

Effects of African Walnut (*Plukenetia conophora*) on Serum Acid Phosphatase, Prostate Specific Antigen and Histological Studies in Male Albino Rats.

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Abstract

The effect of African walnut (*Plukenetiaconophora*) on the serum acid phosphatase and prostate specific antigen levels in male albino rats were investigated. Eighteen wistar albino rats were used for the experiment. The rats test group was fed with a diet prepared with 1% African walnut and 99% of the rat chow. The control group received only the standard feed (rat chow). The baseline group was sacrificed at the onset of the study to ascertain initial conditions. The study lasted for thirty one days after which the serum acid phosphatase and prostate specific antigen levels were estimated using spectrophotometric method. The acid phosphatase kit (Bio systems) and PSA ELISA kit (BioCheck) were used for the assay. The result showed that there was no statistically significant difference ($P>0.05$) in prostate specific antigen level of the test group (3.72 ± 1.243) compared to the baseline (4.08 ± 2.158) and control (5.13 ± 2.416). The total acid phosphatase (19.60 ± 8.148) and prostatic acid phosphatase (15.36 ± 4.836) levels of the test group showed no statistically significant ($P>0.05$) difference compared to the levels in baseline of 32.017 ± 17.406 and 21.23 ± 14.600 respectively. Similarly the test values were also statistically not significant ($P> 0.05$) in comparison to the control values of 28.32 ± 12.204 and 18.63 ± 9.674 respectively for the parameters. The value for the test group (4.33 ± 3.427) showed a statistically significant difference ($P< 0.05$) when compared to the baseline (11.95 ± 5.115) and control (9.69 ± 5.897) for non-prostatic acid phosphatase. The histopathological examination of the liver, lungs and kidney of the different groups is normal for the control contrary to the baseline and test groups which showed diverse histopathological changes. This could be related to environmental or nutritional factors for the baseline group and the presence of phytochemicals and or antinutrients contained in African walnut fed to the test group. These observed effects of *Plukenetiaconophora* have provided an insight into the positive pharmacological potentials of the plant, especially in the management of prostate cancer.

Keywords: *Plukenetiaconophora*, Prostate cancer, Phytochemicals, Acid phosphatase, Prostate Specific Antigen Histological

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I. Introduction

Optimal health is linked to food and nutrition. The use of plant parts as a source of food and medicine to treat diseases is an ancient practice. Nearly all cultures and civilizations from ancient times to the present day have used herbal medicine (Erdemeier *et al.*, 1996; Lino and Deogracious, 2006) to cure infections. Medicinal plants have played a key role in the world health care (Calixto, 2000) with about 80% of Africans depending on it (Elujoba *et al.*, 2005). Medicinal plants have demonstrated its contribution to the reduction of excessive mortality, morbidity and disability due to diseases such as HIV/AIDS, malaria, tuberculosis, sickle cell anemia, diabetes, mental disorders (Elujoba *et al.*, 2005) and microbial infections (Iwuet *et al.*, 1999).

African walnut (*Plukenetiaconophora*) formerly called *Tetracapidiumconophorium*, a tropical rambling perennial woody plant of the family Euphorbiaceae, is widely distributed and consumed by the inhabitants of the Guinea Zone of West and Central Africa (Oyenuga, 1997).

P. conophora is a climbing shrub 10-20ft long. It is known in the Southern Nigeria as Ukpa (Igbo), Western Nigeria as awusa or asala (yoruba). This plant is cultivated principally for the nuts which are cooked and consumed as snacks (Oke, 1995). Nuts (including walnuts) are commonly eaten by man, rodents, domestic and wild animals. Though they are of plant origin, there has been a wealth of fascinating information about many varieties which reveal their amazing health giving potential as major sources of fat, minerals, proteins and vitamins (Okoye and Okobi, 1984).

Prostate cancer is the most frequently diagnosed cancer and the second leading cause of cancer death among men in the United States. Almost 200,000 new cases are diagnosed annually, and more than 30,000 men die every year of this disease. Definitive risk factors for the development of prostate cancer are age (more than 96% of prostate cancer cases occur in men 55 years or older), family history, and African American descent. Possible risk factors include diet and hormonal factors. Since most risk factors for this disease cannot be modified; efforts to reduce mortality related to prostate cancer have focused on early recognition and treatment.

In view of the immense health benefits of walnut it is proper to register an inquiry on its role in the control of prostate cancer.

II. Materials And Methods

PLANT SOURCE AND IDENTIFICATION

African walnut was purchased from Nkwo Igbo market in Awka West Local Government Area, Anambra state, Nigeria. The plant *Plukenetia conophora* was identified by Mr P.O Ugwuozor, a herbarium curator of the Department of Botany, NnamdiAzikiwe University, Awka.

PREPARATION OF WALNUT FLOUR

The walnut sample was cleaned, sorted to remove dirt and immature ones. The cleaned sample was boiled for 45 minutes. The shell was removed, dried in an oven at 50°C and milled into flour using manual blender. The flour was packaged in a polyethylene bag for use.

EXPERIMENTAL ANIMALS

Eighteen male albino rats (65-70 day old) with average mean weight of 110g were obtained from Chris Animal Farm, Awka, Anambra State. They were kept in well ventilated house conditions with free access to standard feed (growers mash from Vital feeds and mills Onitsha, Nigeria) and water before the commencement of the experiment. The albino rats were divided in 3 groups, six albino rats in each group (baseline, control and the test groups). The baseline was sacrificed the day the experiment began; the control group was fed with the standard feed while the test group was given the formulated feed made with 99% of the standard feed and 1% of the sample (African walnut flour) for 31 days.

FEED PREPARATION

The feed was prepared by mixing 1% of the walnut flour with 99% of the standard feed. Distilled water was added to the mixture to help in the mixing and binding process. The mixture was pelletized and dried in an oven at 50°C.

COLLECTION OF SAMPLES

The groups used as baselines were sacrificed on the first day of the experiment when the animals were brought to the laboratory, while the control and test groups were sacrificed after 31 days. The sacrificed animals were anaesthetized with cotton wool dipped in chloroform. Using a 5ml syringe, an average of 5ml of blood sample was collected directly from the heart of the animal and transferred into centrifuge tubes. The blood sample was allowed to clot and centrifuged at 1500 rpm for 10 minutes. The serum was collected using a Pasteur pipette into a plain bottle. Also, using a surgical blade; an animal from each group was dissected ventrally to obtain the kidney, liver and the lungs. Each organ from each group was fixed in 10% formal saline.

