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Liver Regenerative Effect of *Phyllanthus amarus* Linn. Against Alcohol Induced Liver Cell Injury in Partially Hepatectomised Albino Rats

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Abstract: The present study investigates the liver regenerative effect of *Phyllathus amarus Linn* against alcohol induced liver cell injury in partial hepatectomised albino rats. The oral administration of *Phyllanthus amarus* extracts increases the activities of thymidine kinase in regenerating rat liver at 24 h. Levels of DNA and protein analysis showed that the increase in thymidine kinase was caused by comparable increase the DNA and protein. Histopathology confirmed the mitosis counts increases to the activity of DNA. These findings suggested that *Phyllanthus amarus* inducing DNA synthesis by the induction of levels of synthesizing nucleic acid enzymes during liver regeneration. *Phyllanthus amarus* may be used, as a potential liver regenerative herb in hepatic disorders and seems to be beneficial against alcohol induced liver cell damage.

Key words: *Phyllanthus amarus* Linn, thymidine kinase, DNA synthesis, ethanol, Liver regeneration

INTRODUCTION

Phyllanthus amarus Linn. (Euphoriaceae) is widely distributed in India. It is known as a natural remedy for a number of illness like Viral infection. (Thyagarajan *et al*., 1988; Calixto *et al*., 1988; Jayaram *et al*., 1997).

Phyllanthus amarus extracts has been shown to inhibit DNA polymerse of hepatitis B virus and related hepatitis viruses (Venkatswaran *et al*., 1987) and down regulate hepatitis B virus mRNA transcription and translation (Lee *et al*., 1996; Ott *et al*., 1997).

Simultaneous administration of *Phyllanthus amarus* extract along with the carcinogen has been reported to inhibit the hepatocellular carcinoma development induced by N-nitrosodiethylamine (NDEA) (Joy and Kuttan, 1998; Rajeshkumar and Kuttam, 2000). Recently it is reported that ethanolic extract of *Phyllanthus amarus* possess anti-inflammatory (Raphel and Kuttan, 2003; Kiemer *et al*., 2003; Candida *et al*., 2005; Kassuya *et al*., 2003) and antinociceptive properties (Kassuya *et al*., 2003). Earlier work described that *Phyllanthus* species showed significant heapoto protective activity on alcohol induced liver cell damage in non-hepatectomised and partially hepatectomised rats (Agrawal *et al*., 1986). No liver regenerative property of *Phyllanthus amarus* has been so far reported. Here we evaluate the liver regenerative effect of *Phyllathus amarus Linn* against alcohol induced liver cell injury in partial hepatectomised albino rats.

MATERIALS AND METHODS

Plant material: *Phylanthus amarus* was uprooted from the Delhi Institute of Pharmaceutical Sciences and Research campus. It was identified according to the description of The Wealth of India (Council of Industrial and Scientific Research, Government of India, New Delhi, 1985) and macroscopic microscopic comparison to authentic samples at Laboratory of Delhi Institute of Pharmaceutical Sciences and Research where a voucher specimen is deposited.

Extraction of plant: Dried and coarsely powered whole plant of *Phylanthus amarus* were extracted with a mixture of 50% ethanol by Soxlet extractor and the yield was 12.10%. The extract evaporated to dryness in water bath.

Chemicals: The (Methyl-3H) Thymidine (2 mci) was purchased from Board of Radioisotope Technology, Mumbai, India and Durapore membrane filter, from Milipore, USA. Diphenylamine and TRIS purchased from SRL, Mumbai, India. ATP, Marcaptoetahanol, Calf thymus DNA and Phenyl Ethyl Sulphonyl Fluoride were purchased from Sigma (St. Louis, MO, USA). Optiphase Hi safe scintillation cocktail LKB scintillation was purchased from FSA Laboratory (Lough Boorough, England LE 110 RG).

