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# Biochemical Investigation of Standardized *Wattakaka volubilis* Leaf Petroleum Ether Cold Macerated Extract Against Experimentally Induced Diabetes in the Rat

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## ABSTRACT

**Objective:** *Wattakaka volubilis* is widely used in Indian traditional medicine to treat pain, cough, fever, dyspepsia and diabetes. This study explores the antidiabetic potential of Petroleum Ether Cold Maceration Extract (PEME) of *Wattakaka volubilis* in alloxan induced diabetes in rats. **Materials and Methods:** The Petroleum Ether Cold Macerated Extract (PEME) of *W. volubilis* was evaluated to quantify lupeol by HPTLC method. Male Wistar rats were divided into five groups (with six rats each) and fed at libitum: the control (0.9% saline), alloxan treated rats with or without supplementary PEME of *W. volubilis* and metformin (50,100 and 250 mg $kg^{-1}$ b.wt.) for three weeks. The blood-glucose, α-amylase, Alanine Transaminase (ALT), Alkaline Phosphatase (ALP) and bilirubin levels were measured on 7, 14 and 21 day of PEME treatment on alloxan treated rats. **Results:** Histopathological changes in the pancreas, kidney and liver were examined with hematoxylin-eosin staining. The PEME had 382.82 µg of lupeol, treatment of PEME in experimental rats by oral injections for 21 days showed reductions in the levels of serum biochemical markers. Histopathology results showed that PEME administration suppressed the abnormal cellular degenerations in alloxan treated rats. **Conclusion:** These results suggest that PEME has a protective effect over alloxan-induced diabetes.

**Key words:** *Wattakaka volubilis*, PEME, HPTLC, lupeol, alloxan, liver

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## INTRODUCTION

Diabetic mellitus is characterized by multiple metabolic disorders of carbohydrate metabolism resulted in chronic hyperglycemia with the insulin deficiency and resistance in the pancreatic β-cells[1]. It is renowned as one of the leading causes of morbidity and mortality, considered as one of the five leading causes of death in the world. Asia and Africa, where the rate could raise much than the present rate, it would be raised from 171 millions to 366 million in 2020-2030 [2,3,4]. Diabetes has been shown to be a lack of biologically active insulin or increased free radical production that elicits oxidative stress as a consequence of an imbalanced free radical generation and scavenging systems[3]. Current pharmacotherapies are insufficiently reverse hyperglycemia with some serious adverse effects and limited tolerability [5]. Since earliest times, the medicinal plants have been investigated for ethnomedicinal developments. The available literature shows that there are more than 400 plant species showing hypoglycaemic activity[4]. In recent years, numerous Indian traditional medicinal plants were tested and several other Indian medicinal plants still remain to be explored for their antidiabetic potential[6,7]. Previous literature results were highly support, that the *W. volubilis* is a medicinal member of the family Asclepiadaceae well known for their ethnobotanical importance and widely used in Indian traditional medicines. It is abundantly available throughout the hotter parts of India[8]. The plant has been traditionally used as medicines. The leaf paste and powder of the plant are taken orally along with cow's milk for diabetic complications[9]. However, no studies have specifically addressed the efficacy of PEME in alloxan induced diabetes in rats. The present study was undertaken to determine the effect of PEME on alloxan induced diabetes in rats.

## MATERIALS AND METHODS

**Plant material extraction and HPTLC analysis:** Around 2 kg of leaves of *W. volubilis* were powdered and passed through 40 mesh sizes to obtain coarse powdered which is best suited for extraction. Exhaustive extraction was performed with a cold maceration method. The extract was concentrated under vacuum using a rotary vacuum evaporator. The entire extract was taken for the

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HPTLC quantitative analysis for the determination of lupeol and *in vivo* pharmacological study. HPTLC analysis of PEME was performed by the method of Akowuah[10]. Toluene: methanol (9:1) was used as mobile phase. After development, the layers were dried and the components were visualized by UV light at 525 nm.

**Induction of diabetes and treatment schedule:** Wistar rats were fasted for sixteen hours for induction of diabetes by administering 150 mg $kg^{-1}$ b.wt. of alloxan in normal saline, injected through intraperitoneal route. Hyperglycemia was confirmed after 48 h, later fasting serum glucose levels >250 mg $dL^{-1}$ were used for antidiabetic study by GOD-POD method estimation[11]. The rats were divided among five groups comprising six rats: Group 1: Normal control (saline treated), Group 2: Diabetic untreated rats, Group 3 and 4: Diabetic rats treated orally with PEME (50 and 100 mg $kg^{-1}$ b.wt.), Group 5: Metformin (250 mg $kg^{-1}$ b.wt., orally) all for 21 days[12]. Experiments were conducted in accordance with the guidelines of CPCSEA, New Delhi, India (Registration No: 0367/01/C/CPCSEA) and the study permit with the institutional ethical committee of the Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India.

