

***Toxicological investigation of aqueous extract of leaves of a plant
Phyllanthus amarus, in rat***

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Abstract

Phyllanthus amarus (*P. amarus*), a plant species of *Euphorbiaceae* family, is used as a folk medicine for jaundice and other diseases in Malaysia and other countries. The variation in their efficacy with geographical location and varieties has been reported which has been attributed to their constituent composition. But, so far no toxicity studies have been carried out on this plant with clear documentation, especially with those plants growing in Malaysia. So the aim of this preliminary study was to determine the toxic side effects of aqueous extract of leaves of *P. amarus* (grown in Malaysia) following oral administration in rats. Acute administration of *P. amarus* extract, orally at a dose of 5 gram/Kg body weight) did not produce any signs of toxicity or mortality. In the chronic study, no significant differences ($p > 0.05$) were observed between the control and *P. amarus* extract administered (male and female) rats (at the doses of 100mg/kg body wt/day, 400mg/Kg body wt/day and 800 mg/kg body wt for 6 weeks) in the body weight gain during the experimental period as well as in the biochemical markers analyzed (ALT, AST,ALP,LDH and total protein) in serum. The non-toxic nature of *P. amarus* extract administration was confirmed by histological studies [light microscopy, proliferative cell nuclear antigen (PCNA) study and apoptotic study by using ApopTag kit] i.e., no observable changes were found between control and *P.amarus* extract administered rats. Therefore, acute oral administration of *P.amarus* extract is non-toxic to the rat liver, even at a dose of 5 gram /kg body weight and also the chronic toxicity studies of *P. amarus* extracts administration (of 100-800 mg/kg body wt) showed the absence of cumulative toxicity as reflected by the non-significant change in the parameters studied as well as from the results of the histological studies.

Key words: *Phyllanthus amarus*, Aqueous extract, Liver, Toxicity, Marker enzymes, Histological studies

1. Introduction

Herbal medicines has been used since ancient times. Most of the population of the underdeveloped and developing countries depend on some form of traditional medicine. Malaysia is one of the important nations of biodiversity in the world and enriched with natural plant resources (Muhamad bin Zakaria and Mustafa Ali Mohd, 1994).

One of the plant genres widely used traditionally for the treatment of different diseases is *Phyllanthus* (Family: *Euphoribaceae*) and is distributed in most tropical and subtropical countries and comprise approximately of 550-750 species throughout the world. Among them, one of the most studied species is *P.amarus* and is widely used in Malaysia and other countries (Calixto *et al.*, 1998; Muhamad bin Zakaria and Mustafa Ali Mohd, 1994). In Malaysia, the plant is called as "Dukung anak".

P.amarus has bitter, astringent, cooling, diuretic, stomachic, antiseptic, antiviral, antidiabetic, hypotensive, antinociceptive, febrifuge properties and is traditionally used in the treatment of jaundice, diarrhea, dysentery, diabetes, fevers, uro-genital diseases, ulcers and wounds (Santos *et al*, 1995; Calixto *et al*, 1998). Recent years, a growing interest shown towards the *Phyllanthus* with respect to their potential on management of several diseases (Odetola and Akojenu, 2000; Rajeshkumar *et al.*, 2002; Srividya and Periwal, 1995) More research was done on its antiviral effect. Some reports have shown the antiviral effect of this plant by reducing the detectable hepatitis B surface antigen (HbsAg) of HBV

positive patients (Thyagarajan *et al.*, 1988; Ott *et al.*, 1997). But other studies like from China, Thailand , India showed the failure of *P.amarus* in eradicating the HbsAg in patients with chronic hepatitis B virus (Leelarasamee *et al*, 1990; Wang *et al*, 1991, Doshi *et al*, 1997). The variation in the clinical effect of these studies has been attributed to many factors such as different species, differences in growing condition and different processing methods (Thyagarajan *et al.*, 2002; Wang, 2000).

Usually herbal medicines are widely perceived by the public as being natural, healthful and free from side effects, but that is not 100 % true. Plants contain hundreds of constituents and some of them may elicit a toxic side effects. A number of studies exist reporting the toxic effect of herbal medicines (Shaw *et al.*, 1997; Kaplowitz, 1997; Calixto, 2000). Therefore efficacy and safety study should be performed in these herbs.

Eventhough a large number of clinical trials has been done on *P.amarus* and reporting their benefits, so far no systematic toxicological investigation has been reported on this plant, especially the *P. amarus* growing in Malaysia.

Since the efficacy of *Phyllanthus* species varies with geographical location and varieties and because of their constituent composition variation, the *P.amarus* grown in Malaysia (widely used here as the folk

medicine for jaundice) has to be ascertained for their biosafety by conducting acute and chronic toxicity study .

So the aim of this preliminary study was an attempt to determine the toxic effects of aqueous extract of leaves of *P. amarus* (grown in Malaysia) following oral administration in rats by assessing the morphological, biochemical and histological changes.

2. Materials and methods

2.1 Plant material

P.amarus was collected from MARDI (Malaysian Agricultural Research and Development Institute, Telung, Bachok, Kelantan) during the months of July-October and identified by FRIM (Forest Research Institute of Malaysia, Kepong, Selangor). The aqueous extraction of *P.amarus* leaves was carried out at the Department of Pharmacology, School of Medical Sciences, Universiti Sains Malaysia.

2.2 Preparation of plant extract

The plant material was cleaned and the leaves were separated, oven dried at 50°C. Then macerated into dry powder. Approximately 4.5 grams of fine leaves powder of *P.amarus* were extracted with distilled water (2:25) using soxhlet apparatus. After extraction, concentrated by rotary evaporator at 65°C. Then transferred into a suitable container and freeze dried by freeze dryer. The yield of the final crude aqueous *P.amarus* extract was 6-9% (~380 grams). The dried extract was stored in a

desiccator until its use. The extract was dissolved in distilled water to the desired concentration just before the study.

2.3 *Animal and experimental design*

Male and female Sprague-Dawley rats weighing (180-220 grams) were used as the experimental animal in this study and were obtained from Animal House Facility Unit, Health Campus, Universiti Sains Malaysia and acclimatized for one week prior to start the experiment. Animals were housed in a standard cages at a temperature of $23\pm 2^{\circ}\text{C}$, 45-55 % relative humidity with a 12h light/12h dark cycle. The animals were fed with commercial pellet diet and water *ad libitum*.

Our study protocol was approved by the animal ethical committee, Health Campus, Universiti Sains Malaysia.

Two types of studies were carried out (WHO, 1993; Arnold, 1990) Acute and chronic study and in both studies the rats were administered with *P.amarus* extract by gavage (orally).

