# Potential of Rutin and Vildagliptin Combination against Alloxan Induced Diabetic Nephropathy in Mice

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## Abstract

*Objective: The aim of present study was to explore antidiabetic and antioxidant actions of rutin, vildagliptin and their combination in the kidney of alloxan induced (150 mg/kg alloxan monohydrate in normal saline injected by intraperitoneal route) diabetic mice. Methods: Animals were allocated into five groups (each group of six mice, n=6) as: i. control group treated with carboxymethyl cellulose (0.5 %, o.d.), ii. a diabetic control group treated with carboxymethyl cellulose (0.5 %, o.d.), iii. diabetic mice treated with rutin (400 mg/kg, o.d.), iv. diabetic mice treated with vildagliptin (30 mg/kg, o.d.) and v. diabetic mice treated with combination of rutin (400 mg/kg, o.d.) and vildagliptin (30 mg/kg, o.d.). The level of antioxidant enzymes such as lipid peroxidase (LPO), catalase (CAT), superoxide dismutase (SOD) and non enzymatic level of reduced glutathione (GSH) was measured in plasma. Similarly level of protein, albumin, creatinine and blood urea nitrogen (BUN) was measured alongwith urinary output and urinary albumin level before as well as after treatment with drugs. Results: The results obtained after treatment with combination of rutin and vildagliptin were more efficient than those obtained from treatment with either rutin or vildagliptin. Likewise histological damage to glomeruli and tubule (hypertrophy) due to diabetic nephropathy was also improved with combination. Conclusion: Our results suggest that the combination of rutin and vildagliptin is more effective than either of the two drugs alone and can be another effective alternative therapeutic for diabetic nephropathy.*

**Keywords:** Diabetes, lipid peroxidase, nephropathy, oxidative stress, rutin, vildagliptin.

## Introduction

Diabetes mellitus (DM), a chronic metabolic disorder occurs mainly due to deficiency of insulin which is accompanied retinopathy, neuropathy, nephropathy and cardiomyopathy. Diabetic nephropathy (DN) is considered to be among one of the major complication of diabetes[1]. It is the largest growing metabolic disorder of the world that needs more appropriate therapy[2]. As per the prediction of World Health Organization (WHO) around 300 million peoples would be diabetic by the year 2025[3]. DM has several documented prevalent systemic complications[4]. Hyperglycemia, a clinical condition of diabetes mellitus plays a key role in damage mediated through overproduction of reactive oxygen species (ROS) and accumulation of lipid peroxidation by products. Most common symptoms that appear in diabetic nephropathy are edema, proteinuria, anorexia, malaise, fatigue etc. There are several factors that increase the risk of diabetic nephropathy such as smoking, increased blood pressure, steady diabetes and family history of diabetes.

Diabetic nephropathy characterized by nodular glomerulosclerosis and nephritis that are considered as progression of kidney disease. Albuminuria is another confirmatory character for diabetic nephropathy alongwith decreased glomerular filtration and enhanced arterial blood pressure. Diabetic nephropathy is considered as one of the leading cause for kidney failure in western countries. Long term diabetes is one among the cause of mortality in patient[5-10]. In diabetes, major damage occurs to the renal tissues because here, insulin does not participate to regulate the entry of glucose[11]. Diabetes may also be associated with prevalence of obesity and other severe complicated disorders such as coronary heart disease, heart failure, atherosclerosis and diabetic cardiomyopathy[12]. Diabetes also leads to generation of free radicals which may to complicate the situations further. Herbal formulations are mainly recommended for prevention of diabetes as they are thought to be less toxic than oral hypoglycemic such as sulfonylureas, metformin[13,14]. Natural antioxidants plays important role to overcome oxidative damage[15]. Similarly natural products based medicines also play major role in execution of DM[16-18].

Rutin is a flavonoid (polyphenol) glycoside that enhances the antioxidant potential in diabetic rat. It is also considered most consumed in food because of having free radical scavenging potential. Other important uses of rutin are as anti-inflammatory, prevent atherosclerosis and inhibit platelets aggregation. Rutin is also included in various multivitamin and herbal formulations. Vildagliptin is oral hypoglycemic drug that

improves the responses of pancreatic islets toward glucose. It is specially used to treat type 2 diabetes. Vildagliptin is DPP-4 (Dipeptidyl peptidase-4) inhibitor that enhances the insulin secretion[19,20].

