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Reversal of hyperglycaemia and renal alterations in Streptozotocin diabetic rats treated with *Anacardium occidentale* (Anacardiaceae).

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Abstract

Anacardium occidentale (Anacardiaceae) has recently emerged as an effective oral treatment for diabetes. Early morphological alterations in the rat kidney due to experimentally induced diabetes by Streptozotocin are described in order to evaluate the possible therapeutic role of hexane extract of *Anacardium occidentale* (AO). Screening study in our laboratory had shown that hexane extract of AO is more potent in lowering blood glucose levels in diabetic rats than aqueous, ethyl acetate, and methanol fraction. Our findings concern the alterations observed in the rat kidney because this organ (together with retina) mainly involved in the early morphological modifications associated with damage of organs and tissues. At the end of the treatment biochemical parameters (total protein excretion rate, albuminuria, glycosuria, urea and glycemia) were measured. Photographs of kidneys were submitted to qualitative and quantitative analysis of images (using Quantimet 500 Image, Analyser-Leica) equipped with specific software). The following parameters were analysed: Diameter of Bowman's capsule, distribution and total area occupied by glomerular capillaries, PAS positive structures (related in to the intensity of reaction resulting from the different amount of muccopolysccharides). *Anacardium occidentale* hexane fraction (which contents, saponins, alkaloids, and polyphenols) is efficient in preventing these modifications when treatment started earlier before diabetic damage of kidneys.

However, the histological study of mucopolysaccharides in diabetic animals highlighted, a tendency for kidneys to accumulate the substances. *Anacardium* significantly reduced the accumulation. Previous evidence demonstrating a beneficial effect of therapy based on hexane extract of *Anacardium occidentale* in insulin-dependant diabetes mellitus seems to be confirmed by our experimental results in kidneys of adult rats. In fact, *Anacardium* treatment is effective (in our experience) for ameliorating the morphological pattern observed early in some diabetic tissues of rats, above all, in the kidney.

Keywords: *Anacardium occidentale*, Hexane extract, Diabetes mellitus, rats, Nephropathy.

1-Introduction

Insulin-dependant diabetes mellitus (IDDM), also called type 1 diabetes is defined as a chronic disease, characterised by a clinical disorder of sugar, fat and proteins metabolism, caused by absence of insulin to promote sufficient glucose output from the liver [1]. Patients depend on insulin for survival. Without insulin, they develop acute metabolic complications such as ketoacidosis and coma. Diabetic nephropathy is the most important cause of death in type 1 diabetic patients, of whom, 30% - 40% eventually develop end-stage renal failure [2]. Studies have shown that good metabolic control is beneficial in slowing the progression of nephropathy in diabetes, and if the duration of diabetes is prolonged before reinstitution of normoglycemia, nephropathy is not easily reversed [3].

Experimental type 1 diabetes induced with streptozotocin in rats display many of the features seen in human subjects with uncontrolled diabetes mellitus [4]. The development of new therapies that are able to improve glycaemia management and even to cure diabetes is of great interest.

Anacardium occidentale L. (Anacardiaceae), plant originated from Brazil is used as folk medicine in south of Cameroon and other African countries for the treatment of diabetes mellitus, diarrhoea and hypertension [5].

In our laboratory, hypoglycaemic and protective role of this plant has been reported [6, 7]. Moreover, a preliminary test has revealed that hexane fraction is more potent in lowering blood glucose than methanol, ethyl acetate and methylene choride.

Base on our previous findings, the current study aimed to identify biochemical and renal histopathological abnormalities that occur with the evolution of streptozotocin-induced diabetes in rats, and to appreciate their possible reversal after establishment of good metabolic control with the hexane extract of *Anacardium occidentale*.

2-Materials and methods

2-1 Plant

Plant materials of *Anacardium occidentale* were collected, in month of January and were authenticated by comparison with national herbarium (Yaounde-Cameroon) file, voucher specimen (N° 41935 /HNC).