2.4 *Acute study*

Aqueous leaves extract of *P.amarus* at a single dose of 5g/Kg body weight was administered orally to two male rats. Another two male rats served as control (without *P.amarus* extract administration). The animals were observed carefully for any visible signs of toxicity and mortality

immediately after dosing, at 4h, 24h intervals, during the recovery period of 48h and twice daily upto 14 days. After 14 days, the rats were sacrificed under ether anesthesia. A thorough autopsy was carried out and all organs were observed for any macroscopic changes.

2.1 *Chronic study*

2.5.1. *Animal groups*

For the chronic study, the animals both sex were grouped (ten rats in each group) as follows: For the male rats, Group I served as the control, Group II,III and IV as the groups administered with *P.amarus* extract at the doses of 100,400 and 800 mg/Kg body wt/day for 6 weeks by gavage, respectively. For the female rats, Group A served as the control, Group B,C and D as the groups administered with *P.amarus* extract at the doses of 100,400 and 800 mg/Kg body wt/day for 6 weeks by gavage, respectively. The animals were observed daily for any signs of morbidity and mortality and their body weights were measured periodically in the experimental period.

2.5.2. *Collection of serum and liver samples for analysis*

At the end of the experimental period (6 weeks), after an overnight fasting, both the male and female rat controls and *P.amarus* extract administered groups were sacrificed by decapitation. Blood was collected, allowed to clot and then centrifuged at 3,000 rpm for 15 minutes. Serum samples were separated and used for biochemical analysis. The samples were stored at -80°C, if not used immediately.

A portion of the liver tissue of all the (control and *P.amarus* extract administered) groups were fixed in 10% formal saline for the histological studies.

2.5.3. *Biochemical analysis*

In the collected serum, the total protein and the activities of the liver marker enzymes such as ALT,AST,ALP and LDH were assayed by using standard kit (ALT Randox kit , Randox Total protein biuret reagent-Randox Laboratories Ltd, UK; AST,ALP and LDH Roche Kits - Roche diagnostics, GmbH, Germany) in a Hitachi-912 autoanalyser available in our department of chemical Pathology, School of Medical Sciences, Universiti Sains Malaysia.

2.5.4. *Histological studies*

This study was carried out in the department of Pathology, School of Medical Sciences, Universiti Sains Malaysia.

A portion of all the liver specimens fixed in 10% formal saline were processed routinely overnight using histokinette. Then they were embedded in paraffin was. Three sections, each four micron in thickness were cut from each paraffin block.

Light Microscopic study

One section from each sample was stained with Haematoxylin & Eosin (H&E) stain by the standard method for light microscopic histological examination

Proliferative Cell Nuclear Antigen (PCNA) study

Another section from each sample was stained with anti PCNA (DAKO, Denmark) using standard immunohistochemistry procedure and the positivity was visualized with the chromogen diaminobenzidine (DAB) (DAKO, Denmark) and expressed in percentage.

Apoptosis study

The third section from each sample was assessed for apoptosis. They were stained with ApopTag –Apoptosis detection kit (Chemicon, USA) whereby the reagents are designed to label the free 3'OH DNA termini in situ with chemically labelled and unlabelled nucleotides and the procedures are followed according to the manufacturers instructions.

2.6. *Statistical Analysis*

The results of the biochemical analysis are expressed as mean \pm S.D. for ten animals in each group. The difference between the control and P.amarus extract administered groups (for each sex of animals) have been analysed by student's t-test. p value <0.05 , considered as significant.

3. **Results**

3.1. *Acute study*

During the course of the acute study, no treatment related effect was observed on the general condition or behaviour of the experimental animals. All the rats appeared to be normal, survived during the experimental period and none of them showed any visible signs of toxicity.

A thorough autopsy of the *P.amarus* administered rats revealed no treatment related macroscopic changes.

3.2. *Chronic study*

3.2.1. *Body weight*

Mean body weight gain of the *P.amarus* extract administered groups (at the doses of 100,400 and 800 mg/Kg body weight/day) have shown no appreciable difference when compared to their control (both male and female rats) after 6 weeks duration of the study (Data not shown).

The macroscopic appearance of all the organs observed was found to be normal between control and *P.amarus* extract administered groups .

3.2.2. *Biochemical study*

There were no significant differences ($p>0.05$) observed in the biochemical parameters studied (ALT,AST,ALP,LDH and total protein) in the serum between controls and *P.amarus* extract administered groups (of both male and female) (Table 1 & 2).

3.3 **Histological study**

3.3.3. *Light microscopic study*

Histological examination of the liver under light microscope showed no significant pathological changes in the *P.amarus* extract administered groups (at the acute dose of 5g/Kg body weight and at the doses of 100,

400 & 800 mg/Kg body wt/ day, orally, for six weeks to both male & female rats) (figures 3-9) when compared to their controls (Figures 1 & 2).

3.3.2 PCNA Study

Anti PCNA antibody was used to measure the proliferation of hepatocytes by immunohistochemistry. The cells that are in the proliferative pool shows brown colour staining (Figure 10). PCNA of liver from control rat was 1.9% (Figures 11 &12) and for the acute dose (5g/Kg body weight) *P.amarus* extract administered rat was 1.8% (Figure13). For the *P.amarus* extract administered groups at the doses of 100, 400 & 800 mg/Kg body wt/ day , orally, for 6 weeks (both male & female rat livers) showed PCNA of 0.2% -2.1% (Figures 14-19) which are similar to that of their controls.

3.3.3. Apoptosis study

Apoptosis in the liver was measured in the hepatocytes using ApopTag Kit. The positivity was indicated by the brown colour in nucleus (Figure 20). Apoptosis in the livers from control (both male and female rats) were 0.2% (Figure 21 &22). Apoptosis in the livers from *P.amarus* extract administered groups were ranged from 0.2% to 1.5% (Figures 23-29) which are similar to that of their controls.

4. Discussion

Herbal medicines have played a major role in world health and are very popular in developing and underdeveloped countries with a long tradition in the use of medicinal plants. Reports indicate that the idea, herbal drugs are very safe and free from side effect is false (Shaw *et al.*, 1997;

Kaplowitz, 1997; Calixto, 2000). Therefore, clear understanding of potential adverse effect of herbs used by human population is necessary for implementing safety measures to the public.

Since in the case of *P. amarus*, conflicting reports have been obtained regarding the efficacy of the plant grown in different countries and hence, a study on their toxicity is required. In our present work, we have studied the toxicity of *P. amarus* (growing in Malaysia) in rats .

The results of our acute study with the absence of lethality or toxic side effect on oral administration of *P.amarus* extract even at a dose of 5g/kg body wt clearly indicate the non-toxic nature of the plant extract. Toxicologists agree that any test substance that is not lethal on acute administration at a concentration of 5g/Kg body wt, is essentially non-toxic (OECD, 1981; Brock *et al.*, 1995). In the chronic toxicity study, we did biochemical analysis and histological studies to determine the cumulative toxic effect *P. amarus* extract on rat liver .