**Biochemical estimation:** Blood samples were collected for biochemical analysis on 0, 7, 14 and 21 day of normal, diabetic and PEME treated rats for colorimetric estimation using commercial diagnostic kits (Span diagnostic's Ltd, Surat, India) by a biochemistry auto analyzer (Star 21 plus, Rapid Diagnostic Pvt. Ltd., New Delhi) and UV spectroscopy (Elico-SL 159).

**Histopathological studies:** Animals were sacrificed after 21 days and the pancreas, kidney and liver tissues were preserved in formalin (10%) for further processed and fixed in paraffin wax, 5 µm thin sections were stained with hematoxylin and eosin dyes on glass slides for histopathological evaluations.

**Statistical analysis:** The results are expressed as Mean±Standard Deviation (SD), with each group containing six rats. Graph pad prism version 5.04 was used for ANOVA followed by Tukey's multiple comparison to assess the mean differences and significance variation.

## RESULTS

**Quantification of lupeol in PEME:** A triterpene (lupeol) has been identified in this TLC condition, after spraying with anisaldehyde, glacial acetic acid, sulphuric acid and ethanol which produced good separation with Rf values of PEME is 0.54 (Fig.1). The standard lupeol has the good linear relationship ($r^2$ = 0.9822 and 0.9931

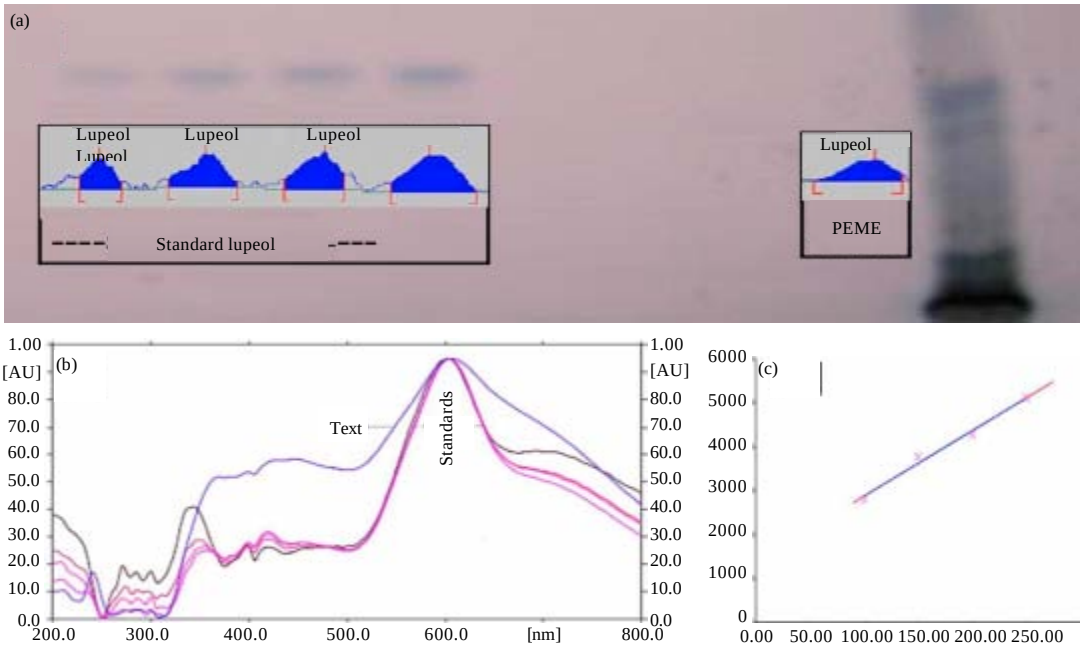


Fig. 1(a-c): HPTLC profiles: (a) TLC plate of standard lupeol and *W. volubilis* (PEME), run in toluene: methanol (9:1v/v) solvent systems and scanned at 525 nm, (b) UV-Visible spectrum of standard and test sample, (c) Linearity of the calibration curve for quantification of standard lupeol (5-25 µg $mL^{-1}$ concentration)

with respective of peak at height and area, respectively) was observed in 5-25 μg $mL^{-1}$. The concentration of lupeol in PEME was found to be 382.82 μg (Table 1).

