme induction may be associated with changes in liver weight, histological evidence of abnormal hepatocytes, and alteration of serum clinical chemistry.[4] Many of infectious diseases are known to be treated with herbal remedies throughout the history of mankind: even today plant materials continue to play a major role in primary health care as therapeutic remedies in many developing countries. *R.tuberosa* belongs to Acanthaceae family. It is a medicinal plant distributed in Asia. In folklore medicine, it is used as diuretic, antidiabetic, antipyretic, analgesic, anti-hypersensitive and antidotal agent.[5] *R.tuberosa* had very good antiproliferative, cytotoxic effect on HepG2 cells.[6] The present study aspires to carry out a systematic investigation on activities of hepatic marker enzymes; AST, ALT, ALP along with histopathological study in the protective influence of the ethyl acetate and ethanolic extract of *R.tuberosa* against N-Nitrosodiethylamine-induced hepato-carcinogenesis in wistar albino rats using doxorubicin as control drug.

MATERIALS AND METHOD

Chemical reagents:

N-Nitrosodiethylamine, Doxorubicin Hydrochloride and Phenobarbital were purchased from Sigma Chemical Company, USA. All other chemicals including solvents were of high purity and of analytical grade purchased from Glaxo Laboratories, Mumbai and Sisco Research Laboratories Pvt, Ltd, Mumbai, India.

Plant materials and extract preparation:

R.tuberosa in Siddha and Tamil it is called as Tapas-kaava, Pattaskai and Local Amukara, in English it is called as Meadow-weed, Cracker plant, in Telugu it is called as Eerra verulu, and in

Marathi it is called as Ruwel. They are low-growing perennial herb with thick finger-like fusi form tuberous roots widely branched, with erect hairy stems. Leaves are simple, opposite, elliptic, short petioled, abruptly narrowed at the base, with undulate margins, mostly basal, finely pubescent and petiolate. Cymes dichotomous bisexual flowers, with funnel shaped having 5-lobed corolla, tube abruptly expanded above, up to 5cm across and 4-6cm long; bracts are narrow; calyx lobes linear having 12-20mm long, hispid; mauve or light bluish purple in colour. The plant flowers only after the start of the rainy season. The ripe fruits, in a pod with 7-8 seeds each, burst open, when they get wet and the black thin, discoid seeds are hurdled away.[7,8]

Fresh plant materials of *R.tuberosa w*ere collected from Tiruvallur district of Tamilnadu. The plant materials were identified and authenticated by botanist of this institute using the Flora of Presidency of Madras[9,10] and voucher specimen (No: 00628) was deposited in the museum of Captain Srinivasa Murti Drug Research Institute for Ayurveda, Arumbakkam, Chennai. The shade dried and coarsely powdered plant material (100g) was successively extracted with ethyl acetate and ethanol successively using Soxhlet apparatus, filtered and concentrated to dryness.[11] One gram of ethyl acetate and ethanolic extract from whole plant of *R.tuberosa* was weighed in dry weighing bottle. The working concentration of the each extract was diluted with Tween-80 to make a concentration of 100mg/ml and then the diluted solution was used for further chemopreventive study.

Experimental animals:

The procedure for animals experiments were reviewed and approved by Institutional Animal Ethics Committee (IAEC) of CCRIS (Approval No: 109/PHARMA/SCRI, 2011). Wistar albino male and female rats weighing 160-180g were purchased, from Tamilnadu Veterinary and animal Science University (TANUVAS), Madhavaram, Milk colony, Chennai, Tamilnadu, India, for this study. The animals were maintained under standard conditions of humidity, temperature (25 ± 2ºC) and light (12hr light and 12hr dark). The animals were acclimatized and maintained over husk bedding in polypropylene cages in central animal house facility of the institution for one week before use. The animals were fed with commercial pelleted diet (Hindustan lever Ltd, Bangalore, India with composition of 5% fat, 21% protein, 55% nitrogen free extract and 4% fibre (w/w) with adequate mineral, vitamin levels and free access to water throughout the experimental period. Experimental animals were handled according to the University and Institutional legislation, regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

Experimental design:

The experimental animals were divided into five groups with six animals in each group as shown in the table 1.