The plant leaves were dried in the laboratory at room temperature and powdered in a mixer grinder. The powder-dried leaves (3kg) were macerated in 8 l of methanol at room temperature. After filtration, the solution was concentrated under reduced pressure. The resulting extract (262 g) was eluted in hexane (1l) and concentrated to dryness to afford viscous mass of hexane extract (35.67g) with the extraction yield of 13.6 %. 1.5 g of this extract were dissolved in 3 ml of dimethyl sulfoxide (DMSO) and solution adjusted to 97 ml with distiller water to obtain a solution of 100 ml.

2-2 Animals

Male wistar rats weighing 150-230g from the animal house of the faculty of science, University of Yaounde I, were used. The animals were fed with standard laboratory diet, and given tap *ad libitum*. After randomisation into various groups, the rats were acclimated for a period of 6-7 days in new environment before the initiation of experiment. Animals described as fasted were deprived of food for at least 14 h, but had free access to water. A group of 5 non-diabetic (ND) rats receiving distiller water, was considered as control group whereas another group of non-diabetic rats receiving extract of AO 300mg/kg was considered as control treated group (NDT).

2-3 Streptozotocin-induced diabetic rats

Diabetes was induced in rats by single iv. injection of streptozotocin, Sigma chemical Co (St Louis, Mo, USA) at a dose of 55 mg/kg (freshly dissolved in physiological saline), while non-

diabetic rats were injected with saline only. 72 h following streptozotocin injection, the blood glucose was monitored, using glucometer Accutrend GC (Boeringer Mannheim, Germany). Animals with plasma glucose levels higher than 200 mg/dl were selected for the study.

Diabetic untreated rats were sub-divided into three groups: group I with two weeks duration of diabetes (DC-2W), group II with 8 weeks duration of diabetes (DC-8W), and group III with twelve weeks duration of diabetes (DC-12W).

In the other hand, diabetic-treated rats were sub-divided into four groups: group IV, rats given AO 150 mg/kg (D-2W+AO150), 2 weeks after diagnosis of diabetes; group V, rats given AO, 300 mg/kg (D-2W+AO300), 2 weeks after diagnosis of diabetes; group VI, rats given AO, 300 mg/kg of BW immediately after diagnosis of diabetes (D-0W+AO 300); group VII, rats given Insulin, 2 weeks after diagnosis of diabetes (D-2W+In).

All animals were housed individually, with free access to food and water. The plant extract was given once daily for duration of 7 weeks.

2-4 Determination of blood glucose level and other biochemical parameters.

To determine the blood glucose level, all animals were overnight fasted. Blood was obtained from the tail vein and glycaemia was monitored once per week as previously described.

At the end of the treatment, all rats were sacrificed , blood and organs were collected for further analysis. Blood was centrifuged at 3000 XrPM at 4°C for 10 min to separate the serum. The level of glucose, albuminuria, glycosuria, and urea was measured using Randox Kits.

2-5 Renal histological assessment

Nephropathy was assessed after treatment by measurements of 24-h urinary albumin, total protein excretion rate, glycosuria and urea. For urinary collection, rats were housed in metabolic cages for 24 h. Several drop of toluene were added to the urine collection beaker to inhibit microbial growth [8]. At the end of treatment, rats were sacrificed, kidneys were removed, fixed in 10 % buffered formalin, dehydrated in gradual ethanol (80-100°), cleared in xylene, and embedded in paraffin. Section (3 µm thick) were prepared and then stained with hematoxylin-eosine, Trichome of Masson, and PAS dye for photomicroscopic observation. Photographs of kidneys were submitted morphometric analysis of images, using Quantimet 500 image, Analyser-Leica.

2-6 Statistical analysis

All values are expressed as means ± SEM. Statistical analysis were evaluated using student's t test. Findings were considered to be statistically significant at a p value (*p) of less than 0.05.

3-Results

The hexane extract exhibited antidiabetic property in streptozotocin-induced diabetic rats as shown by glycemia and biochemical parameters levels measured in urine.