Liver is an organ involved in many metabolic functions and is prone to xenobiotic-induced injury because of their central role in xenobiotic metabolism (Sturgill and Lambert, 1997). Liver contains a host of enzymes such as ALT,AST,LDH and ALP. The activities of these enzymes are used to assess the functional status of the liver and as the biochemical markers of liver damage (Moss and Ralph Handerson, 1999). Hepatotoxic drugs

cause damage to the liver cell membrane and these enzymes are leaked out into serum and shows increased activities (Kumar et al, 2004; sturgill and Lambert, 1997). Our study results showed that there were no increased activities of ALT,AST,LDH and ALP on P.amarus extract administration indicating that the plant is non-hepatotoxic in nature. The results of our biochemical estimations were confirmed by the histological studies (light microscopy, PCNA study and apoptosis study).

Histological examination of liver specimen is useful in identifying the type of injury present in it (Friedman *et al.*, 1996). Our light microscopic study showed no observable changes between control and P.amarus administered groups.

Researchers assessed the role of cell proliferation and compensatory tissue repair in response to initial liver injury by PCNA study (Wang *et al.*, 2000). The expression of PCNA by immunohistochemistry is a reliable indicator of both the proliferation rate and location of the compartment (Yamada *et al.*, 1992). We also found no difference between control and P.amarus administered groups in the PCNA study.

In assessing cell proliferation, a strong correlation was found between the PCNA study and ³H-Thymidine incorporation study. PCNA expression identifies more cells than just those in S-Phase (Yamada *et al.*, 1992). Since there were no difference between control and P.amarus

administered groups in the PCNA study, the necessity to carryout the ³H-Thymidine incorporation study did not occur.

Experimentally induced T-cell mediated hepatic injury is assessed by apoptosis (Tiegs *et al.*, 1992). In our study, the assessment of apoptosis by using Apop Tag Kit showed similar pattern between control and *P.amarus* administered groups.

5. Conclusions

Acute oral administration of *P. amarus* extract is non-toxic to the rat liver, even at a dose of 5 gram /kg body weight. The chronic toxicity studies of *P. amarus* extracts administration (of 100-800 mg/kg body wt) showed the absence of cumulative toxicity as reflected by the non-significant change in the parameters studied as well as from the results of the histological studies. So, further studies can be carried out in this plant (grown in Malaysia) to assess for their antihepatotoxic, antidiabetic, anticancerous and antidiarrheal action.

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Table 1

Level of total protein and activities of serum marker enzymes of control and *phyllanthus amarus* administered groups of male rats

Parameters	Group I (Control)	Phyllanthus amarus extract administered groups		
		Group II	Group III	Group IV
Total Protein	75.2 ± 3.75	74.4 ± 3.06 ^{NS}	75.7 ± 2.45 ^{NS}	72.3 ± 3.37 ^{NS}
ALT	38.2 ± 3.08	36.5 ± 3.51 ^{NS}	36.4 ± 2.76 ^{NS}	36.4 ± 3.38 ^{NS}
AST	38.8 ± 3.17	38.4 ± 2.81 ^{NS}	38.2 ± 2.67 ^{NS}	38.5 ± 3.50 ^{NS}
ALP	90.3 ± 8.58	88.3 ± 8.55 ^{NS}	87.5 ± 5.76 ^{NS}	85.7 ± 7.15 ^{NS}
LDH	173.9 ± 9.46	173.0 ± 6.55 ^{NS}	170.6 ± 4.93 ^{NS}	170.0 ± 6.88 ^{NS}

Values are mean ± S.D., n = 10 rats in each group.

Group I was the control, Group II, III and IV were the plant extract *phyllanthus amarus* administered groups at the doses of 100 mg/kg body weight/day, 400mg/Kg body weight/day and 800mg kg body weight/day for 42 days respectively.

Group II,III & IV are compared with Group I (Control) by student's t-test and p value <0.05, considered significant.

^{NS} p>0.05 , not significant.

Table 2

Level of total protein and activities of serum marker enzymes of control and *phyllanthus amarus* administered groups female rats

Parameters	Group A (Control)	Phyllanthus amarus extract administered groups		
		Group B	Group C	Group D
Total Protein	74.0 ± 6.96	73.8 ± 3.29 ^{NS}	75.0 ± 5.44 ^{NS}	75.9 ± 3.96 ^{NS}
ALT	30.8 ± 2.65	30.6 ± 2.72 ^{NS}	30.9 ± 2.51 ^{NS}	31.1 ± 2.36 ^{NS}
AST	38.2 ± 3.52	37.9 ± 3.65 ^{NS}	37.3 ± 2.60 ^{NS}	38.0 ± 3.59 ^{NS}
ALP	84.0 ± 5.42	83.8 ± 3.43 ^{NS}	84.6 ± 2.96 ^{NS}	85.1 ± 6.35 ^{NS}
LDH	146.0 ± 5.42	143.2 ± 5.85 ^{NS}	142.6 ± 5.5 ^{NS}	141.9 ± 4.89 ^{NS}

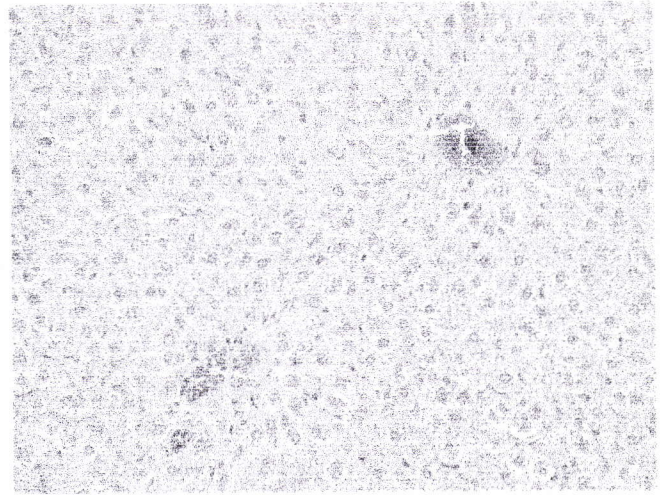
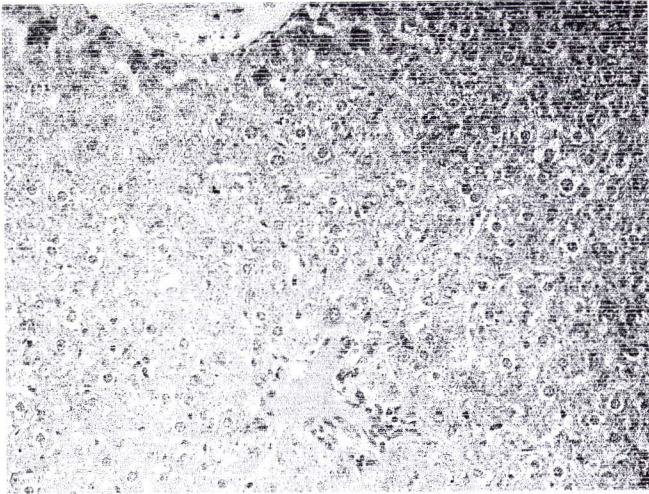
Values are mean ± S.D., n = 10 rats in each group.