Table 1: Experimental design

Treatment	No. of rats	Study period
Group I - Normal control animals	(3 Male and 3 Female)	20 weeks
Group II - Control animals with single intra-peritoneal injection of N-Nitrosodiethylamine 200mg/kg body weight followed by phenobarbital of 0.05% mixed with drinking water daily for 20 weeks.	(3 Male and 3 Female)	
Group III - Animals with single intra-peritoneal injection of N-Nitrosodiethylamine 200mg/kg body weight, followed by phenobarbital of 0.05% mixed with drinking water daily for 16 weeks and ethyl acetate extract of *R.tuberosa* 400mg/kg body weight for 4 weeks.	(3 Male and 3 Female)	
Group IV - Animals with single intra-peritoneal injection of N-Nitrosodiethylamine 200mg/kg body weight, followed by phenobarbital of 0.05% mixed with drinking water daily for 16 weeks and ethanolic extract of *R.tuberosa* 400mg/kg body weight for 4 weeks.	(3 Male and 3 Female)	
Group V - Animals with single intra-peritoneal injection of N-Nitrosodiethylamine 200mg/kg body weight, followed by phenobarbital of 0.05% mixed with drinking water daily for 16 weeks and standard doxorubicin drug 5mg/kg body weight of one dose per week for 4 successive weeks.	(3 Male and 3 Female)	
Total number of rats	30	

Tumour induction and drug treatment:

The experimental rats were fasted overnight and induced by a single intraperitonial injection of N-Nitrosodiethylamine at a dose of 200mg/kg body weight in saline to induce liver cancer. N-Nitrosodiethylamine was dissolved in freshly prepared saline (0.9%). The control rats were similarly injected with saline. Neither death nor any other aggressive effect was observed. Two weeks after the administration of N-Nitrosodiethylamine, Phenobarbital at a concentration of 0.05% was incorporated into drinking water for about 14 successive weeks to promote the liver cancer.[12,13] The changes in body weight in all groups of rats were recorded at regular intervals (every week). After the 16th week the animals with liver cancer was confirmed by testing the level α-fetoprotein measured quantitatively by solid phase enzyme linked immunosorbent assay, and γ-glutamyl transferase in serum. The plant extract of 400mg/kg/body weight of ethyl acetate

extract from *R.tuberosa* whole plant for group III animals and 400mg/kg/body weight of ethanolic extract from *R.tuberosa* whole plant for group IV animals were treated for 4 weeks, the group V animals were treated with standard doxorubicin 5mg/kg body weight of one dose per week for 4 successive weeks. After the end of the drug treatment the animals were fasted overnight and sacrificed by cervical dislocation. The plasma/serum sample was collected with and without anticoagulant. The serum was separated and stored at -20ºC.

Collection of blood sample for hematology:

At the end of the experimental period animals were fasted overnight and anaesthetized with ether. Blood was collected from retro orbital vein in one tube with anticoagulant ethylene diamine tetra acetic acid (EDTA) which was used for hematological studies.

Collection of blood sample for biochemical test:

At the end of the experimental period animals were fasted overnight and anaesthetized with ether. Blood was allowed to coagulate before being centrifuged and the serum was separated. Assay of Aspartate transaminase (AST),[14] Assay of Alanine Transaminase (ALT),[14] and Assay of Alkaline Phosphatase (ALP).[15]

Preparation of tissue homogenate:

The liver tissue was excised, washed in ice-cold saline; 1g of tissue was weighed and homogenized in 0.1M cold Tris-HCl buffer of pH 7.4 in a potter-Elvehjam homogenizer fitted with a Teflon plunger at 600 rpm for 30min. The liver homogenate was prepared in cold 50mM potassium phosphate buffer of pH 7.4. The unbroken cells and debris were removed by centrifugation at 10,000 rpm for 15min at 4°C using a REMI cooling centrifuge and the supernatant was used for the biochemical assays.