## Material and Methods

**Animals:** The animals (mice of either sex) were procured from Pinnacle Biomedical Research (PBRI) animal house (Bhopal, India) and housed in standard laboratory conditions ($21\pm2$ºC, 55% RH) and, alternate light and dark cycle of 12 h each were maintained during the study. The care of animals was taken according to the principles of the guide for care and use of experimental animals. Animals were fed with standard rat pellet and allowed free access to tap water.

**Drugs:** Rutin was purchased from Hi-Media Laboratories (Mumbai, India) and vildagliptin from Novartis (Mumbai, India). The experimental protocol approved by Institutional Animal Ethical Committee (IAEC) was followed.

**Induction of diabetes:** After acclimatization (for two weeks) mice were induced diabetic nephropathy by intraperitoneal administration of alloxan monohydrate (150 mg/kg). After 6 h mice were given 20% (5-10 ml) glucose solution as alloxan may develop severe hypoglycemia. For next 24 h, 5% glucose was given to vent hypoglycemia and animal with moderate diabetes (blood glucose level approximately 300 mg/dl) were selected for experimentation.

**Experimental design:** Animals used were randomly classified into five groups with six animals in each group (n=6) as normal control group orally treated with freshly prepared 0.5 % carboxy methylcellulose (CMC), a diabetic control group orally treated with 0.5 % CMC, diabetic mice orally treated with 400 mg/kg/day rutin alone, diabetic mice orally treated with 30 mg/kg/day vildagliptin alone and diabetic mice orally treated with combination of both 400 mg/kg/day rutin and 30 mg/kg/day vildagliptin with the use of catheter everyday in morning.

**Preparation of homogenate:** Kidneys from the sacrificed animals were homogenized in ice cold 0.15 M Tris-HCl (pH 7.4) to prepare 10 % (w/v) homogenate which was centrifuged at 15000 rpm for 15 min (at 4 C). The supernatant was then used for determination of antioxidant activity.

**Collection of blood samples:** Animals treated with drugs were fasted overnight and blood samples were collected in vesiculature precoated with anticoagulant (EDTA) by retro-orbital puncture under mild anesthesia. The collected samples were centrifuged (7000 rpm) for 15 min (at $4^0$C) to separate plasma as supernatant for biochemical analysis that was stored at $-20^0$C.

**Collection of urine sample:** Animals were kept in metabolic cage after treatment and urine samples were collected in urine collecting bottles after 24 h.