PROSTATE SPECIFIC ANTIGEN (PSA) IMMUNOASSAY

This was carried out using the method of Ambruster, (1993). PSA ELISA kit (BioCheck) was used for the assay

PRINCIPLE: The PSA ELISA test is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes a goat anti-PSA antibody directed against PSA for solid phase immobilization (on the microtiter wells). A monoclonal anti-PSA antibody conjugated to horseradish peroxidase is in the antibody-enzyme conjugate solution. The test sample is allowed to react first with the immobilized goat antibody at room temperature for 60 minutes. The wells are washed to remove any unbound antigen. The monoclonal anti-PSA-HRP conjugate is then added and allowed to react with the immobilized antigen for 60 minutes at room temperature resulting in the PSA molecules being sandwiched between the solid phase and enzyme-linked antibodies. The wells are washed with water to remove unbound-labelled antibodies. A solution of TMB Reagent is added and incubated at room temperature for 20 minutes resulting in the development of blue colour. The colour development is stopped with the addition of stop solution, changing the colour to yellow. The concentration of PSA is directly proportional to the colour intensity of the test sample. Absorbance is measured spectrophotometrically at 450nm.

TEST PROCEDURE: The desired number of coated wells in the holder was secured. Then a quantity (50µl) of standards, baseline, test and control was transferred into the wells. Zero buffer (50µl) was dispensed into each well, mixed thoroughly for 30 seconds and incubated at warm temperature (18-25°C) for 60 minutes. The incubation mixture was removed by emptying the plate content into a waste container, and the micro-titer wells were rinsed and emptied (5 times) with distilled water. The wells were stricken sharply onto absorbed paper to remove all residue water droplets. Enzyme conjugated reagent (100µl) was then dispensed into each well which is mixed gently for 10 seconds and incubated at a warm temperature (18-25°C) for 60 minutes. Resultant incubation mixture was removed by emptying plate contents into a waste container and the micro-titer wells rinsed and emptied (5 times) with distilled water which was further stricken sharply onto absorbent paper to remove residue water droplets. TMB(3,3',5,5'-Tetramethylbenzidine) reagent (100µl) was dispensed into each well, mixed gently for 20 minutes and incubated at room temperature for 20 minutes. The reaction was stopped by the addition of stop solution (100µl) to each well and mixed gently for 30 minutes. A micro plate reader was used to obtain the optical density at 450nm within 15 minutes.

ACID PHOSPHATASE ACTIVITY ASSAY

This was carried out using the method of Knowles and Plaxton, (2013). The acid phosphatase kit (Bio systems) was used.

PRINCIPLE: Acid Phosphatase catalyzes the hydrolysis of the phosphate group from α -naphthyl phosphate in acid medium. The α -naphthol formed react with diazonium salt (Fast Red TR) originating a chromogen. The catalytic concentration is determined from the rate of chromogen formation, measured at 405nm. Pentanediol accelerates the rate of reaction by acting as phosphate acceptor. Tartate is used as a specific inhibitor of the prostatic fraction in order to obtain the value of the non-prostatic acid phosphatase. The difference between the value of total acid phosphatase and non-prostatic acid phosphatase is the measure of the prostatic acid phosphatase.

PROCEDURE FOR PREPARATION OF THE WORKING REAGENT:(Biosystems kit) The working reagent was prepared by adding 10ml of Reagent AT(containing sodium citrate 110mmol/L, 1,5-pentanediol 220mmol/L,pH 5.2 for Total Acid Phosphatase) or 10ml of Reagent A1 (containing sodium citrate 110mmol/L, 1,5-pentanediol 220mmol/L sodium tartrate 110mmol/L,pH 5.2 for non-prostatic acid phosphatase) to the Reagent B1(containing α -naphthylphosphate 12.5 mmol/L). The suspension was capped and dissolved by shaking. This solution was added to Reagent B2(containing Fast Red TR 1.25mmol/L). This mixture was capped and shaken until dissolved. The working reagent was ready for use (stable for 10 days at 2-8°C).

TEST PROCEDURE: Using a micro pipette, the working reagent (1.0ml) and the sample (0.1ml) were measured into a cuvette. They weremixed, the cuvette was inserted into the photometer with stop watch started, the initial absorbance was recorded after 5 minutes and at a minute interval, thereafter for 3 minutes. The differences between consecutive absorbance's were calculated and the average absorbance difference per minute ($\Delta A/\text{min}$) was measured.

The total acid phosphatase or non-prostatic phosphatase concentrations in the sample was calculated using the following general formula:

$$\frac{(\Delta A/\text{min}) \times V_t \times 10^6}{\epsilon \times I \times V_s} = U/L$$

ϵ = the molar absorbance of the azoic chromogen at 405nm = 13033

I = Light path = 1cm

V_t = the total reaction volume = 1.1

V_s = the sample volume = 0.1

[U/L] = 16.67nKat/L

III. Results

The results of the investigations are presented in the figures and plates below.

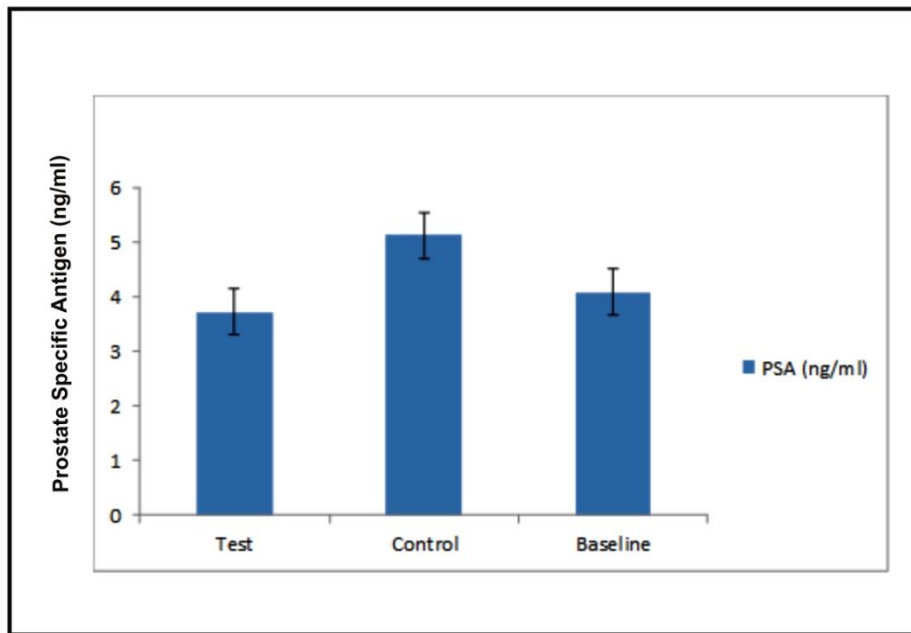


Figure 1: Shows the mean concentrations of Prostate Specific Antigen in Test, Control and Baseline groups. The test had a lower value than control which obtained a higher value than baseline