**Serum biochemical parameters:** Alloxan treated rats caused a significant elevation in serum glucose levels after 48 h estimations in fasting animals. Administration of PEME (50,100 mg $kg^{-1}$ b.wt.) and metformin (250 mg $kg^{-1}$ b.wt.) caused significant reduction ($p<0.0001$) in serum glucose levels during 21 days treatment. Higher dosed 100 mg $kg^{-1}$ b.wt. of PEME showed the better effect than the PEME 50 mg $kg^{-1}$ b.wt. treated group. Standard metformin showed improved anti-hyperglycemic activity than the PEME treated rats (Table 2). Determined activities of the serum α-amylase when assayed it was found that the enhanced serum α-amylase levels in alloxan treated rats relative to non diabetic rats. Table 3 obviously showed that the activity of α-amylase in PEME group and metformin group was decreased compared to the diabetic groups. From the data represented at the Table 3, an inhibition in α-amylase activity was noticed in metformin more than that in PEME group (100 mg $kg^{-1}$ b.wt.) and became around of normal levels during 21 day administration. The results of the biochemical parameters revealed the elevation of serum marker enzyme in alloxan treated groups, indicating the significant increases in ALT levels. Table 4 showed the PEME treated groups were successfully caused lowered ALT levels as (4.12, 10.23, 13.47 U $L^{-1}$ in 50 mg $kg^{-1}$ b.wt., 10.79, 17.73 and 23.46 U $L^{-1}$ in 100 mg $kg^{-1}$ b.wt.) and metformin as (8.98, 13.47 and 16.13 U $L^{-1}$) showed significant ($p<0.0001$) decreased in ALT level near to the pre-diabetic levels. There was an increase in serum ALP level after the onset of diabetes. The PEME successfully lowered ($p<0.0001$) the ALP levels near to the pre-diabetic levels (Table 5). As it is apparent to the current results, the diabetic rats manifested elevated bilirubin levels during the study period. However, the doses of PEME caused a significant decreased as the serum level of bilirubin in diabetic rats. Table 6, demonstrates the levels of bilirubin were found to be reduced significantly on 14 and 21 days after PEME treatment in diabetic rats.

**Histopathological evaluation:** Pathologically, pancreas histological structure was normal in the healthy control group (Fig. 2). Islets of langerhans from the pancreas of

Table 1: HPTLC analysis of PEME on system precision, linear regression equation and $R_f$ values of the developed method

| Samples | $R_f$ | Regression equation | r* | SD | Area calibration results of standard and test (Area) | Amount of standard |
|---|---|---|---|---|---|---|
| Lupeol | 0.54 | Height: Y = 31.89+0.394 | 0.9822 | 5.91 | 2786.8 | 100.00 μg |
| | | | | | 3766.7 | 150.00 μg |
| | | Area: Y = 1372+14.91 | 0.9931 | 3.50 | 4265.1 | 200.00 μg |
| | | | | | 5105.9 | 250.00 μg |
| *W. volubilis* (PEME) | 0.54 | | | | 7818.63 | 382.82 μg |

r*: Correlation coefficient, SD: Standard deviation

Table 2: Influence of PEME of *W.volubilis* on fasting blood glucose level in hyperglycemic and non-hyperglycemic rats

| Group | Normal | Diabetic | Day 7 | Day 14 | Day 21 |
|---|---|---|---|---|---|
| Control | 72.03±0.07 | ----- | 74.93±0.36 | 73.67±1.32 | 76.01±0.47 |
| Diabetic (alloxan) | 67.94±0.20 | $409.78\pm1.18^{a1}$ | $391.30\pm1.97^{a1}$ | $361.35\pm2.49^{a1}$ | $371.97\pm2.18^{a1}$ |
| Alloxan+PEME 50 mg $kg^{-1}$ | 78.64±0.43 | $394.10\pm1.62^{a1}$ | $369.11\pm1.63^{b1}$ | $264.00\pm2.21^{b1}$ | $236.13\pm1.53^{b1}$ |
| Alloxan+PEME 100 mg $kg^{-1}$ | 87.00±0.24 | $360.16\pm1.75^{a1}$ | $183.08\pm1.15^{b1}$ | $176.25\pm1.18^{b1}$ | $145.08\pm1.19^{b1}$ |
| Alloxan+Metformin | 83.06±0.61 | $287.90\pm1.32^{a1}$ | $165.96\pm1.29^{b1}$ | $157.99\pm1.07^{b1}$ | $134.99\pm1.38^{b1}$ |

Values are expressed as Mean±SD, (n = 6), [a]normal group versus diabetic group, [b] diabetic group versus treated groups, [1]$p<0.0001$

Table 3: Influence of PEME of *W. volubilis* on serum α-amylase level in hyperglycemic and non-hyperglycemic rats

| Group | Normal | Diabetic | Day 7 | Day 14 | Day 21 |
|---|---|---|---|---|---|
| Control | 122.94±0.35 | ----- | 124.62±0.10 | 123.35±0.39 | 121.00±0.19 |
| Diabetic (alloxan) | 136.35±0.24 | $223.62\pm0.40^{a1}$ | $234.54\pm0.16^{a1}$ | $248.16\pm0.13^{a1}$ | $259.08\pm0.34^{a1}$ |
| Alloxan+PEME 50 mg $kg^{-1}$ | 106.27±0.67 | $218.17\pm0.63^{a1}$ | $212.36\pm0.42^{bns}$ | $202.14\pm0.55^{bns}$ | $170.94\pm0.20^{b2}$ |
| Alloxan+PEME 100 mg $kg^{-1}$ | 95.43±0.20 | $213.62\pm0.25^{a1}$ | $167.27\pm0.36^{b1}$ | $148.16\pm0.23^{b1}$ | $137.27\pm0.17^{b1}$ |
| Alloxan+Metformin | 121.81±0.29 | $259.08\pm0.17^{a1}$ | $220.89\pm0.21^{b1}$ | $170.81\pm0.30^{b1}$ | $134.54\pm0.28^{b1}$ |