Tissue processing for histopathological studies:

The organs like liver, kidney, brain, spleen and heart were rapidly dissected out. They were washed, dehydrated. The cleaned tissues from the control group and treated group were embedded with molten paraffin at 58ºC. Tissues were fixed in 10% formal saline, processed routinely embedded in paraffin, consecutive sections were taken at 7µ thickness using a razor blade and then stained with haematoxylin and eosin. The change in the liver tissues was observed under light microscope. Liver was divided into two pieces, one piece for biochemical studies and the other for histopathological studies.[16]

Statistical analysis:

Hypothesis testing methods included one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison method was used to compare the means of different groups by

using SPSS version 16.0 software (Chicago, USA), comparisons were made between N-Nitrosodiethylamine induced cancer group animals with other group animals. P<0.05 was considered as an indicator for the significant difference between study groups. All the results are expressed as mean ± S.D for six rats in each group.

RESULTS AND DISCUSSION

The table 2 shows the activities of AST, ALT and ALP which are significantly elevated ($p < 0.01$) in hepatocellular carcinoma of group II animals when compared with group I animals. Administration of N-Nitrosodiethylamine in rats leads to elevation in the activities of serum AST, ALT and ALP which is indicative of hepatocellular damage. This elevation could potentially be due to the release of these enzymes from the cell cytoplasm into the blood circulation after rupture of the plasma membranes.[17] It indicates the cancerous condition in N-Nitrosodiethylamine induced hepatocellular carcinoma of group II animals. AST and ALT activities in serum are considered as the index of liver damage. Aminotransferases are liver marker enzymes that has leaked into the circulation during hepatocyte injury and liver damage, hence it was assumed that elevation in the activities of ALT, AST and ALP are considered as indicator of cancer.[18]

Table 2: Effect of extract of *R.tuberosa* on tumor marker enzymes like AST, ALT and ALP in U/L in serum of control and experimental animals

Parameters	Group I	Group II	Group III	Group IV	Group V
AST	81.1 ± 4	290 ± 12 ^{b}	102 ± 5 ^{a}	89 ± 5 ^{a}	79.1 ± 3 ^{c}
ALT	48.3 ± 2	80.1 ± 5 ^{c}	70 ± 2 ^{c}	55.9 ± 2 ^{b}	51.2 ± 2.5 ^{c}
ALP	484 ± 12	589 ± 17 ^{b}	500 ± 19 ^{b}	487 ± 18 ^{c}	489 ± 21 ^{c}

Each value is expressed as mean ± S.D, for six rats in each group. The activities of AST, ALT and ALP are expressed as U/L. Group I- control animals, Group II- cancer bearing animals, Group III- ethyl acetate extract post treated, Group IV- ethanolic extract post treated and Group V- standard Doxorubicin treated animals. Statistical significance (a = $p < 0.05$, b = < 0.01, c = $p < 0.001$) and non significant (NS), when Group II compared with Group I, when Group III, IV and V compared with Group II.

On treatment of ethyl acetate extract of *R.tuberosa* the level of AST and ALP are significantly reduced ($p < 0.01$) and the level of ALT is significantly reduced ($p < 0.001$) in group III animals. On treatment of ethanolic extract of *R.tuberosa* the level of AST, ALT and ALP significantly reduced in group III animals. The level of hepatic marker enzymes are reduced in the sequence order (AST > ALP > ALT) with ethyl acetate extract of *R.tuberosa* treated in group III animals.

Ethanolic extract of *R.tuberosa* was found to be highly significant than ethyl acetate extract. Ethanolic extract of *R.tuberosa* has significant anticancer activity. It indicates that extracts of *R.tuberosa* are capable of preventing and protecting the hepatocellular damage.