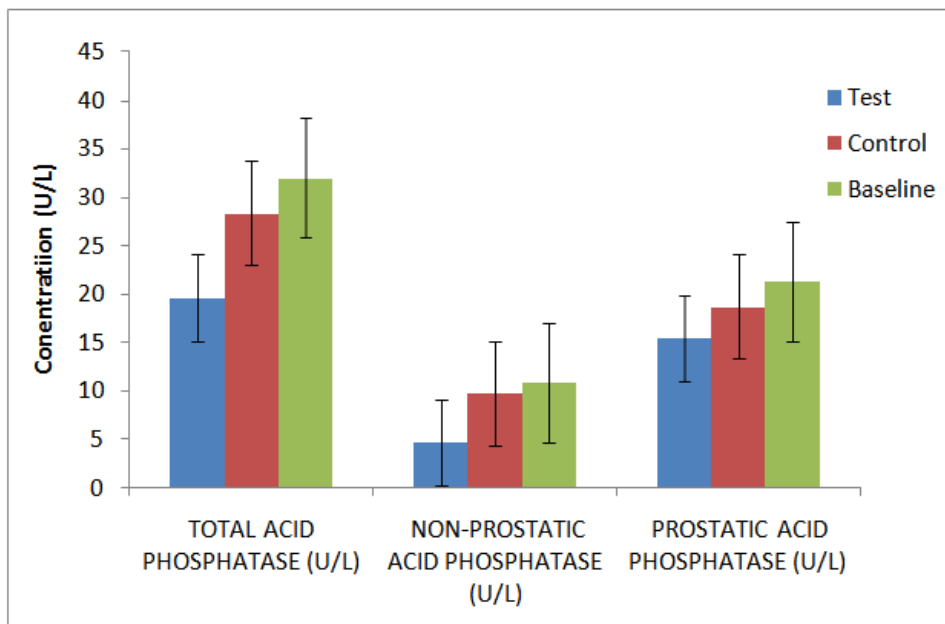


Figure 2: Shows the mean concentration of Total Acid phosphatase, Non-prostatic Acid phosphatase, and Prostatic Acid Phosphatase of the test, control and baseline groups. The test had lower values than the control while the control values were lower than the baseline

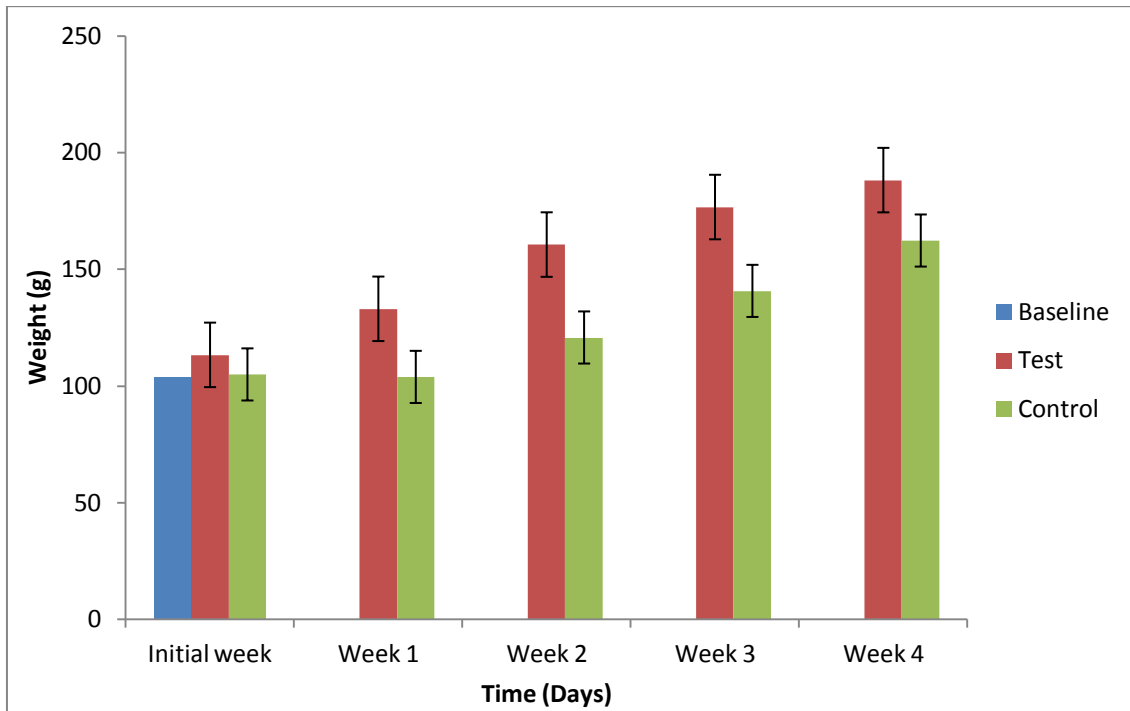


Figure 3: Shows the mean of the rat weight in the different groups (baseline, test and control). The test maintained higher weights than the control all through the weeks

HISTOPATHOLOGICAL EXAMINATION FOR DIFFERENT ORGANS

The histological studies for the control group showed normal micro-architectures for the lungs, kidney, and liver.

THE LUNGS (CONTROL GROUP)

The lungs showed normal bronchiole (B) with patent lumen, normal patent alveoli (A) and normal pulmonary interstitium (arrow). H&EX40

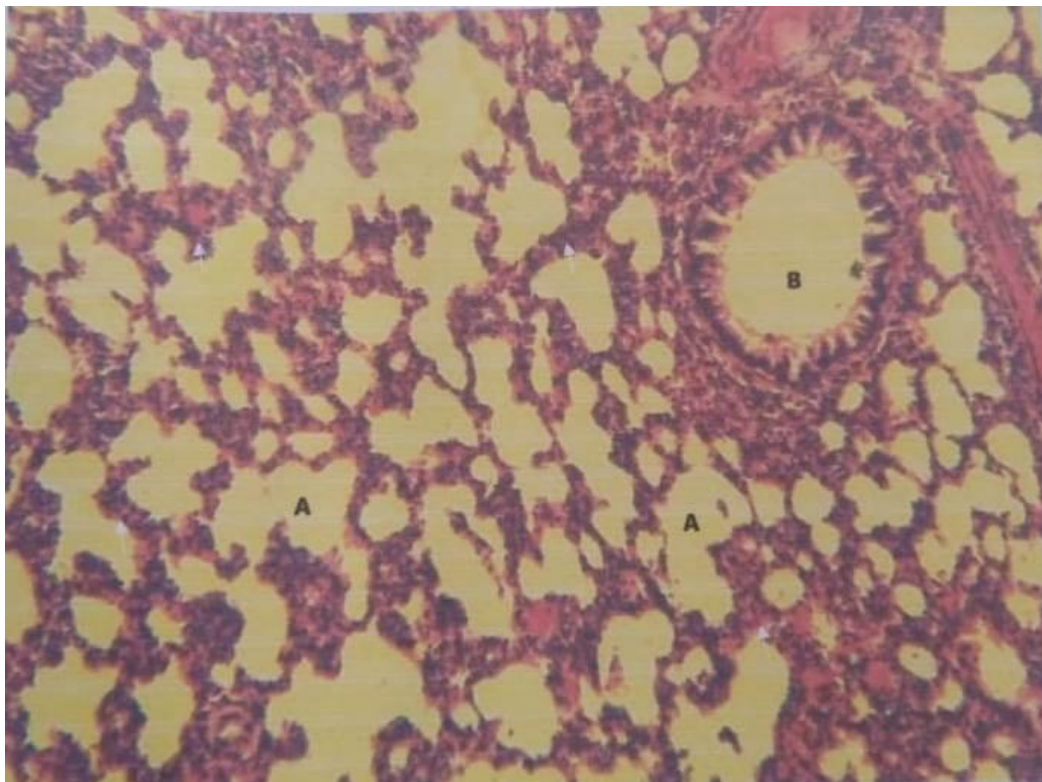


Plate 1: Lung (Control).