Values are expressed as Mean±SD, (n=6), [a]normal group versus diabetic group; [b] diabetic group versus treated groups; [1]$p<0.0001$, [2]$p<0.05$, ns: Non significant

Table 4: Influence of PEME of *W. volubilis* on serum alanine transaminase (ALT) level in hyperglycemic and non-hyperglycemic rats

| Group | Normal | Diabetic | Day 7 | Day 14 | Day 21 |
|---|---|---|---|---|---|
| Control | 24.08±0.10 | ----- | 24.57±0.30 | 24.71±0.40 | 24.28±0.15 |
| Diabetic (alloxan) | 17.55±0.12 | $48.24\pm0.20^{a1}$ | $47.75\pm0.32^{a1}$ | $45.54\pm0.25^{a1}$ | $45.13\pm0.09^{a1}$ |
| Alloxan+PEME 50 mg $kg^{-1}$ | 18.27±0.92 | $46.34\pm0.35^{a1}$ | $42.22\pm0.24^{bns}$ | $36.11\pm0.16^{b1}$ | $32.87\pm0.36^{b1}$ |
| Alloxan+PEME 100 mg $kg^{-1}$ | 16.42±0.09 | $45.07\pm0.17^{a1}$ | $34.28\pm0.06^{b1}$ | $27.34\pm0.20^{b1}$ | $21.61\pm0.34^{b1}$ |
| Alloxan+Metformin | 21.06±0.11 | $51.02\pm0.65^{a1}$ | $42.04\pm0.17^{b1}$ | $37.55\pm0.22^{b1}$ | $34.89\pm0.19^{b1}$ |

Values are expressed as Mean±SD, (n=6), [a]normal group versus diabetic group, [b] diabetic group versus treated groups, [1]$p<0.0001$, ns: Non significant