The effects of the extract on body and kidney weight in streptozotocin-induced diabetic rats are given in table 1. The body weight significantly decreased ($p<0.05$) in diabetic rats compared to normal controls at the end of the experiment. The kidneys weight were also decreased in diabetic rats (19.54 %) as compared to healthy rats.

In insulin as well as extract (300 mg/kg given earlier or 2 weeks after diabetes diagnosis), there were no significant decreased of the body and kidneys weight (Table 1).

The results of biochemical measurements used to evaluate renal function are shown in Fig 1. By the end of study, total 24h urinary protein, albumin, glycosuria, and urea were approximately, 7, 12, 100, and 4 times higher, respectively in diabetic controls animals compared with non-diabetic rats. Regarding the effective dose (300 mg/kg) given immediately after diagnosis of diabetes, albuminuria, and urea were decreased with 24%, and 44%, as compared to diabetic untreated-rats. With the same comparison, we also observed a decrease of proteinuria and glycosuria with 9% and 26%, respectively.

The effects of the plant extract on serum glucose levels in streptozotocin diabetic rats are shown in Fig 2. The initial blood glucose levels of the diabetic rats selected for the study were in range of 208 and 380 mg/dl. In the untreated control rats, the blood glucose level increased by 328.8 mg/dl on the 14 days and 2/11 diabetic rats died during this period. There after 4/9 animals died in the second period of experiment and the mean glucose levels on the last days of treatment in 5 animals, which survived was 400 mg/dl.

In diabetic-treated rats, D-2W+AO150 and D-2W+AO300, the blood glucose levels steadily decreased and it was 226.4 and 123.6 mg/dl, respectively at the end of the treatment. Thus, the hexane extract of AO restored (in the dose-dependant manner) the glycemia almost nearer the normal values at the dose of 300 mg/kg. The effect of the plant extract (300 mg/kg) is there for, comparable to that of insulin (standard drug). In the other hand, there were no significant decreased of blood glucose levels in non-diabetic treated rats (NDT) with AO 300 mg/kg compared to non-diabetic rats receiving only distiller water.

Phytochemical analysis revealed the presents of alkaloids, saponins and polyphenols in hexane extract of our plant.

The photographs in Fig 3B, C, and D show the histopathological lesions in glomeruli and tubules. We observed an osmotic nephrosis, desquamation and destruction of tubular epithelium, pathologies, which increased with the duration of diabetes (Fig 3D). We also remarked an atrophy of glomerular capillaries with Bowman's space dilated in morphometric study (Table 1). Diabetic untreated-rats (D-8W), exhibited a significant atrophy of total area occupied by glomerular capillaries, $3.5\mu m^2$ versus $6.38\mu m^2$ for non-diabetic rats. These structures tended to be normal in *Anacardium*-treated animals. Periodic acid schiff (PAS) staining revealed that mucopolysaccharide were abundant in diabetic rats kidneys. Although AO efficiently restored the histopathological and morphometric alterations (Fig 3E), it failed to recover all these failures when treatment started two weeks after diabetic state).

4-Discussion

The main function of the kidneys is to excrete the waste products of metabolism, to regulate the body concentration of water and salt in other to maintain the appropriate acid-base balance of the plasma, and serves as an endocrine organ secreting erythropoietin, renin and prostagladins [9].

In this study, we have evaluated biochemical parameters determinants of progression to nephropathy. Our observations confirm that microalbuminuria is a very good predictor of the developing diabetic kidneys disease. However, the development of diabetic nephropathy is characterised by a progressive increase in urinary protein particularly albumin, and late decline in glomerular filtration rate, leading eventually to end-stage renal failure [10]. Physiologically, the upper normal rang of urinary protein and albumin excretion is 150 mg/24h and 30 mg/24h, respectively [11-12]. Recently, Warram and *al* [13] studied a large cohort of approximately 300 microalbuminuric type 1 diabetic subjects for 4 years and found and increasing risk of developing nephropathy for patients with proteinuria.