Section from the lungs showed a severe, widespread, haemorrhage. The alveoli are filled with red blood cells (arrow). Bronchiole (B).H&EX100, 400.

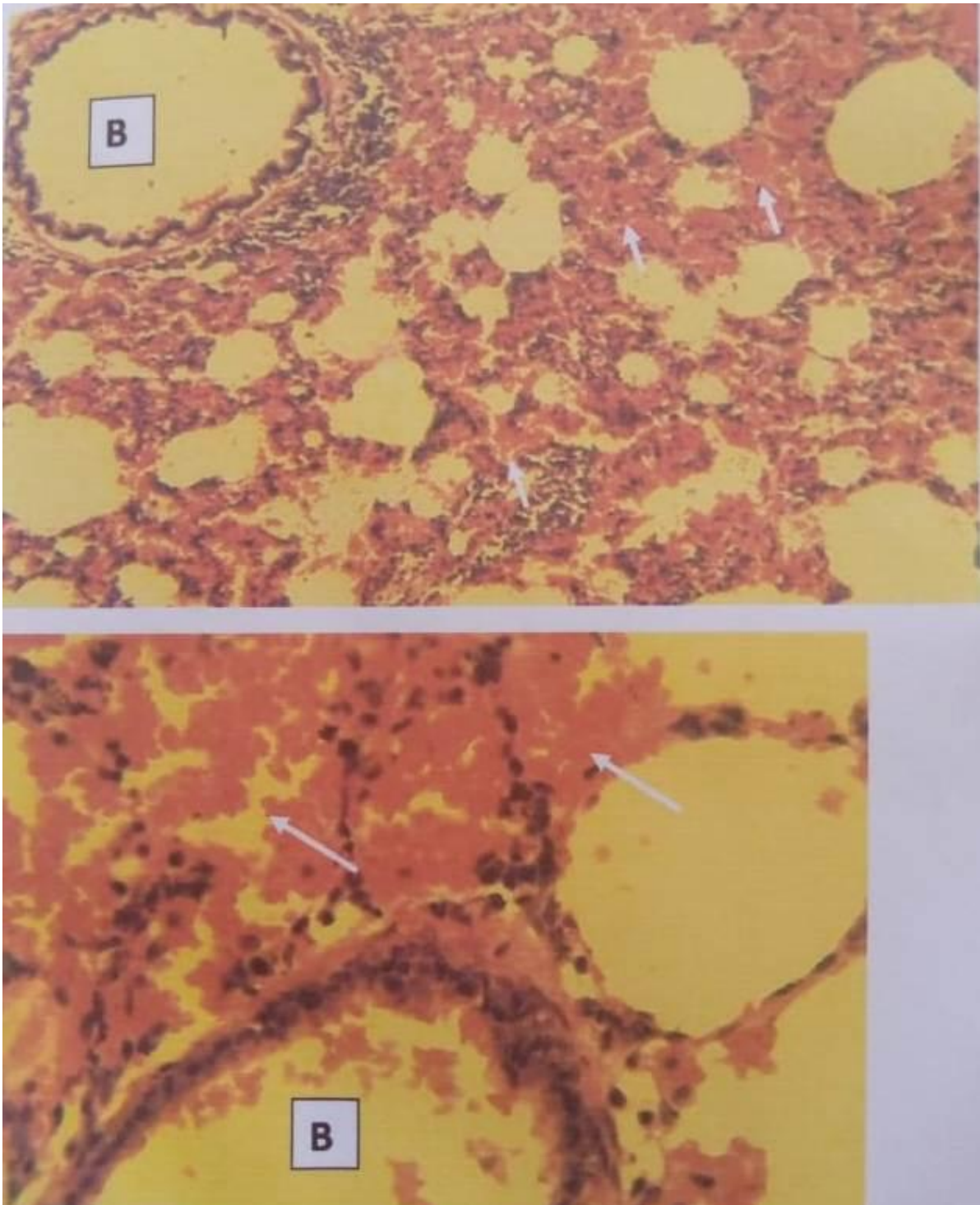


Plate 2: Lung (Test)

Section of the lung showed a pyogranuloma (P) and perivascular accumulation of lymphocytes. Bronchioles (B), Alveolus (A). H&EX400

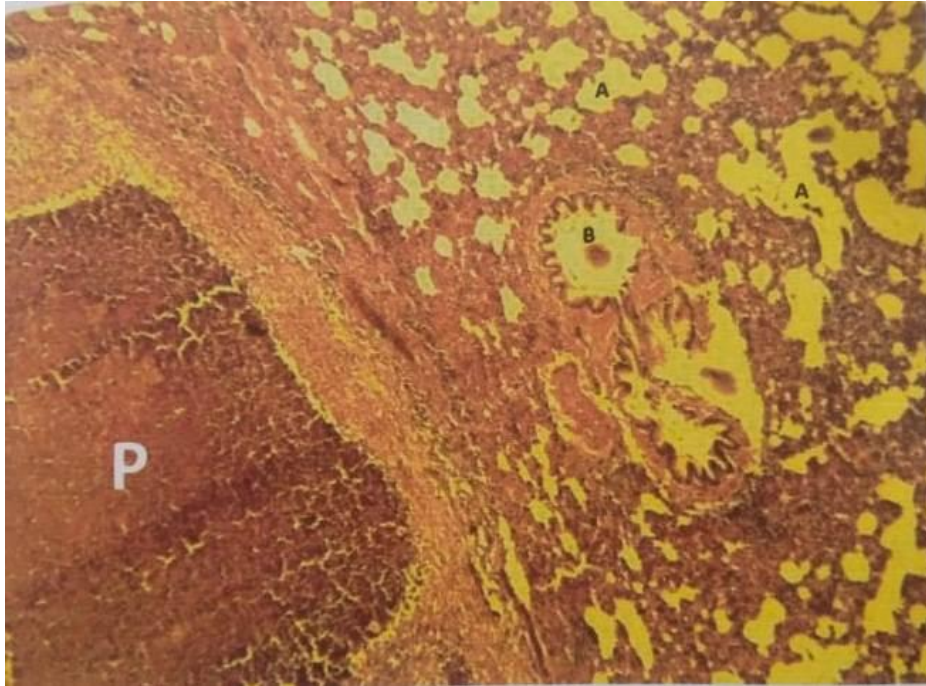


Plate 3: Lung (Baseline).