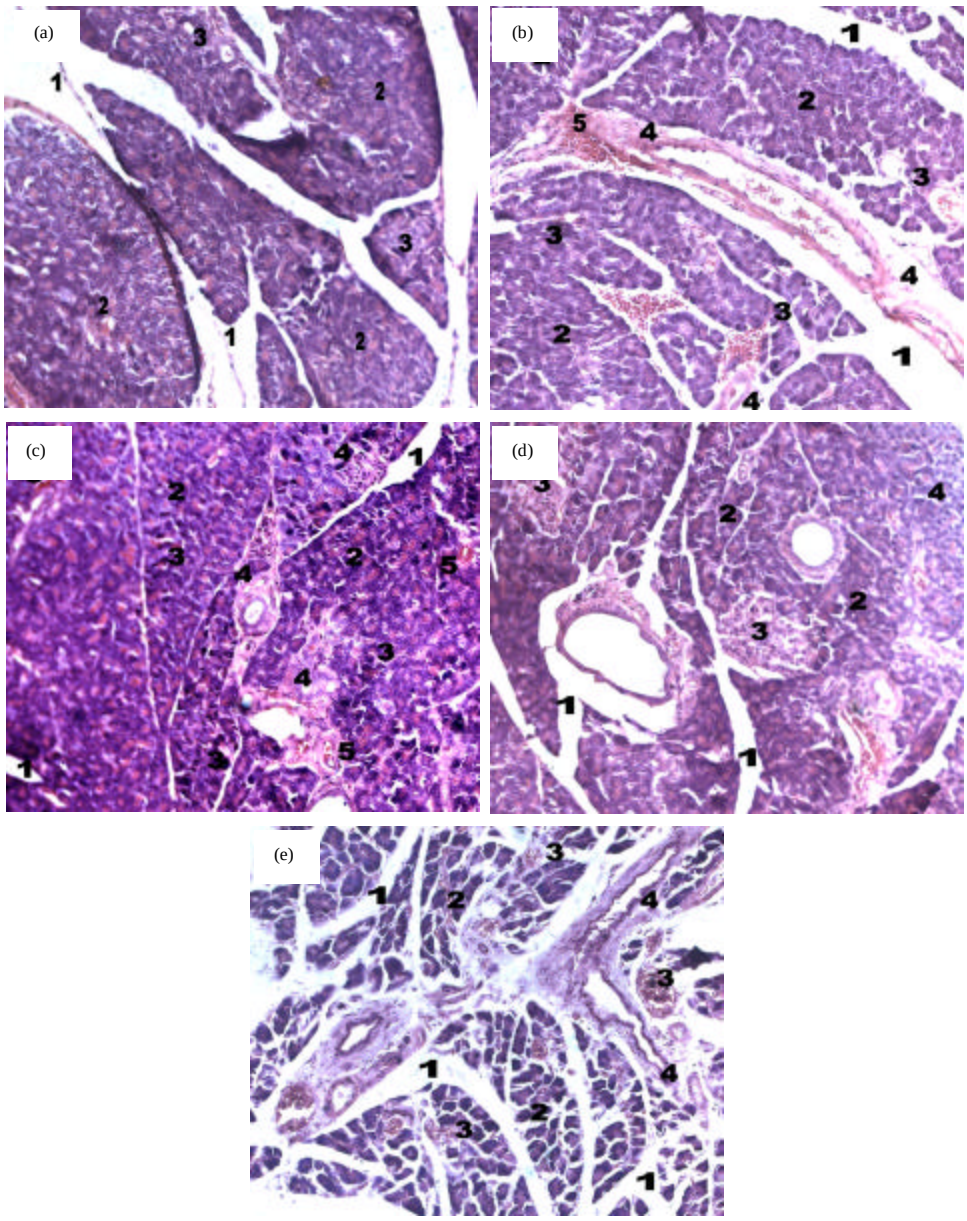


Fig. 2(a-e): (a) Histopathology examination of pancreas section of control rat (1-Interlobular septae; 2-Exocrine glands; 3-Endocrine islets), (b) Histopathology examination of pancreas section of diabetic rat (1-Interlobular septae; 2-Exocrine glands; 3-Atrophied islets; 4-Vascular congestion; 5-Infiltration of lymphoid follicles). (c) Histopathology examination of pancreas section of PEME treated diabetic rats (50 mg $kg^{-1}$ b.wt.). 1-Interlobular septae; 2-Exocrine glands; 3-Atrophied islets; 4-Islets congestion; 5-Tissue haemorrhage), (d) Histopathology examination of pancreas section of PEME treated diabetic rats ( 100 mg $kg^{-1}$ b.wt.). 1-Interlobular septae; 2-Exocrine glands; 3-Islets regeneration; 4-Activated lymphoidal follicles), (e) Histopathology examination of pancreas section of metformin treated diabetic rats (250 mg $kg^{-1}$). 1-Interlobular septae; 2-Exocrine glands; 3-Activated lymphoidal follicles; 4-Vascular regeneration)

Table 5: Influence of PEME of *W. volubilis* on serum alkaline phosphatase (ALP) level in hyperglycemic and non-hyperglycemic rats

| Group | Normal | Diabetic | Day 7 | Day 14 | Day 21 |
|---|---|---|---|---|---|
| Control | 24.08±0.10 | ----- | 24.57±0.30 | 24.71±0.40 | 24.28±0.15 |
| Diabetic (alloxan) | 17.55±0.12 | $48.24\pm0.20^{a1}$ | $47.75\pm0.32^{a1}$ | $45.54\pm0.25^{a1}$ | $45.13\pm0.09^{a1}$ |
| Alloxan+PEME 50 mg $kg^{-1}$ | 18.27±0.92 | $46.34\pm0.35^{a1}$ | $42.22\pm0.24^{bns}$ | $36.11\pm0.16^{b1}$ | $32.87\pm0.36^{b1}$ |
| Alloxan+PEME 100 mg $kg^{-1}$ | 16.42±0.09 | $45.07\pm0.17^{a1}$ | $34.28\pm0.06^{b1}$ | $27.34\pm0.20^{b1}$ | $21.61\pm0.34^{b1}$ |
| Alloxan+Metformin | 21.06±0.11 | $51.02\pm0.65^{a1}$ | $42.04\pm0.17^{b1}$ | $37.55\pm0.22^{b1}$ | $34.89\pm0.19^{b1}$ |

Values are expressed as Mean±SD, (n = 6), [a]normal group versus diabetic group, [b] diabetic group versus treated groups, [1]$p<0.0001$, ns: Non significant

Table 6: Influence of PEME of *W. volubilis* on serum bilirubin level in hyperglycemic and non-hyperglycemic rats

| Group | Normal | Diabetic | Day 7 | Day 14 | Day 21 |
|---|---|---|---|---|---|
| Control | 0.31±0.02 | ----- | 0.34±0.02 | 0.31±0.04 | 0.33±0.05 |
| Diabetic (alloxan) | 0.22±0.03 | $1.13\pm0.20^{a1}$ | $1.77\pm0.01^{a1}$ | $1.60\pm0.01^{a1}$ | $1.71\pm0.38^{a1}$ |
| Alloxan+PEME 50 mg $kg^{-1}$ | 0.26±0.00 | $1.04\pm0.07^{a1}$ | $0.97\pm0.26^{bns}$ | $0.92\pm0.03^{bns}$ | $0.76\pm0.12^{bns}$ |
| Alloxan+PEME 100 mg $kg^{-1}$ | 0.23±0.05 | $0.95\pm0.11^{a1}$ | $0.86\pm0.06^{bns}$ | $0.73\pm0.03^{b2}$ | $0.48\pm0.04^{b1}$ |
| Alloxan+Metformin | 0.17±0.04 | $0.93\pm0.02^{a1}$ | $0.71\pm0.01^{b1}$ | $0.54\pm0.02^{b1}$ | $0.42\pm0.03^{b1}$ |