Group A was the control, Group B,C and D were the plant extract *phyllanthus amarus* administered groups at the doses of 100 mg/kg body weight/day, 400mg/Kg body weight/day and 800mg/kg body weight/day for 42 days respectively.

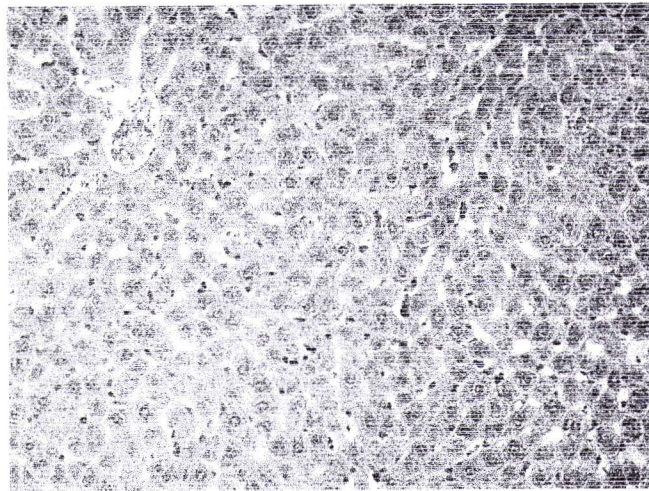
Group B,C and D are compared with Group A (Control) by student's t-test and p value <0.05, considered significant.

^{NS} p>0.05, not significant.

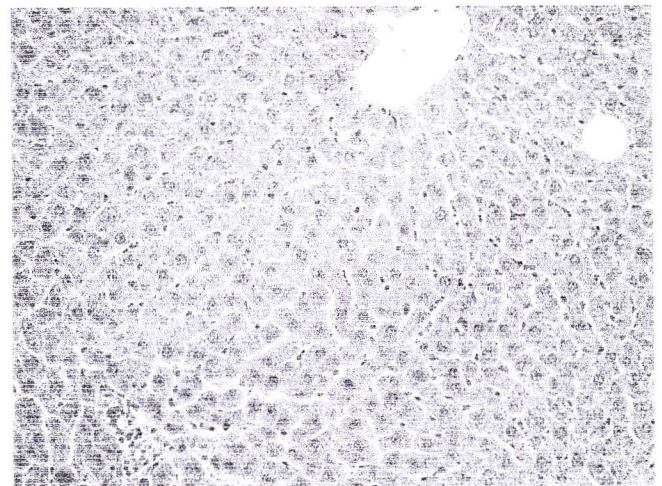
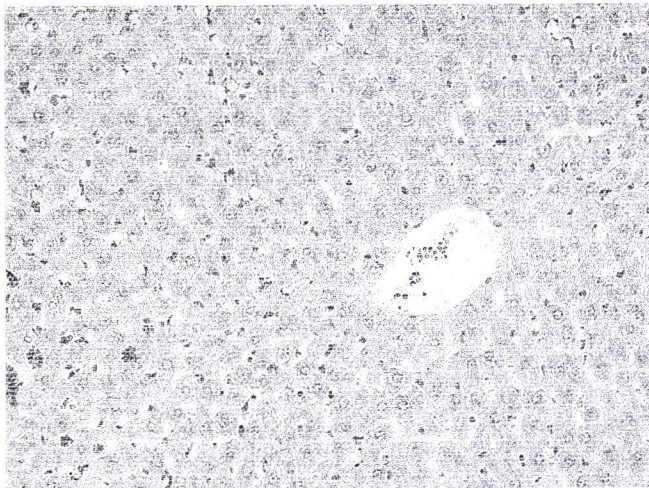
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Fig 1 & 2



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Fig. 3

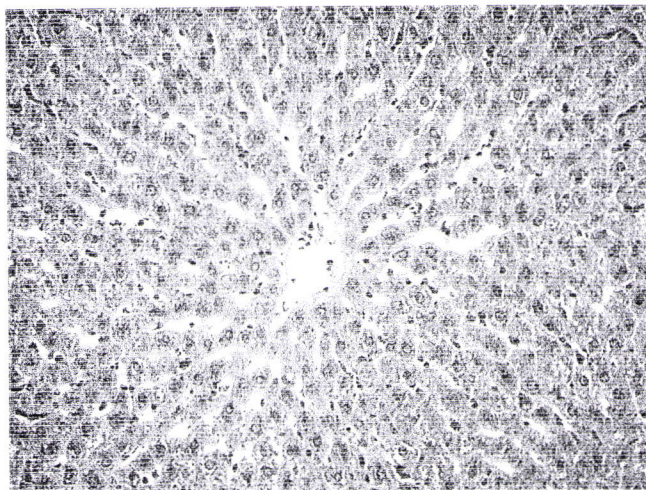
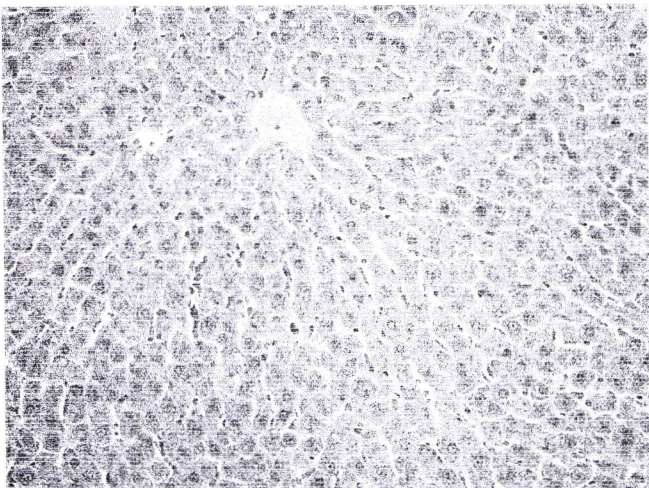