Histopathological examinations plate (1-5):

Histopathological examinations of the liver tissues provided supporting evidence for the result obtained in the biochemical analysis. The plate 1 shows the section of liver tissue with normal architecture from (group I) normal animals. The plate 2 shows that the sections of liver tissue with parenchyma with extensive fatty changes as indicated by clear white cytoplasm as against pink eosinophilic cytoplasm. Hepatocytomegaly with multifocal clear cell areas with foci of necrosis were seen in liver. It also shows dysplastia and neoplastic, pleomorphic hepatocyte with nuclear enlargements, condensed chromatin, increased eosinophilia of the cytoplasm, in collectively hepatocytomegaly as detected in (group II) N-Nitrosodiethylamine induced hepatocellular carcinoma animals. The plate 3 shows that the section of liver tissue with almost uniform appearing hepatocytes with regular vesicular nuclei for (group III) ethyl acetate extract of *R.tuberosa* (400mg/kg body weight) treated animals. The plate 4 shows that the sections of liver tissue are normal for (group IV) ethanolic extract of *R.tuberosa* (400mg/kg body weight) treated animals. The plate 5 shows that the section of liver tissue shows normal architecture for (group V) animals.

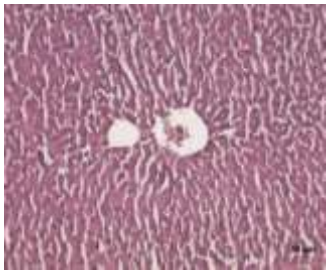

Plate 1: Group I

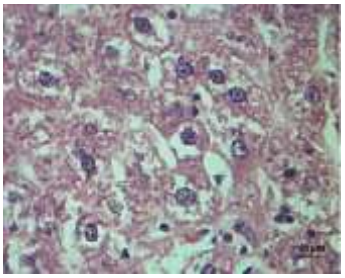

Plate 2: Group II

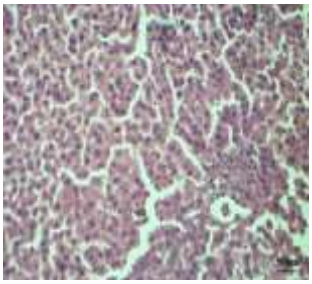

Plate 3: Group III

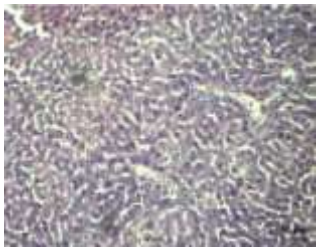

Plate 4: Group IV

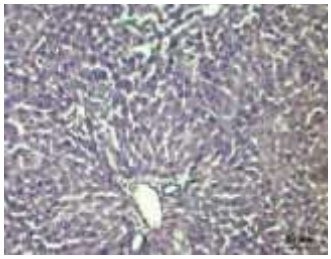

Plate 5: Group V

Plate (1-5): Histopathological sections of Liver from the control and experimental animals.

i. Group I-Normal architecture is seen in the section of liver tissue of control animals.

ii. Group II-The section of liver tissue shows parenchyma with extensive fatty changes as indicated by clear white cytoplasm as against pink eosinophilic cytoplasm. Hepatocytomegaly with multifocal clear cell areas with foci of necrosis were seen. It also shows dysplastia and neoplastic, pleomorphic hepatocyte with nuclear enlargements, condensed chromatin, increased eosinophilia of the cytoplasm, in collectively hepatocytomegaly is detected.

iii. Group III-The section of liver tissue shows almost uniform appearing hepatocytes with regular vesicular nuclei.

iv. Group IV-The section of liver tissue are normal.

v. Group V- The section of liver tissue shows normal architecture and no abnormalities were observed.

CONCLUSION

The 400mg/kg dose of ethanolic extract of *R. tuberosa* effectively decreased the activities of serum ALT, AST and ALP. Further, in histopathological studies liver damage due to neoplastic conditions in N-Nitrosodiethylamine induced hepatocellular carcinoma animals were also recovered after *R.tuberosa* treatment. In conclusion it is confirmed that ethanolic extract of *R.tuberosa* has potential chemopreventive property which in turn reduces the hepatocellular carcinoma and liver damage caused by N-Nitrosodiethylamine than ethyl acetate extract in *in-vivo* system. This indicated that the *R.tuberosa* effectively blocks the secondary malignancy and thereby increases the life span and reduces the tumor growth. However further studies are required to isolate the active compounds from ethanolic extract of *R.tuberosa* to confirm these properties for safe, efficacious and cost effective anticancer drug for the poor and under privileged people of India.

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