Pharmacological studies

Animals and treatments: Male Wister rats weighing 200-250 g from Laboratory Animal Resources, Indian

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Veterinary Research Institute, Izzatnagar, UP, India and were maintained under temperature-controlled rooms with 12 h alternating light and dark cycles were given adequate nutrition and water *ad libitum*. All experimental protocols using animals were performed according to the "Principles of Laboratory Animal care" (NIH publication 85-23, revised 1985) adopted. Two-third partial hepatectomy (PH) was performed under diethyl ether anesthesia following the procedure of (Higgins and Anderson, 1981). The rats were divided into four groups with 6 animals each and studies were made 24 h, 48 h and on 7th day post operation intervals. Group I served as vehicle control, Group II served as only partial hepatectomy (Control Group) (PH) and Group III received extract of *Phyllanthus amarus* equivalent to 200 mg kg^{-1} body weight/daily by oral route. Group IV received extract of *Phyllanthus amarus* equivalent to 200 mg kg^{-1} body weight/daily by oral route and alcohol equivalent to 8 g kg^{-1} body weight/daily by oral route respectively. Group IV received only alcohol equivalent to 8 g kg^{-1} body weight/daily by oral route. At 24 h, 48 h and on 7th day liver samples collected from each group were divided in two parts. One part was immediately preserved in 10% formaldehyde solution for histopathological examination and the other part used for the determination of enzyme activity and contents of DNA and Protein.

Analytical procedure: Excised liver was homogenized with 5 volumes of 50 mM Tris-HCl buffer (PH 7.3) containing 0.25 (M) sucrose, 10 mM β-mercaptoethanol, 1 mM phenylmethylsufonyl fluoride and 1 mM EDTA. The supernatant fractions of liver homogenate centrifuged at 36000 g for 30 min at 4°C, was used for determining the enzymatic activity. Activity of TK was measured (Nakata *et al*., 1985a) and expressed as pmol of product formed/min/ mg of protein at 37°C DNA contents of the liver were measured by Diphenylamine (Burton, 1956) reactions. Protein concentration was measured by the method of Lowry (Lowry *et al*., 1951) using bovine albumin as standard. The degree of cellular injury and the miotic index were assessed histologically after hematoxylin and eosin staining (H and E). The percentage of cells in metaphase was determined across 1000 hepatic cells.

Statistics: Statistical analysis of data was performed with student's-test. The level of significance was $p<0.05$.

RESULTS

Effect of *Phyllanthus amarus* on the activity of thymidine kinase, DNA, protein in regenerating liver: The results of TK, DNA and protein are shown in (Table 1-3), respectively. The TK actions increased significantly when compared with normal activity (resting in Go state) at 24 h in all groups.

Table 1: Effect of *Phyllanthus amarus* in TK level on regenerating liver after partial hepatectomised at 24, 48 h and on 7th day

Groups	TK at 24 h	TK at 48 h	TK at 7th day
Normal (non-hepatectomised)	33.84±3.01	-	-
PH(Control)	245.09±7.63*	174.22±5.43*	97.952±0.72*
PH+*P. amar*	305.31±8.07*	201.23±6.95*	109.913±14.47
PH+*P. amar*+Alco.	229.84±3.15	155.44±10.03	86.830±2.93*
PH+Alco.	197.06±1.45	117.13±6.46*	64.603±8.64

Where * indicate significant differences from the control ($p<0.05$)

Table 2: Effect of *Phyllanthus amarus* in DNA level on regenerating liver after partial hepatectomised rats at 24, 48 h and on 7th day

Groups	DNA at 24 h	DNA at 48 h	DNA at 7th day
Normal (non-hepatectomised)	3.3±1.17	-	-
PH(Control)	3.49±0.24*	4.48±0.33*	2.98±0.108*
PH+*P. amar*	4.275±0.16	5.57±0.21*	3.35±0.124
PH+*P. amar* +Alco.	3.450±0.24*	4.32±0.88*	2.94±0.126*
PH+Alcohol	2.87±0.71*	3.41±0.33	1.99±0.177*

Where * indicate significant differences from the control ($p<0.05$)

Table 3: Effect of *Phyllanthus amarus* in protein level on regenerating liver after partial hepatectomised at 24, 48 h and on 7th day

Groups	Protein at 24 h	Protein at 48 h	Protein at 7th day
Normal (non-hepatectomised)	95.61±2.30*	-	-
PH	125.44±3.71*	135.87±7.40*	74.84±1.804
PH+*P. amar*	87.43±3.34	154.44±8.38*	88.76±5.441
PH+*P. amar* +Alco.	71.74±3.55	113.433±3.86*	72.23±2.124
PH + Alco.	148.32±6.29*	87.10±3.64*	52.33±2.103

Where*indicate significant differences from the control ($p<0.05$)