Morphological study showed an atrophy of glomerular capillaries, interstitial fibrosis and tubular necrosis, which increased with a duration of diabetes. Moreover, chemical analysis of diabetic glomeruli indicates increased carbohydrate content in basement membrane. These results are consistent with those of other authors analysing renal biopsies of human diabetic patients, but with diffuse basement membrane thickening of capillaries, glomerulosclerosis, and exudative lesions even in diabetes with relatively short duration. [13].

At the dose employed, we were able to demonstrate significant protection of hexane extract of AO against early nephropathy (Proteinuria, albuminuria, and glycosuria). All interventions of treatment slowed and sometimes inhibited the morphological alterations observed. However, the excellent recovery of renal function expected with treatment of AO can be explained by the regenerative capability of the renal tubules [14]. The action by which the extract lowered the blood glucose is not wellknown. It could act by increasing glycogenesis and/or decreasing glycogenolysis in diabetic rat liver, there for, the mode of action of AO could be compared to that of *Cassia kleinii* leaf in STZ-induced diabetic rats [15]. In another hand, the chemical substances could be mediated by stimulation of regeneration process and revitalisation of remaining β cells [16].

Phytochemical analysis had revealed the presents of alkaloids, polyphenols and saponins in the plant extract. Based on increasing number of reports on blood glucose reduction associated with some saponins [17] and alkaloids [18], isolated from other medicinal plants it is likely that the active principle (s) could be present in one or the two families chemical substances. Accelerated chemical modification of proteins by glycoxidation and accumulation of AGE (Advanced Glycation End products) in tissue are implicated in pathogenesis of diabetic nephropathy. The increase in AGE formation is sometimes attributed to increase oxidative stress, which can be inhibited with polyphenols [19].

Streptozotocin-induced diabetic rats are insulin deficient, hyperglycaemic, and have a reduction in glucose uptake in adipose and skeletal muscle [20]. The effect of plant extract is equal to (urea and albuminuria) or less than (proteinuria and glycosuria) that of insulin. But in all case, the hexane extract decreases the urinary parameters in the dose-dependant manner.

Insulin as a standard drug causes hypoglycaemia when taken in excessive doses and overt hypoglycaemia is the most worrisome effect of this drug. *Anacardium occidentale* did not cause hypoglycaemic effect and is not toxic, there for, could serve for optimal protection of renal function during diabetic nephropathy.

5-Conclusion

The goal of these studies was to evaluate the effect of variety period therapies on development of renal complications in streptozotocin-induced diabetic rats. The experiments indicated that the more effectively a therapeutic intervention limits the progression of nephropathy. Our data shows that, the hexane fraction expressed the best protection against renal function during nephropathy, and that alteration can be preventing by earlier treatment. Therefore, further

studies are necessary to isolate the active component and to clarify in more detail the pathway concerning the protection.

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6-References

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	ND	ND-AO300	D-8W	D2W+AO150	D2W+AO300	D-0W+AO300	D-2W+In
Relative percentage of the kidneys weight	0.38	0.37	0.38	0.44	0.37	0.38	0.35
UER ml/24h	3.6±1.02	8.6±0.52	62.8±4,7*	45.8±8,4*	30.8±0.8*	18.75±1.1	20.00±1.1
Total surface of glomeruli n=5 (μm^2)	8.71±0,3	8.5±1.04	5.78±1.2*	6.6±0.38	7.6±1.06	7.05±0.14	7.17±0.49
Total area occupied by glomerular capillaries n=5 (μm^2)	6.38±0,13	6.44±1.02	3.50±0.8	5.10±0.43	4.37±0.73	4.85±0.31	5.22±0.39

Table 1 Physical parameters in fasted rats, 35 days after treatment with hexane extract of *Anacardium occidentale*. Diabetic control rats (D-8W) showed a significant decrease in total area occupied by glomerular capillaries, compared with non diabetic rats (ND). Values shown are mean ± SEM, *P< 0.05, significant different compared to control rats. UER: Urinary Excretion Rate. n: number of glomeruli measured.