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Fig 4 & 5



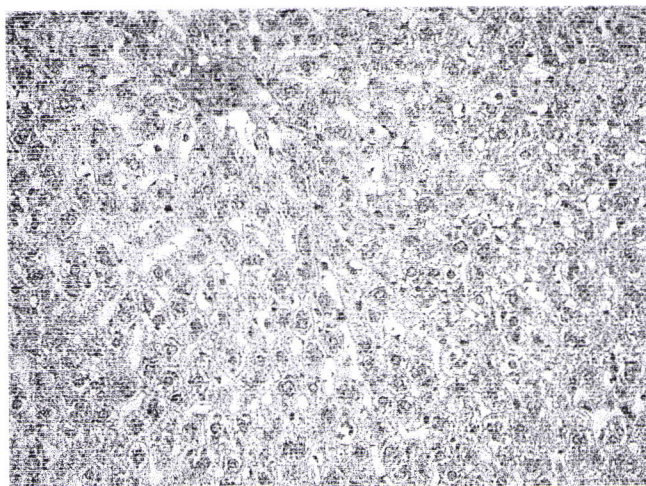
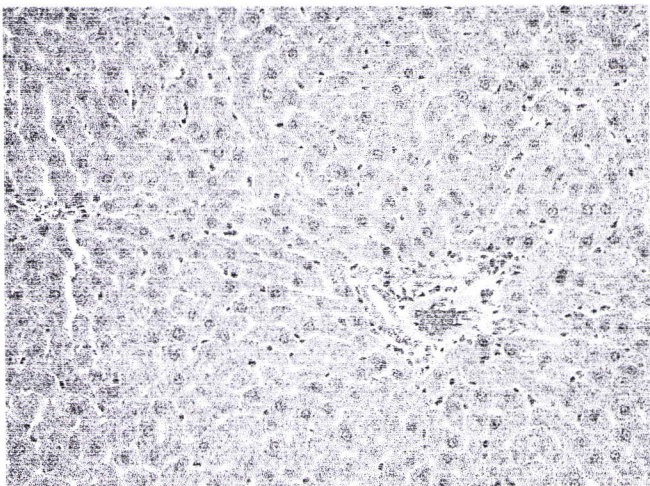
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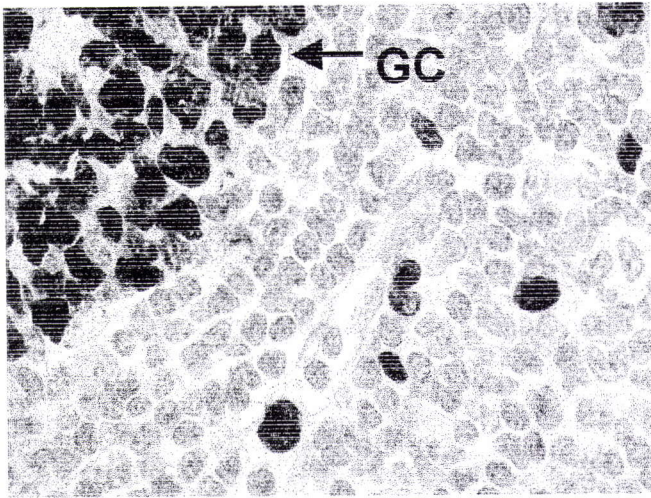
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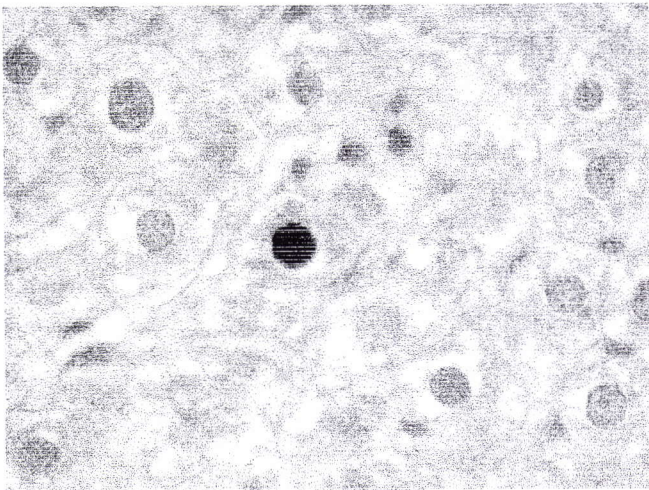
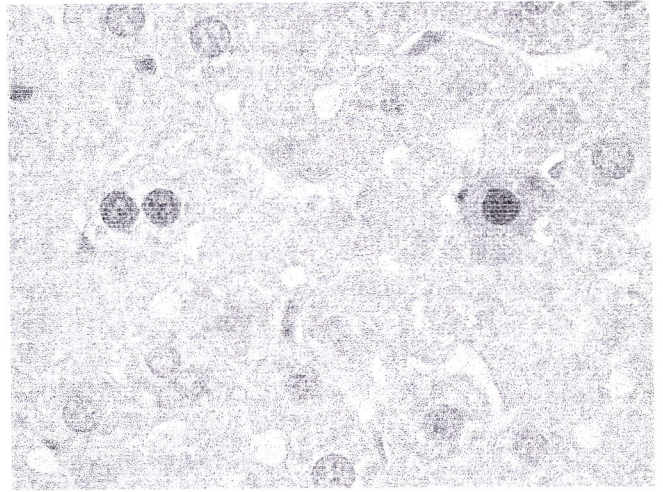
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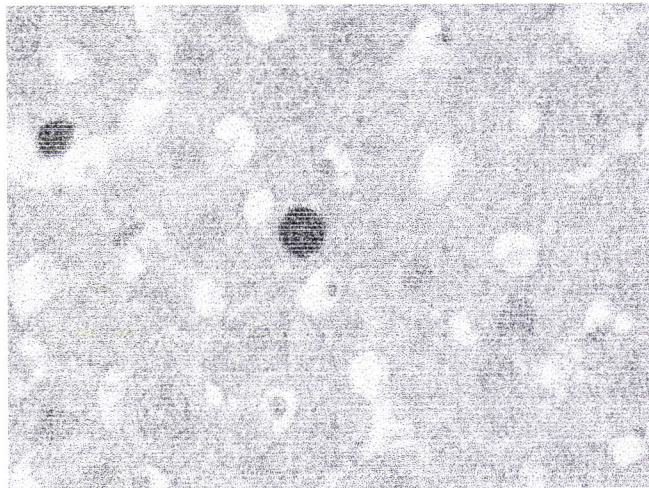
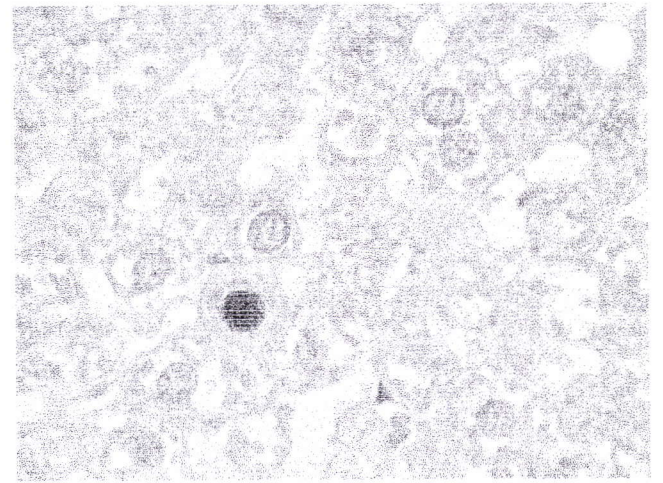