Section of the kidney showed normal glomeruli (G) in Bowman's capsule (white arrow), normal renal tubules (arrow) and normal renal interstitium. H&Ex100, 400 and 400.

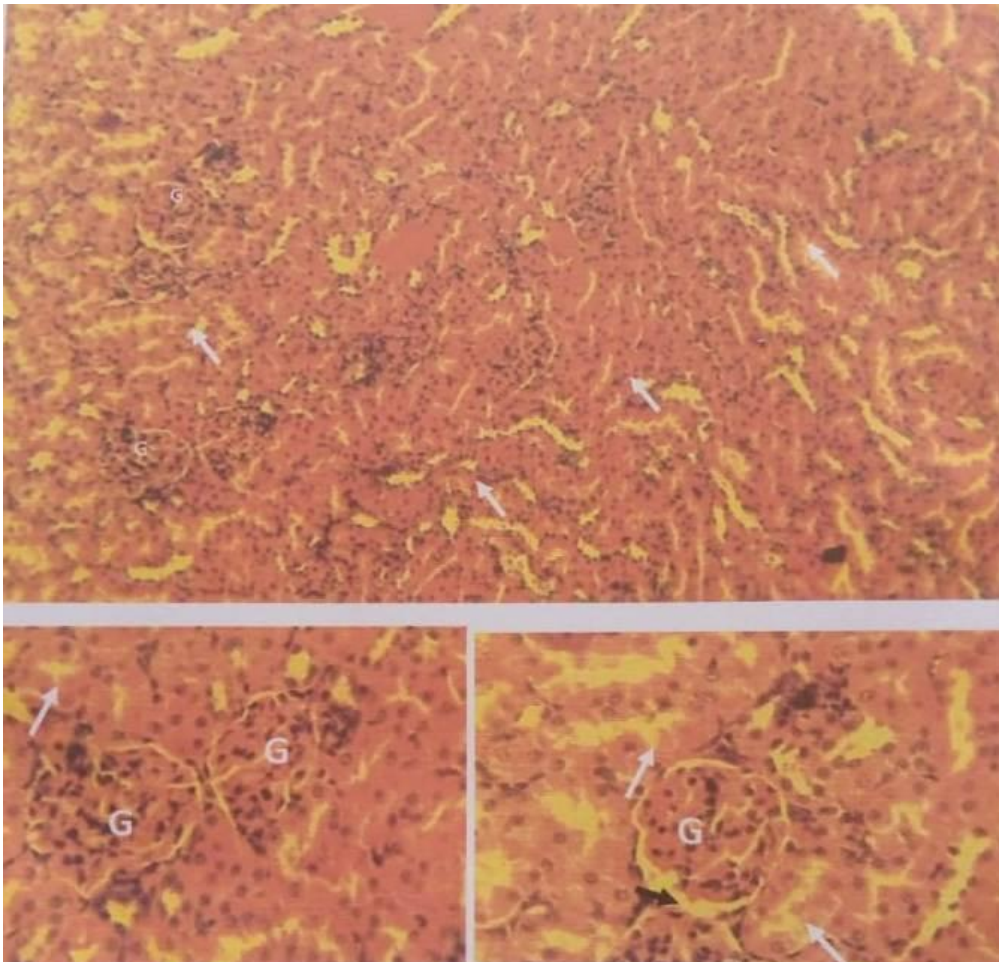


Plate 4: Kidney (Control).

Sections from the kidney showed normal renal micro-architecture. However, some of the renal tubules contained eosinophilic tubular casts (arrow), suggestive of an increased glomerular filtration rate. Glomerulus (G).H&Ex100, 100, 400.

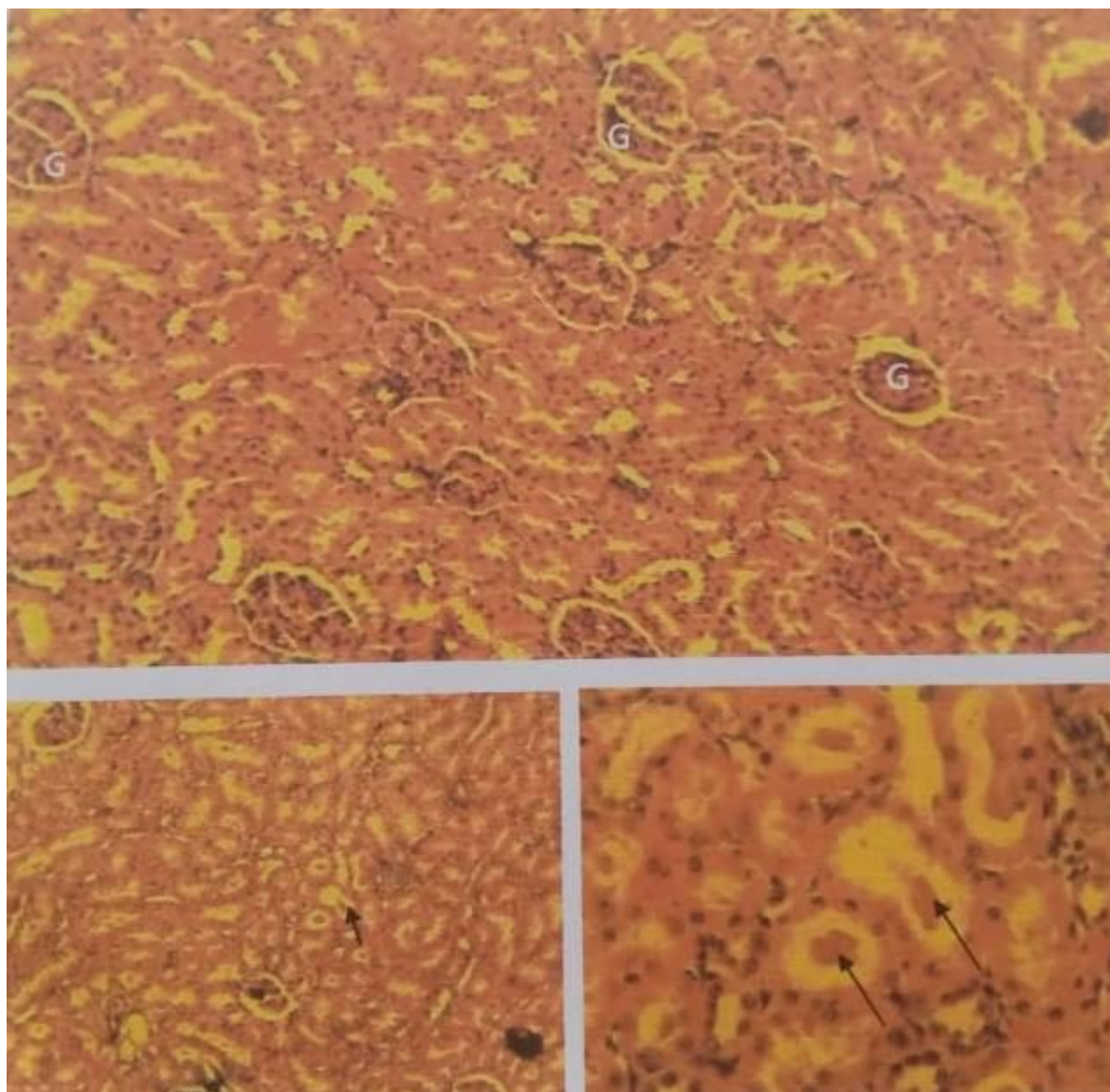


Plate 5: Kidney (Test)