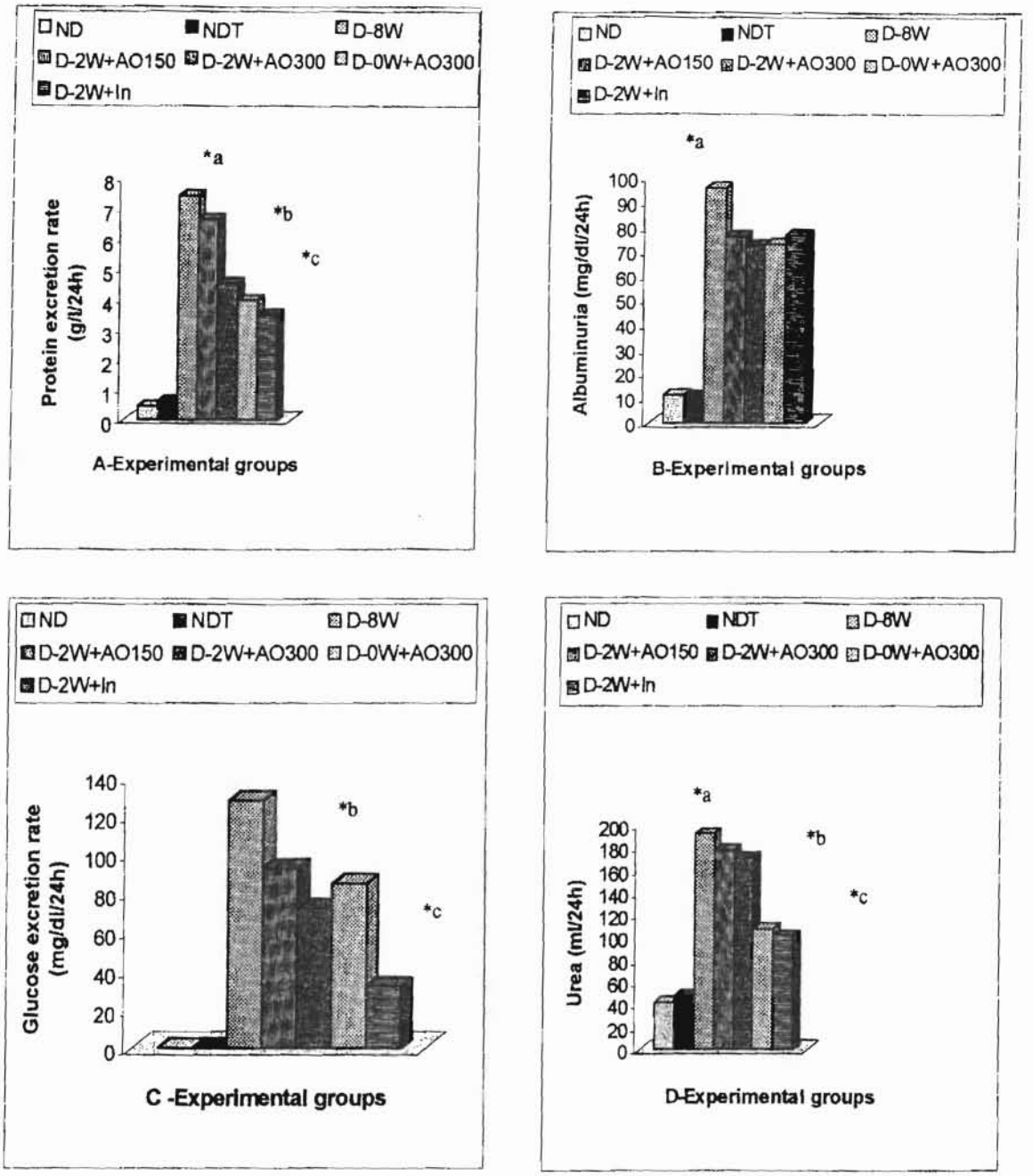

Fig. 1 Metabolic parameters in urine collection of fasted rats, 35 days after treatment with *Anacardium occidentale*. A : Proteinuria ; B : Albuminuria ; C: Glucose excretion rate ; D : Urea. Diabetic control rats (D-8W) showed a marked increase in all metabolic parameters compared with non-diabetic rats (ND). Values shown are the mean ±SEM, *P<0,05, Significant difference as compared to, control (a), *Anacardium*-treated group (b) and Insulin (c).