Table 4: Effect of *Phyllanthus amarus* in Mitosis count/1000 hepatic cells on liver regenerating after partial hepatectomised at 24, 48 h and on 7th day

Groups	24 h	48 h	7th day
Normal (non-hepatectomised)	0	0	0
PH	1 to 4	1 to 5	0
PH + *P. amar*	2 to 5	4 to 8	1 to 3
PH + *P. amar*+Alco.	1 to 4	2 to 5	0 to 2
PH + Alco.	0 to 2	1 to 2	0

At 24 h, the TK activity was found to be at peak level when compared with 48 h and on 7th day. However, TK activities were found to be depressed ($p<0.05$) by the ethanol when compared with control group.

DNA and protein activity increased significantly ($p<0.05$) at 48 h in *Phyllanthus amarus* treated group (Group III) as compared to PH group (Group II) (Table 2, 3). Ethanol alone administered group (Group V) was compared with PH (Group II) and it was found that DNA and protein activity reduced markedly ($p<0.05$). However, alcohol and *Phyllanthus amarus* treated (Group IV) animals showed rise in raised the DNA and protein content.

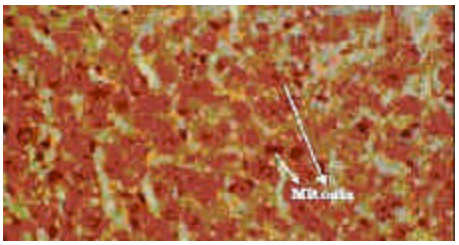

Fig. 1: Bi-Nucleoli, mitosis karyomegali after 24 h of PH in *Phyllanthus amarus* administered groups (H/E×320)

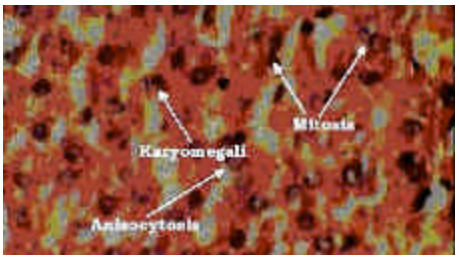

Fig. 2: Hepatocytes Karyomegali, anisocytosis and mitosis after 48 h of *Phyllanthus amarus* administered groups (H/E×320)

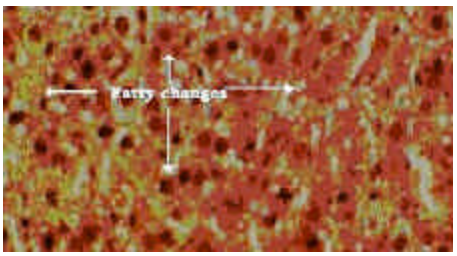

Fig. 3: Diffuse fatty changes in periphery region after PH on 7th day groups (H/E×320)

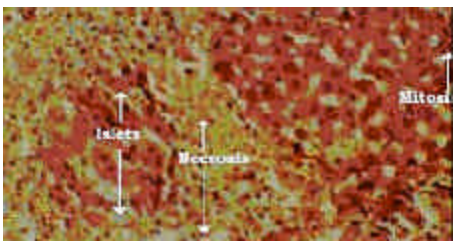

Fig. 4: Necrosis, organization islets pattern hepatocytes, died hepatocytes and mitosis, Regenerative in nature after 24 h PH in *Phyllanthus amarus* and alcohol administered groups (H/E× 320)

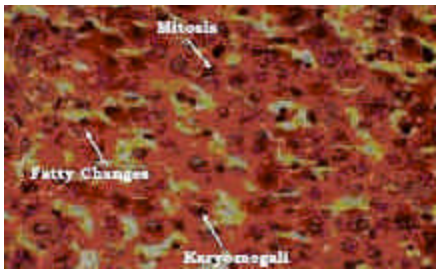

Fig. 5: Hepatocytes are bi-nuclear regenerative, mitosis, Hepatocytes swollen, occasionally showing fatty vacuoles karyomegaliafter PH 48 h in *Phyllanthus amarus* and alcohol administered groups (H/E×320)]

Effect of *Phyllanthus amarus* on the histopathology of regenerating liver: Administration of *Phyllanthus amarus* showed increased mitotic division significantly (Table 4) ($p<0.05$) and regenerative appearance of hepatocytes in the form of karyomegali, anisocytosis (Fig. 1 and 2) when compared to PH group (Group II) at 48 h.