Values are expressed as Mean±SD, (n = 6), [a]normal group versus diabetic group; [b] diabetic group versus treated groups; [1]$p<0.0001$; [2]$p<0.05$; ns: Non significant

the diabetic control group showed varying degree of damage. Regular arrangement of islets has disturbed with atrophied islet's appearance, vascular congestion as well as infiltration of lymphoid follicles. PEME 50 mg $kg^{-1}$ treated rats showed moderate islets congestion. Vascular haemorrhages with some section's necrosis and fibrosis of islets remained unchanged. On the other hand, Islets from PEME (100 mg $kg^{-1}$) treated groups showed substantial recovery. The lost granules of islets β-cells reappeared. Positive effects of metformin were conformed by normal pancreatic acini cells as well as initial stages of regenerating islet's cells. Photomicrography depicts the glomeruli and tubular of the kidney of rat in different groups. Kidney slides of diabetic rats with severe destruction in glomerular as well as dilated glomerular sclerosis atrophy appearances. Oral administration of PEME showing a reduction in severity of thickening glomerular membrane, much improved tubulointerstitial lesions were shown quite normal appearance in metformin groups (Fig. 3). Histological study was carried by light microscopy in order to detect whether this PEME had an effect on the alloxan treated diabetic liver tissues. Pathologically, liver histological structure was normal in the healthy control group. Figure 4 shows a liver showing complete (severe) destruction of hepatocytes in severe congestion with nuclear condensation, loss of hepatic lobules and congested hepatic inflammation was observed in diabetic rats. PEME and metformin treated rats showed no hepatic abnormalities were observed and the arrangements of the hepatocytes were almost normal detectable in the liver (Fig. 4.). Administrations of PEME on diabetic rats showed the majority of the organs were protected. PEME administration showed improvement in the previous morphological changes in the treated group.

## DISCUSSION

The presence of lupeol in PEME was confirmed by comparing the UV-Vis absorption spectra and peak characteristics with those of standard using a CAMAG HPTLC scanner. Alloxan induced hyperglycemia is a commonly used experimental model, by this method onset of diabetes arises by the destruction of the β-islet cells in the pancreas with the reduction of insulin secretion. Present study showed that the PEME possesses considerable hypoglycemic activity in alloxan rats, the antidiabetic property maybe possibly insulinomimetic. These results might be correlated with the previous evidence of lupeol rich plant extracts treatment attenuate the development of diabetes in the experimental diabetic rats[13]. Overall, the study exposed a concentration-dependent cause of the PEME and standard metformin in suppressing blood-glucose levels. Serum α-amylase is a key enzyme in the hydrolysis of starch into simple sugars which further absorbed into cells and reach in the blood stream as glucose. The analysis of serum α-amylase enzyme was suggested to provide additional informative parameters for the assessment of chronicity and illness as of the response to therapy in diabetes[14,15]. The findings of our present study showed that the administration of PEME and standard metformin to diabetic rats significantly reduced serum α-amylase levels. Assessment of liver function is made by estimating the activities of elevated serum alanine transaminase, alkaline phosphatase and bilirubin. The serum levels of these molecules were increased markedly after the onset of diabetics, these molecules leak into the blood stream in compliance with the extent of liver damages[16,17]. ALT which mediates the conversion of alanine to pyruvate and glutamate, is specific for liver and is a suitable indicator of hepatic injuries. Increased levels of this enzyme are an indicator of cellular infiltration and