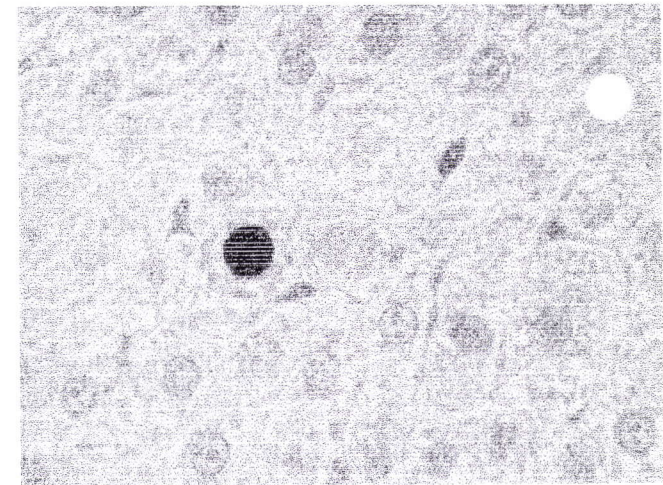
Sirajudeen
Fig 10 & 11

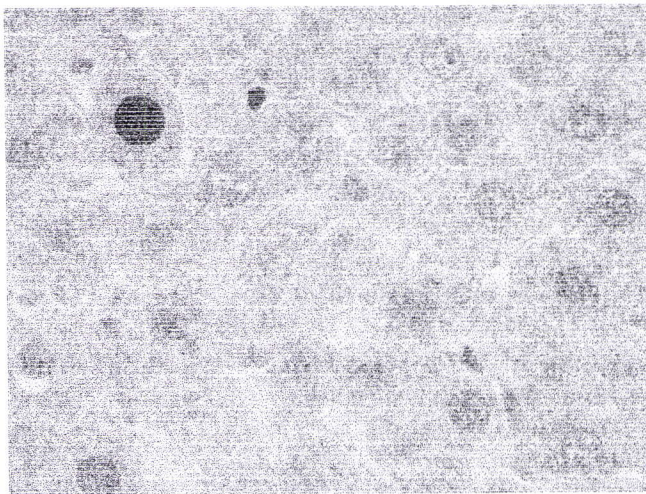


Sirajudeen
Fig 12 & 13

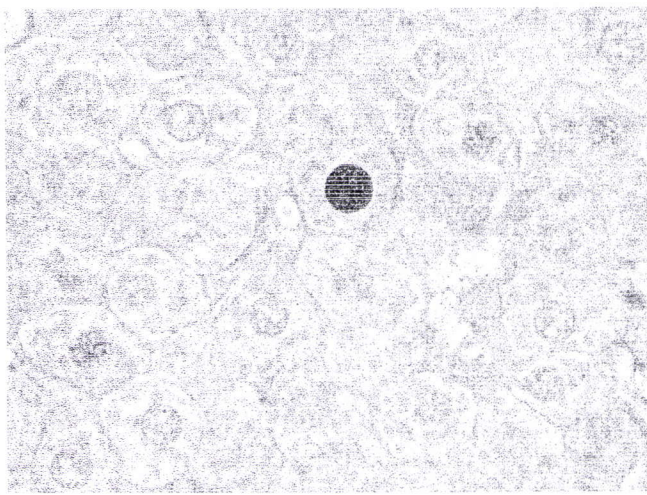
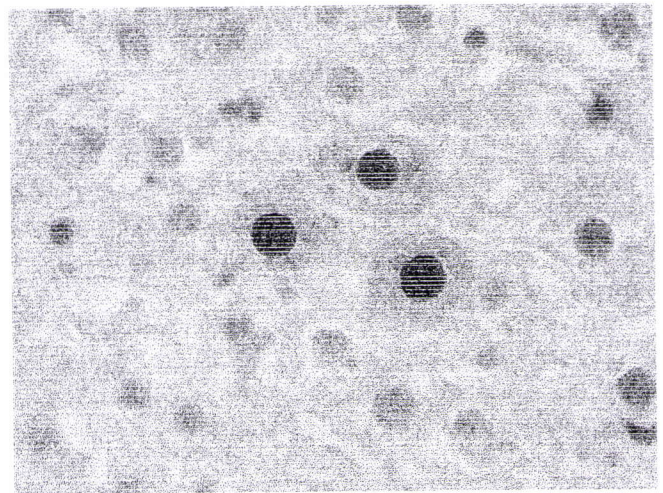


Sirajudeen
Fig 14 & 15





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18219

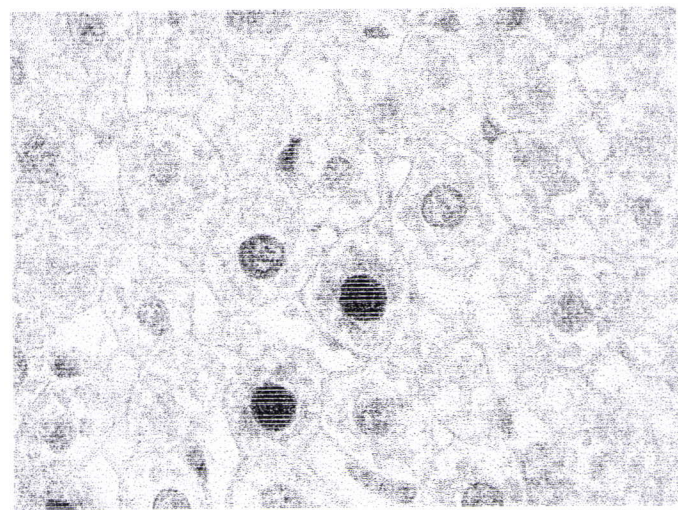


Fig 20 & 21

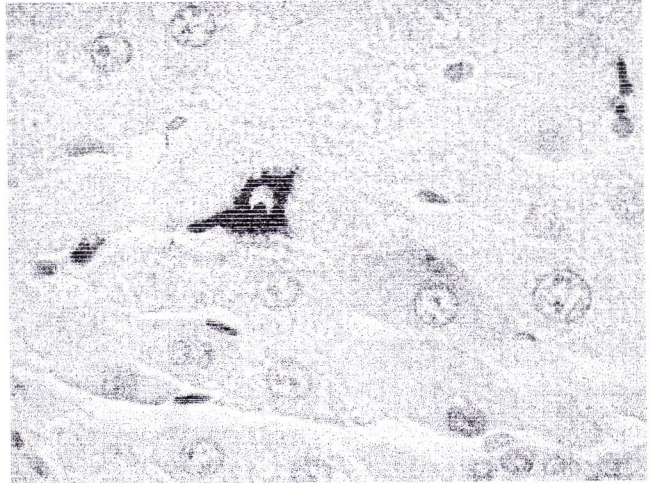
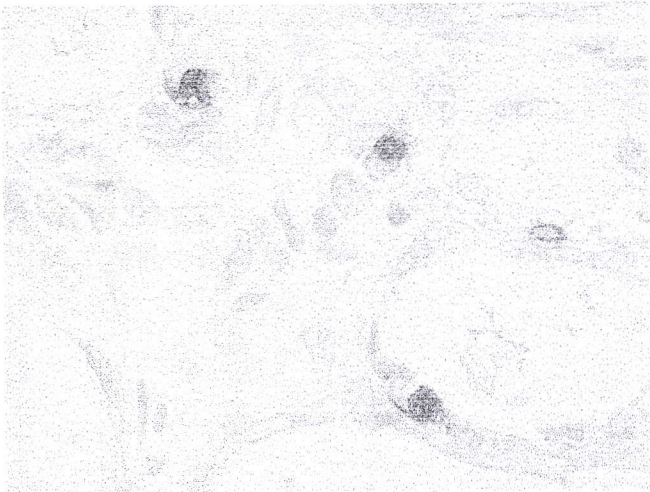