However, alcohol treatment alone (Group V) showed degenerative changes like diffuse fatty changes. kufer cells hyperplasia (Fig. 3). However, treatment with both *Phyllanthus amarus* and alcohol the degeneration appearance of hepatocytes reduced significantly ($p<0.05$) (Fig. 4 and 5).

DISCUSSION

In mechanism regulating liver regeneration following 70% partial hepatectomy has been used as an ideal model to investigate the regulatory mechanism of mammalian cell proliferation *in vivo*. Thymidine kinase (TK:EC:2.7.1.21) which catalyses the formation of thymidilate through the *denovo* and salvage path way and reflected DNA synthesis (Nakata *et al*., 1985, 1987).

In a variety of cell types and tissues examined so far, adverse effect of ethanol on cellular proliferation has been described. The effects of ethanol on the abilities of the liver to regenerate have also been extensively studied *in vivo*. Several studies have demonstrated that acute and chronic ethanol administration impairs the [^{3}H] thymidine incorporation into hepatocytes DNA in rats subjected to partial hepatectomy (PH) with or without the decrease of total DNA control (Wands *et al*., 1979; Levy and Chen, 1979).

Thymidine kinase, the enzyme responsible for phosphorylation of thymidine before incorporation into DNA has been used in cell proliferation studies and

changes in the enzyme have been correlated with DNA synthesis as measured by labeled thymidine incorporation and thymidine kinase activity. (Morley and Royse, 1981). Thymidine kinase is almost undetectable in non-proliferating tissues, such as normal liver cells but after partial hepatectomy the activity increased markedly after a short lag period. It has been shown that this enzyme is closely correlated with DNA biosynthesis. Its activity increases dramatically in rapidly proliferating cells, such as those of regenerating liver and cancer cells (Khan *et al*., 1980).

Positive regeneration activity in liver has been observed following *Phyllanthus amarus* administration (Group III) as compared to PH group (Group II) studies at 24, 48 h and on 7th day post operation intervals. The study revealed increased DNA synthesis as a result of stimulation TK activity during regeneration process at 24, 48 h and on 7th day of PH following treatment of *Phyllanthus amarus* (Group III). Alcohol and *Phyllanthus amarus* administration (Group IV) also showed significant ($p<0.05$) regenerative activity as compared to PH group (Group II) whereas only ethanol administration (Group V) revealed degenerative changes as compared to PH group (Group II). These observations are consistent with the common observation of inhibition of cell proliferation by ethanol *in vitro* studies. *Phyllanthus amarus* and alcohol suppressed degenerative activity which is otherwise is a consequence of ethanol administration (Group V). Interestingly, in rats with partial hepatectomy, alcohol induced degenerative changes were found to be suppressed by the administration of *Phyllanthus amarus* in PH rats. The kinetics of the regenerative response after PH has been well described. It is apparent that first 12 h after PH, hepatocytes move from a state of quiesence, enter the cycle and progress to cause DNA synthesis. DNA synthesis occurred 12-16 h after PH and reached a peak at about 24 h, followed by a mitotic wave 6-8 h later. Thus, the proliferative activation process is synchronized with DNA synthesis and is a marked event the first 24 h after PH (Khan *et al*., 1980).

DNA and protein study showed that increased content of DNA and protein marked the first 48 h after PH. Hepatic Stimulator Substance (HSS) an important factor for liver regeneration Pretreatment with Cadmium arrest the liver regeneration in partially hepatectomised albino rats by suppression of HSS biological activity (Tzirogiannis *et al*., 2003). Therefore, seems to be a potential liver regenerative drug. It, therefore, also appears that *Phyllanthus amarus* stimulate the signal transduction system, which is required to stimulates the explosion of TK activity and content of DNA during liver regeneration.

Histopathological study revealed that *Phyllanthus amarus* induces mitotic divisions/1000 hepatic cells. These findings revealed that *Phyllanthus amarus* may be used, as a potential liver regenerative herb in hepatic disorders and seems to be beneficial against alcohol induced liver cell damage.

Further studies to elucidate the molecular mechanism of action of *Phyllanthus amarus* on the expression of these particular genes during liver regeneration needs to be conducted.

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