Section of the kidney showed multiple foci of tubular degeneration with mild interstitial infiltration of mononuclear leucocytes (arrow) involving the cortex and the outer medulla. Eosinophilic tubular casts (blue arrow) were also present in some of the renal tubules. H&Ex100, 400.

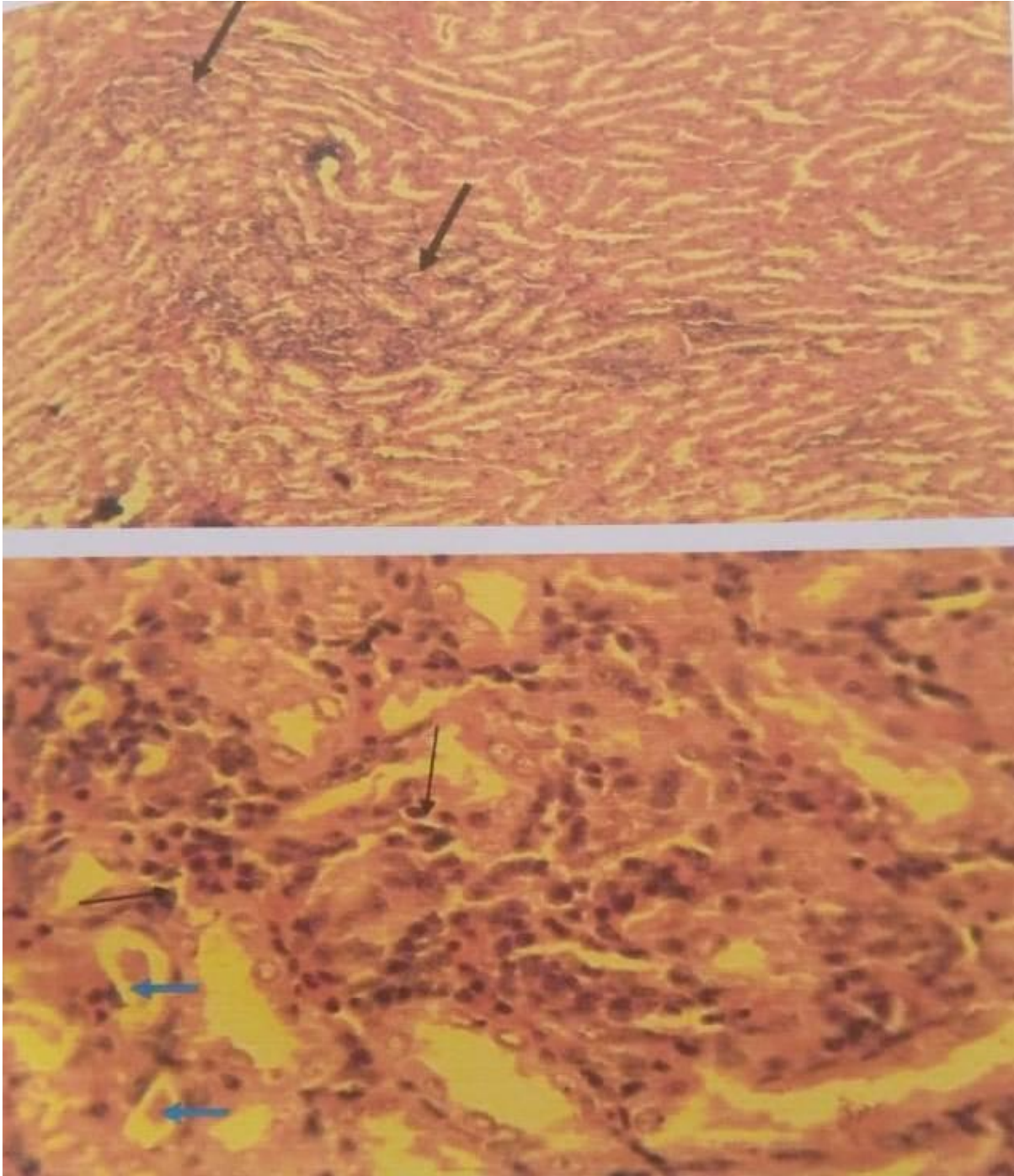


Plate 6: Kidney (Baseline).

Sections from the liver showed normal hepatocytes (arrow) arranged in chords around a central vein (V), radiating towards the portal areas (P). H&Ex100.