Fig 22 & 23

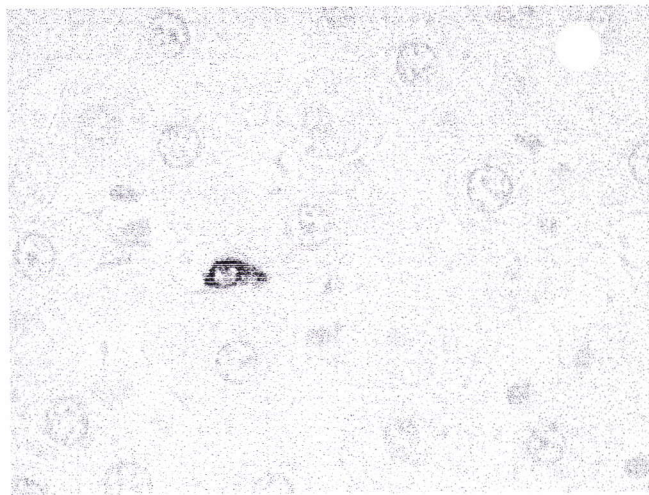
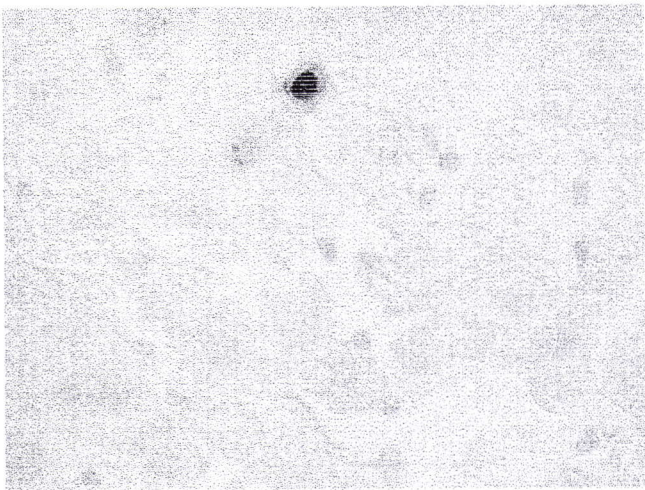


Fig 24 & 25

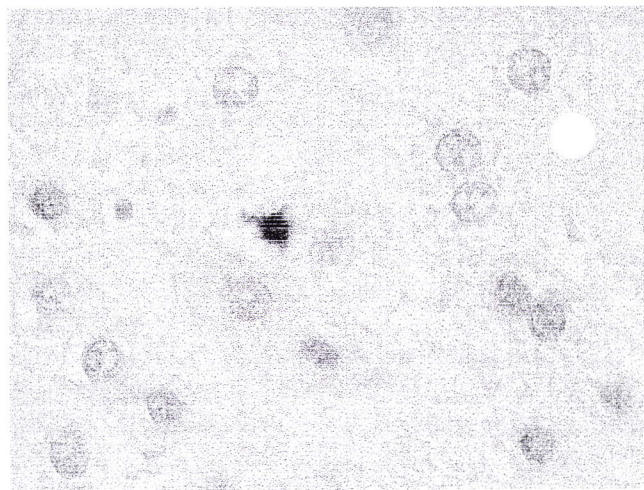
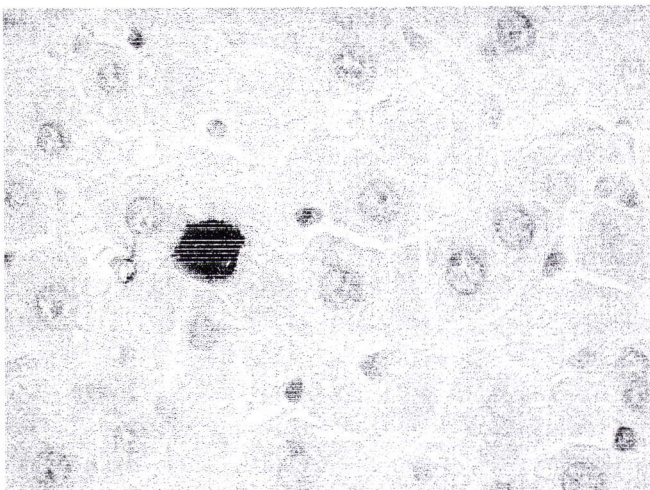