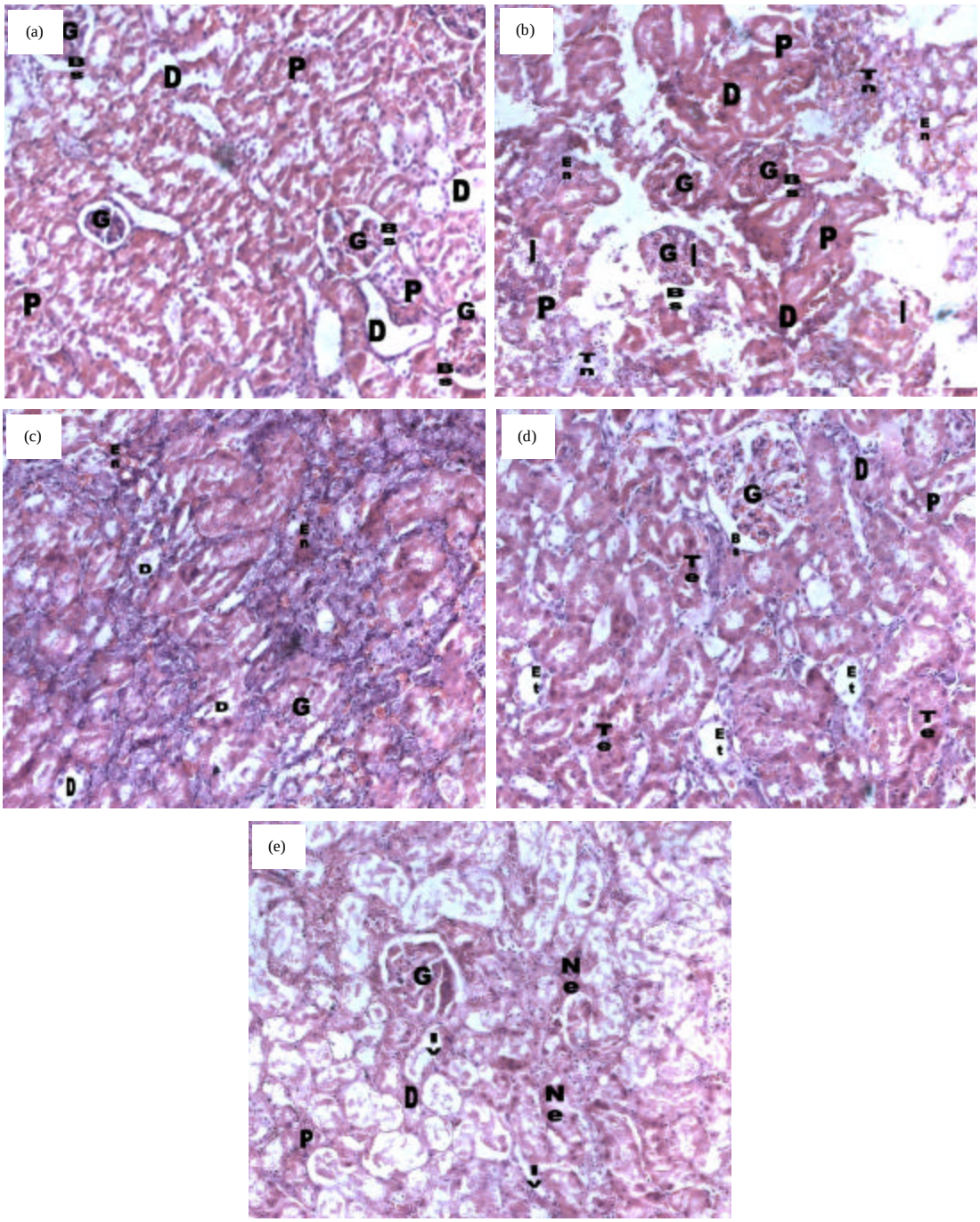


Fig. 3(a-e): Histopathology examinations of rat kidney sections of (a) Control rat, (b) Diabetic rat, (c) PEME treated rats (50 mg $kg^{-1}$ b.w.t), (d) PEME treated rats (100 mg $kg^{-1}$ b.wt.) and (e) Metformin treated rat (250 mg $kg^{-1}$ b.wt.). G: Glomerulus; B: Bowman space; P: Proximal tubule; D: Distal tubule; Et (Te): Epithelium of tubules: Iv: Interstitial vessel; I: interstitium hyperemic and edematous: Tn: tubular necrosis; En: Epithelial necrosis; Ne: Normal epithelium