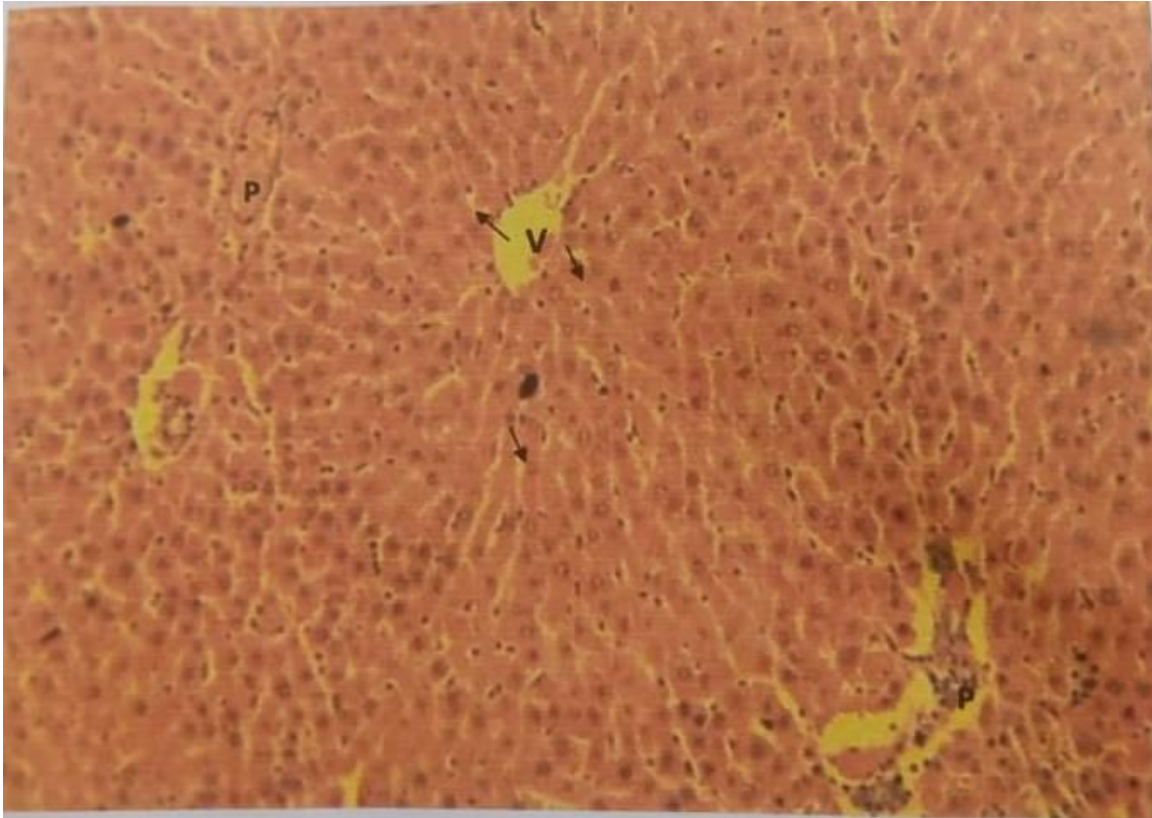


Plate 7: Liver (Control)

The liver showed a moderate periportal mononuclear leucocyte infiltration (arrow) and occasional, random mononuclear cellular infiltration. Central vein (X), Bile duct (B), hepatic vein (V).H&Ex100, 400.

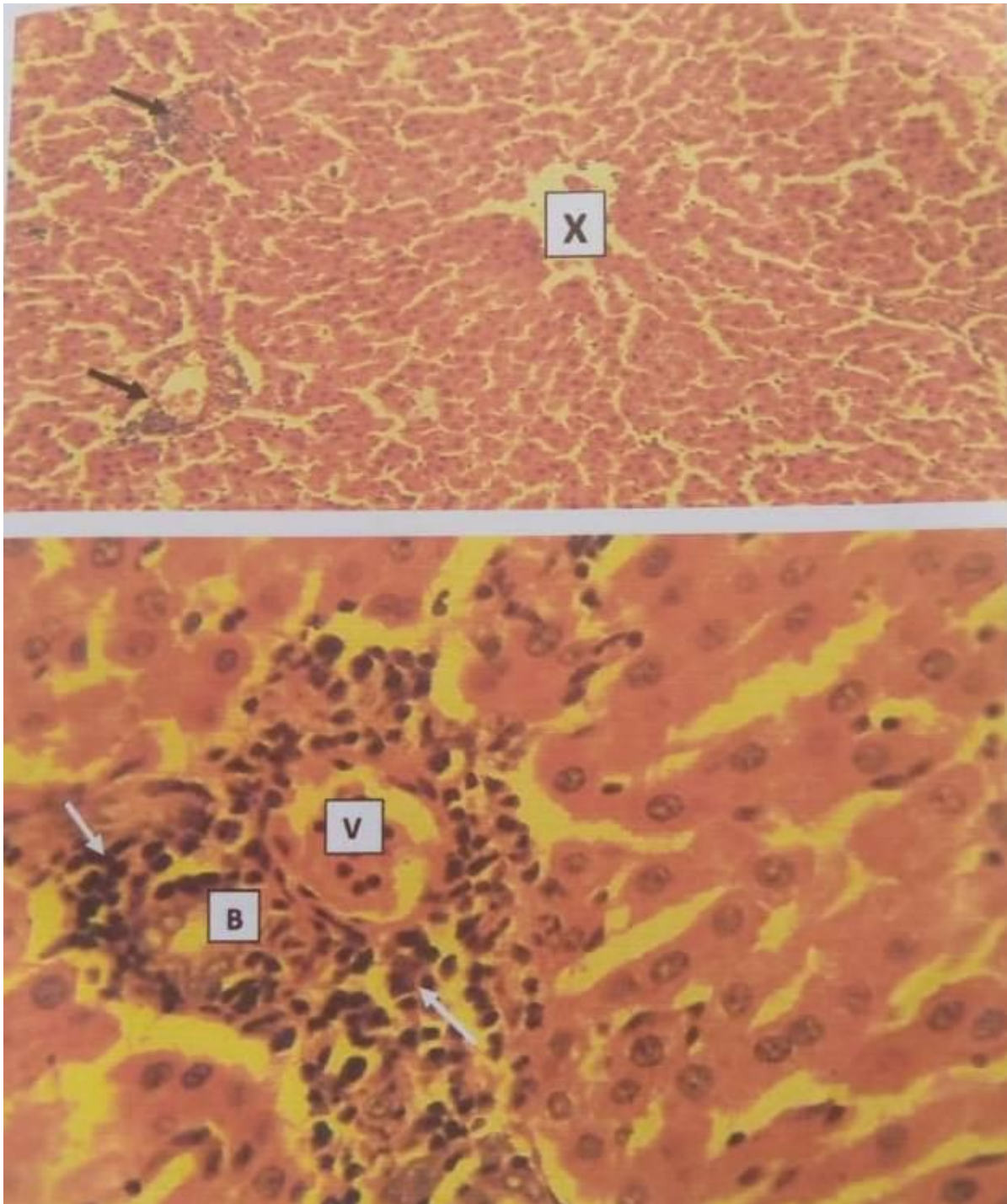


Plate 8: Liver (Test)

Section from the liver showing severe periportal accumulation of mononuclear leucocytes (white arrow), and random hepatocellular necrosis with infiltration of mononuclear leucocytes (black arrow). Hepatic vein (V), Bile duct (B), Central vein (X). H&E x100, 100, 400.