Fig 26 & 27

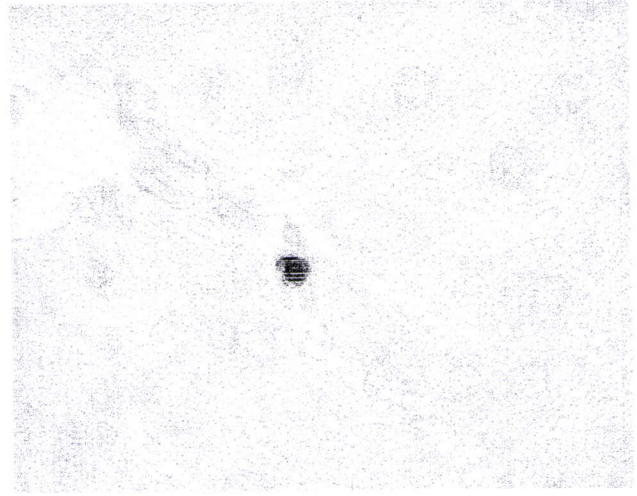
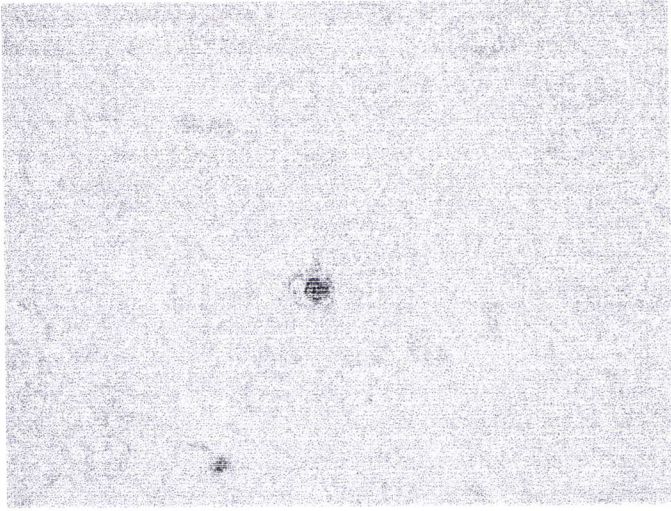
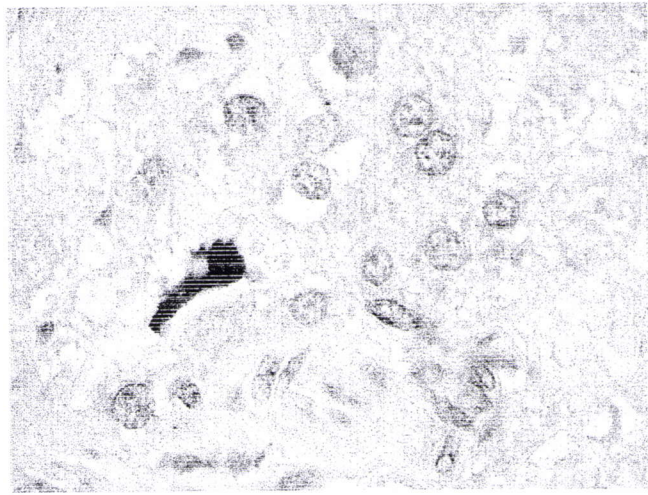
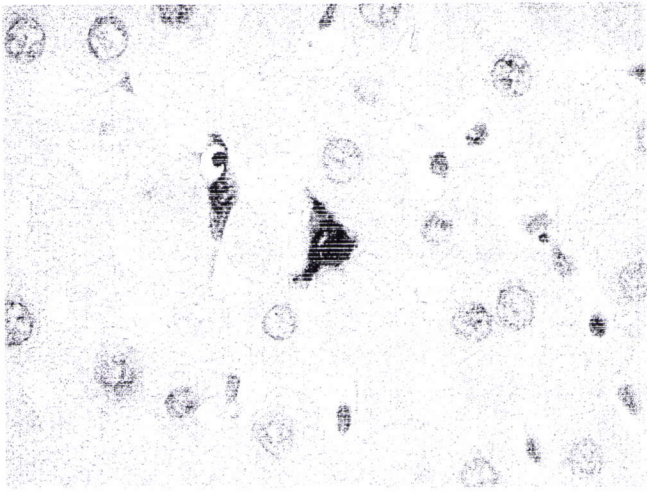


Fig 28 & 29



- Fig. 1. Normal liver from control male rat (H&E)
- Fig. 2. Normal liver from control female rat (H&E)
- Fig. 3. Liver appears normal in male rat with acute administration of *P. amarus* extract 5g/Kg body wt. (H&E)
- Fig. 4. Liver from male rat administered with *P. amarus* extract 100mg/Kg body wt/day for 6 weeks (H&E)
- Fig. 5. Liver from female rat administered with *P. amarus* extract 100mg/Kg body wt/day for 6 weeks (H&E)
- Fig. 6. Liver from male rat administered with *P. amarus* extract 400mg/Kg body wt/day for 6 weeks (H&E)
- Fig. 7. Liver from female rat administered with *P. amarus* extract 400mg/Kg body wt/day for 6 weeks (H&E)
- Fig. 8. Liver from male rat administered with *P. amarus* extract 800mg/Kg body wt/day for 6 weeks (H&E)
- Fig. 9. Liver from male rat administered with *P. amarus* extract 800mg/Kg body wt/day for 6 weeks (H&E)
- Fig. 10. Lymph node –positive control-shows increased proliferation in the generinal center (GC) (anti PCNA)
- Fig. 11. Liver from control male rat shows only occasional cells in proliferative cycle (anti PCNA)
- Fig. 12. Liver from control female rat shows only occasional cells in proliferative cycle (anti PCNA)
- Fig. 13. Liver from male rat with acute administration of *P. amarus* extract 5g/Kg body wt. Shows only occasional cells in proliferative cycle (anti PCNA)
- Fig. 14. Liver from male rat administered with *P. amarus* extract 100 mg/Kg body wt/day for 6 weeks shows only occasional cells in proliferative cycle (anti PCNA)
- Fig. 15. Liver from female rat administered with *P. amarus* extract 100 mg/Kg body wt/day for 6 weeks shows only occasional cells in proliferative cycle (anti PCNA)

- Fig. 16. Liver from male rat administered with *P. amarus* extract 400 mg/Kg body wt/day for 6 weeks shows only occasional cells in proliferative cycle (anti PCNA)
- Fig. 17. Liver from female rat administered with *P. amarus* extract 400 mg/Kg body wt/day for 6 weeks shows only occasional cells in proliferative cycle (anti PCNA)
- Fig. 18. Liver from male rat administered with *P. amarus* extract 800 mg/Kg body wt/day for 6 weeks shows only occasional cells in proliferative cycle (anti PCNA)
- Fig. 19. Liver from female rat administered with *P. amarus* extract 800 mg/Kg body wt/day for 6 weeks shows only occasional cells in proliferative cycle (anti PCNA)
- Fig. 20. Apoptotic bodies are stained brown in a positive control slide (Apop Tag kit)
- Fig. 21. Normal control male liver shows occasional apoptotic bodies (Apop Tag kit)
- Fig. 22. Normal control female liver shows occasional apoptotic bodies (Apop Tag kit)
- Fig. 23. Male rat liver administered with acute *P. amarus* extract at a dose of 5g/Kg body wt. , shows occasional apoptotic bodies (Apop Tag kit)
- Fig. 24. Liver from male rat administered with *P. amarus* extract 100 mg/Kg body wt/day for 6 weeks shows occasional apoptotic bodies (Apop Tag kit)
- Fig. 25. Liver from female rat administered with *P. amarus* extract 100 mg/Kg body wt/day for 6 weeks shows occasional apoptotic bodies (Apop Tag kit)
- Fig. 26. Liver from male rat administered with *P. amarus* extract 400 mg/Kg body wt/day for 6 weeks shows occasional apoptotic bodies (Apop Tag kit)
- Fig. 27. Liver from female rat administered with *P. amarus* extract 400 mg/Kg body wt/day for 6 weeks shows occasional apoptotic bodies (Apop Tag kit)
- Fig. 28. Liver from male rat administered with *P. amarus* extract 800 mg/Kg body wt/day for 6 weeks shows occasional apoptotic bodies (Apop Tag kit)
- Fig. 29. Liver from female rat administered with *P. amarus* extract 800 mg/Kg body wt/day for 6 weeks shows occasional apoptotic bodies (Apop Tag kit)