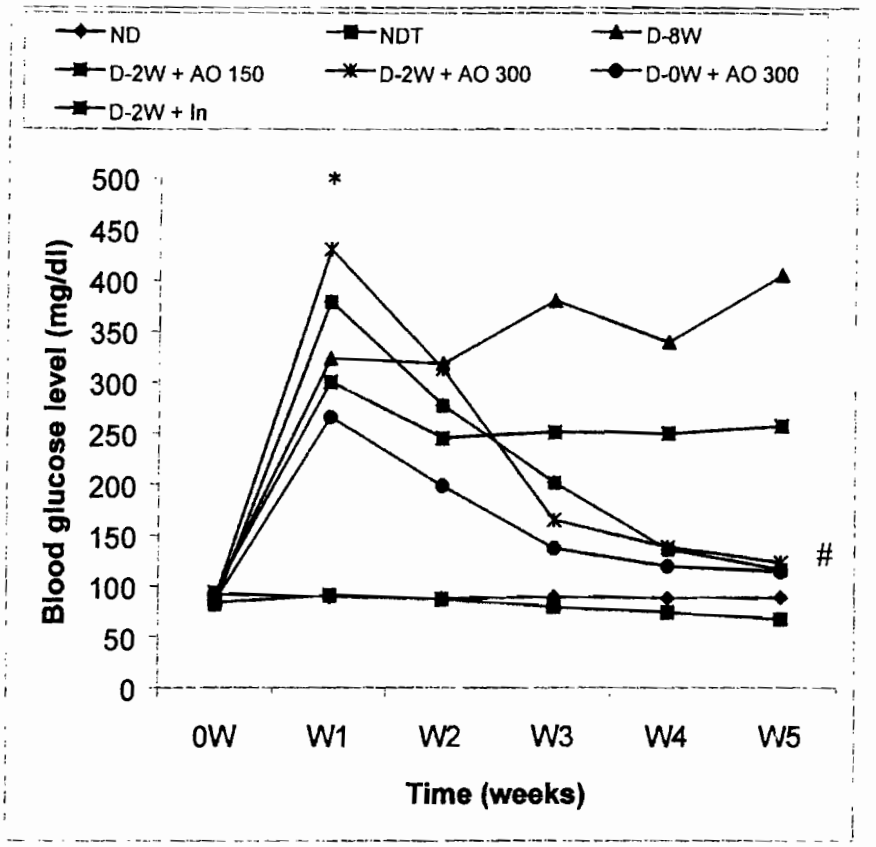

Fig 2 Effects of hexane extract of *Anacardium occidentale* on blood glucose levels of streptozotocin-diabetic rats. Data are shown as means ± SEM. *$p<0.05$, significant different compared to non diabetic (ND) rats, and #, significantly different from the initial day of treatment, $p<0.05$.