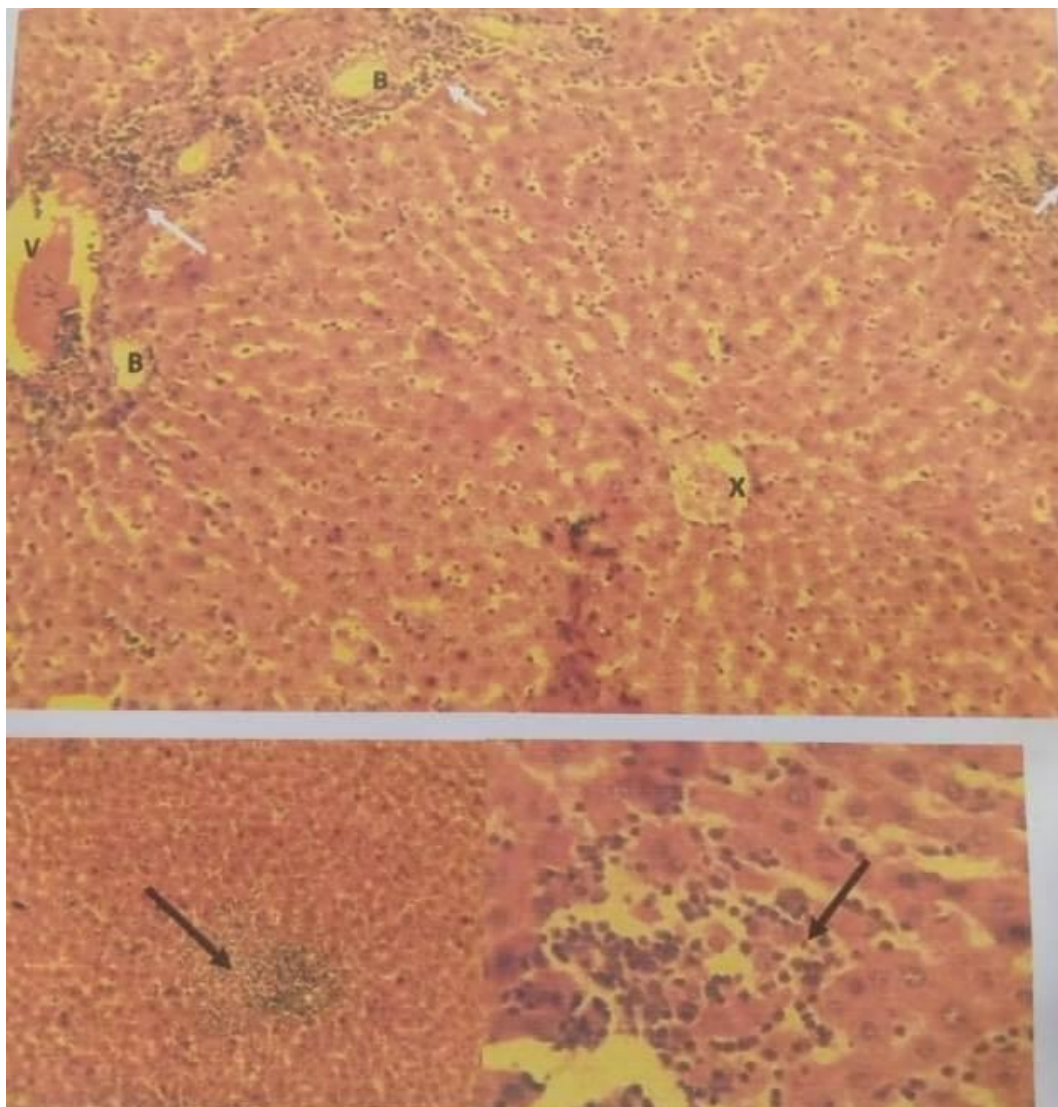


Plate 9: Liver (Baseline)

IV. Discussion

Serum PSA is a biomarker for prostatic carcinoma and it is widely used in diagnosis and clinical monitoring of patients with prostate cancer (Oesteling, 1991). A decrease in the serum prostate specific antigen level of test group ($3.72 \pm 1.243 \text{ ng/ml}$) compared to control (5.13 ± 2.416) and baseline group (4.08 ± 2.158) is a good indication that *Plukenetia conophora* may reduce prostate cancer. A study with Pomegranate (*Punicagranatum L.*) has been reported to have anticarcinogenic properties (Bhandari, 2012).

The weight of the rats in the test group, showed a 66.12% increase when compared to the control group with 54.71% increase. This could be because the test group consumed more feed than the control group. The increase in feed consumption by the test group may be due to the minerals and vitamins found in African walnut (Ihemejeet *al.*, 2012).

The total acid phosphatase, prostatic acid phosphatase and non-prostatic acid phosphatase for the test were 19.60 ± 8.148 (U/L), 15.36 ± 4.836 , and 4.33 ± 3.427 respectively. They were lower than the control values which were 28.32 ± 12.204 , 18.63 ± 9.674 and 9.69 ± 5.897 respectively. Similarly the test values were also lower than the baseline values which were 32.017 ± 17.406 , 21.23 ± 14.600 and 11.95 ± 5.115 respectively. Prostatic acid phosphatase (PAP) was first used as tumor marker in 1938 by Gutman and associates. It is synthesized by the lysosomal fraction of the epithelial prostate cells. Elevation of PAP is found in 60% of men with prostate carcinoma (Sridevi, 2013). Therefore, its decrease in level as shown by this study has given us an insight that *Plukenetia conophora* may be of great importance in the control and management of prostate cancer. Cabbage extract has been reported to cause a decrease in prostatic acid phosphatase activity (Onwuka *et al.*, 2010).

The histopathological studies showed normal micro-architectures for the lungs, kidney, and liver for the control. The baseline showed diverse histopathological changes. In the Kidney of the baseline group, there were

multiple foci of tubular degeneration with mild interstitial infiltration of mononuclear leucocytes involving the cortex and the outer medulla. Eosinophilic tubular casts were also present in some of the renal tubules. Section of the lung (baseline) showed a pyogranuloma and perivascular accumulation of lymphocytes. Also the liver (baseline) showed severe periportal accumulation of mononuclear leucocytes and random hepatocellular necrosis with infiltration of mononuclear leucocytes. These histopathological changes may be due to environmental and nutritional factors.

The test group also expressed different histopathological changes. In the kidney, there were normal renal micro-architecture. However, some of the renal tubules contained eosinophilic tubular casts, suggestive of an increased glomerular filtration rate. Section from the lung (test) showed a severe, widespread, haemorrhage. The alveoli were filled with red blood cells. The liver (test) showed a moderate periportal mononuclear leucocyte infiltration and occasional, random mononuclear cellular infiltration. These changes may be due to the presence of phytochemicals and or antinutrients in African walnut.

V. Conclusion

The findings from this study show that *P. conophora* reduces serum acid phosphatase and prostate specific antigen level and maybe recommended for the prevention and management of prostate cancer. Also, reduction in serum acid phosphatase shows that African walnut may be used for the prevention or treatment of diseases associated with elevation in Acid phosphatase. Further studies on the processing of African walnut are required which will retain its positive effect on PSA and ACP and simultaneously reduce the toxicity which may be associated with the presence of phytochemicals and or antinutrients, thereby preventing tissue or organ damage. The histological studies show that walnut has the potential to increase the glomerular filtration rate and so could be of benefit to renal patients with compromised or reduced glomerular filtration rate. Dietary habits involving the consumption of properly cooked or processed African walnut should be encouraged because of its health and nutritional benefits.

ETHICAL APPROVAL

The animals were handled following standard ethical guidelines on animal handling and research.

COMPETING INTERESTS

Authors have declared that no competing interest exists.

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