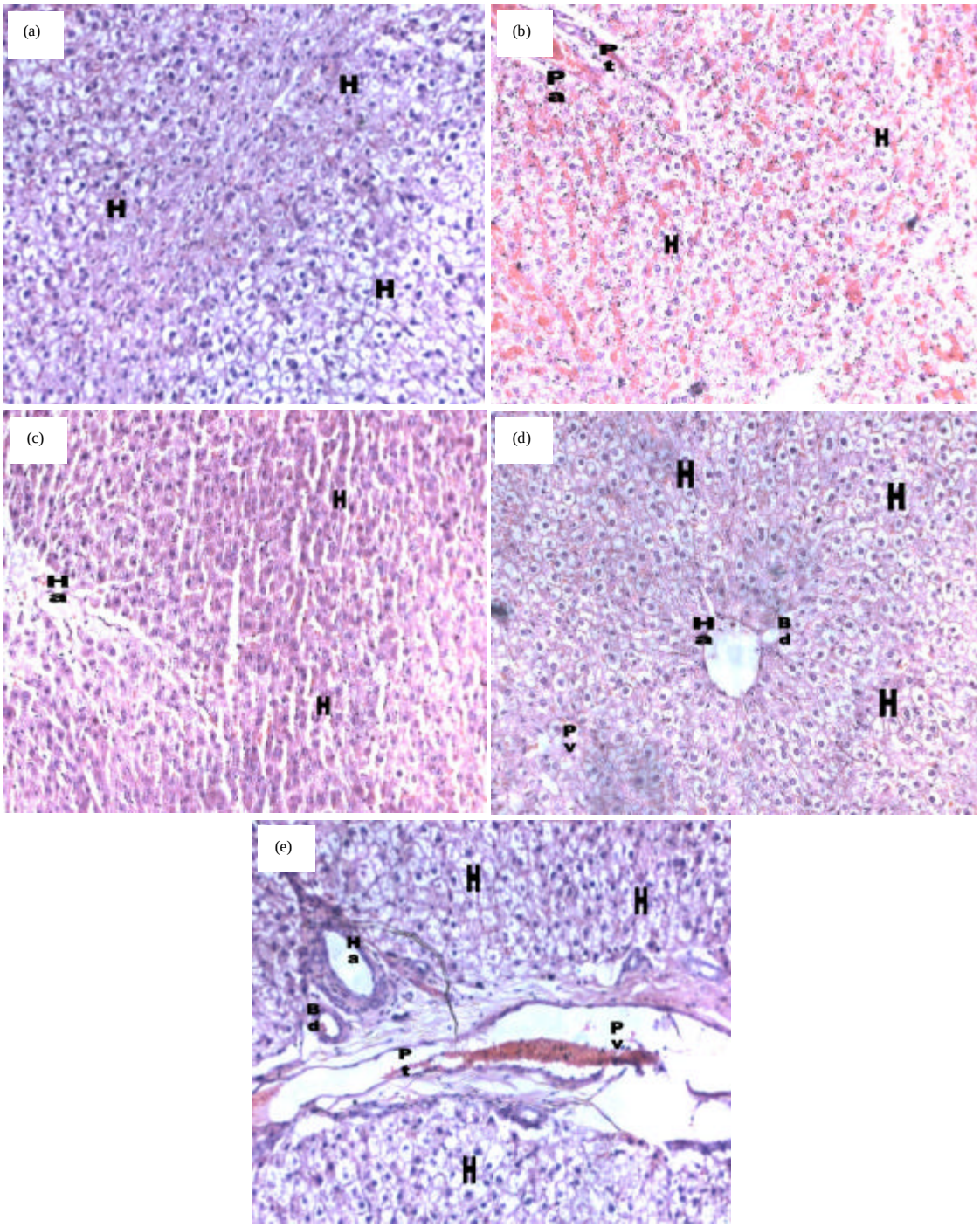


Fig. 4(a-e): Histopathology examinations of rat liver sections of (a) Control rat, (b) Diabetic rat, (c) PEME treated rats (50 mg $kg^{-1}$ b.wt.), (d) PEME treated rats (100 mg $kg^{-1}$ b.wt.) and (e) Metformin treated rat (250 mg $kg^{-1}$ b.wt.). H: Hapatocytes; Ha: Hepatic artery; PV: Portal vein; Pt: Portal track; Bd: Bile duct

functional disturbance of liver cell membranes. ALP is membrane bound and its alteration is likely to affect the membrane permeability and produce derangement in the transport of metabolites. On the other hand, bilirubin is associated with the function of hepatic cells. In the current study, a significant elevation in markers of liver injury (ALT, ALP and bilirubin) reflects the hepatocyte's injury in experimental diabetes. In diabetes, the levels are directly related to changes in metabolism or increased activities of transaminases, due to the absence of insulin secretion also increased gluconeogenesis and ketogenesis. Return of the above enzymes to normal serum values following PEME treatment may be due to prevention of intracellular enzyme leakage resulting from cell membrane stability or cellular regeneration. Effective control of bilirubin with the treatment of PEME shows improvement of functional and secretory mechanism of hepatic cells. Histopathology studies supported our findings. The pancreas, kidney and liver specimens also supported and demonstrated that abnormal histological signs in structure of the organs were exaggerated with prominent changes with alloxan injected groups which were restored to be near normal upon treatment with higher concentration of PEME. The rejuvenation response in treated groups has been mainly attributed to a compensation of some intracellular mechanisms involved in cellular damages during the treatment of diabetic rats.

## CONCLUSION

In conclusion, the present study observation was corroborated with the beneficial effects of PEME in attenuating hyperglycemia, by down regulating elevated levels of serum biochemical parameters. The hypoglycemic action observed could be owed to direct or indirect effects on insulin biosignaling pathway for glucose metabolism, inhibition of gluconeogenesis, peripheral glucose utilization, intracellular electrolyte homeostasis, also the effect of antihypeglycemic phytomarker lupeol in PEME. Histopathology result from this study also demonstrated that PEME is effective in reducing the cellular damages caused by alloxan.

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