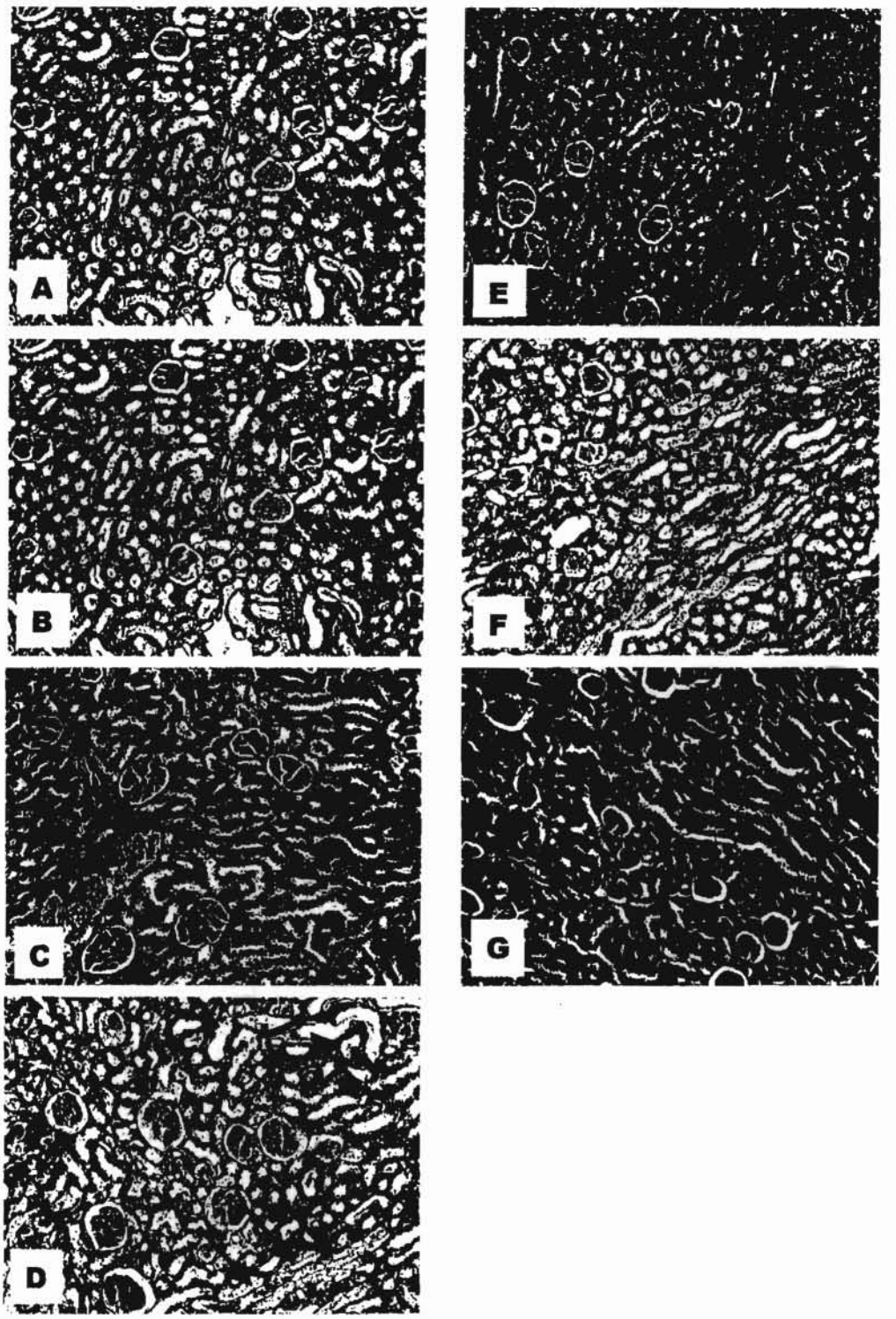

Fig.3: PAS stain of kidneys of rats at 35 days and 28 days of treatment age with hexane extract of *Anacardium occidentale* and Insulin, respectively.
A: Non diabetic (ND) rat. **B**: Diabetic control rat on 2 weeks (DC-2W) after diagnosis of diabetes. **C**: Diabetic control rat on 8 weeks (D-8W). **D**: Diabetic control rat on 3 months (DC-3M). **E**: Diabetic rat on zero day, given AO 300mg/kg/day. **F**:Diabetic rat on 2 weeks,given AO 300mg/kg (D-2W+AO 300).**G**: Diabetic rat on 2 weeks, given insulin, 5µU/kg/day (D-2W+In) (⟶) arrows indicate epithelium tubules nephrosis. (⟶)arrow indicates positive structures from the different amount of muccopolysaccharides