**Biochemical estimations:** i. Thiobarbituric acid-reactive substances (TBARS, by products formed in secondary lipid peroxidation) were the measure of extent of lipid peroxidation. Tissue homogenate (2 ml), 8.1% sodium dodecylsulphate (SDS, 2ml), 20% acetic acid (5 ml) and 8 % TBA (1.5 ml) were mixed upto the volume of 4 ml (diluted with distill water) followed by heating at $95^0$C for 1 h on a water bath. 0.5 ml mixture butanol: pyridine (15:1) was added and centrifuged (3000 rpm) for 10 minutes. OD read of supernatant was taken at 532 nm against blank[21,22]. ii. For estimation of GSH the protocol of Ellman et al., 1959 modified by Jollow et al., 1974 was followed. The method was based on the production of yellow colour by the addition of 5,5-dithiobis-2 nitro benzoic acid (DTNB) to compounds containing sulfhydryl group. Tissue homogenate was prepared in 10%, 0.1 M phosphate buffer (pH 7.4) followed by addition of (0.2 ml each) 20% TCA and 1 mM EDTA to 0.2 ml of tissue homogenate that was centrifuged at 2000 rpm for 10 min. 200 µl of supernatant transferred to a tube containing 1.8 ml Ellman's reagent (5,5-dithio-bis- 2-nitrobenzoic acid) previously prepared in 0.3 M phosphate buffer (pH 7.0) with 1% sodium citrate. Volume was made up to 2 ml with distilled water and OD read was taken at 412 nm against blank[21,23]. iii. CAT activity (in terms of nmol $H_2O_2$ consumed/min/mg of protein) was assessed by method of Aebi, *et al.*. Enzymatic reaction was initiated by adding 100 µl aliquot of homogenized tissue to substrate ($H_2O_2$) to the concentration of 30 mM (1 ml) in 50 mM phosphate buffer (pH 7.4) and change in absorbance at 240 nm were recorded[24]. iv. For estimation of superoxide dismutase (SOD) tissue homogenate (10%) prepared in phosphate buffer (0.1mM) was centrifuged at 15000 rpm for 15 minutes (at $4^0$C). 1.2 ml; 0.052 M sodium pyrophosphate buffer (pH 8.3), 0.1 ml; 186 µM phenazine methosulphate, 0.3 ml; 300 µM nitroblutetrazolium and 0.3 ml; 750 µM NADH were added to 0.1 ml of supernatant and incubated for 90 seconds at $30^0$C followed by further addition of 0.1 ml glacial acetic acid and 4.0 ml of n-butanol. It was again centrifuged (to separate layer of butanol) and OD read was taken at 560 nm[25]. v. Albumin and protein were measured with the Span and Ranbaxy diagnostic kit by autoanalyzer, Echo, Logotech Pvt. Ltd. (India). Formation of green colour complex with bromocresol green dye was the basis of estimating plasma albumin concentration. The mixture of 10 µl serum/plasma and 1000 µl reagent 1 was incubated at room temperature (15-$30^0$C) for 1 min. The auto analyzer (programmed as per assay protocol) was blanked with reagent blank and absorbance was measured at 630 nm. Similarly protein was estimated using Bradford reagent[26,27]. vi. The level of creatinine was estimated spectrophotometrically using diagnostic creatinine kit, Coral Clinical System (Goa, India). Estimation of creatinine was based upon formation of picrate that produces yellow colour and absorb at 492 nm. vii. The level of blood urea nitrogen (BUN) in plasma was estimated spectrophotometrically using BUN GLDH kit, Bhat Bio-tech Pvt. Ltd. (Bangalore, India).

**Histopathological examination:** The kidney of sacrificed animals was isolated, washed and stored at $-20^0$C (fixed in 10 %

buffered formalin) to process for histopathological examinations. The tissues were embedded in paraffin and were stained hematoxylin-eosin followed by mounting finally in DPX mountant (mixture of distyrene, plasticizer and xylene). Blind quantitative histological analysis was done using light microscope (400X) to evaluate tubular and glomerular injury and necrosis.

**Statistical analysis:** All the data obtained was analyzed statistically using Stat view 5 software for Window (SAS Institute, Berkley, CA). Statistical analysis between different groups was performed with one-way ANOVA followed by student t-test. The results were expressed as mean± standard deviation and p value <0.06 was considered significant.

## Results and Discussion

**Lipid peroxidase:** The plasma LPO level of normal control group was 1.867±0.263 nM/mg protein that increased to 6.947±0.396 nM/mg protein in diabetic group. Treatment with combination of rutin and vildagliptin reduced it to 2.165±0.268 nM/mg protein while rutin to 4.822±0.378 nM/mg protein and vildagliptin to 5.499±0.386 nM/mg protein (table 1 and figure 1).
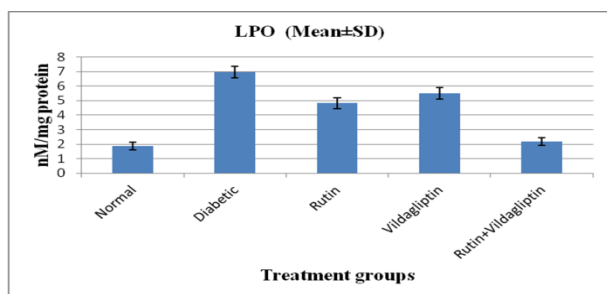


**Figure-1**
**Plasma LPO level (nM/mg protein)**

**Reduced glutathione:** The GSH level of normal control group was 25.586±2.276 nM/mg wet tissue while in diabetic group it was decreased drastically to 6.520±0.786 nM/mg wet tissue. Treatment with rutin, vildagliptin and combination of both caused the plasma level of GSH to increased upto 16.173±0.390 nM/mg wet tissue, 15.741±0.504 nM/mg wet tissue and 21.947±1.089 nM/mg wet tissue respectively (table 1, figure 2).
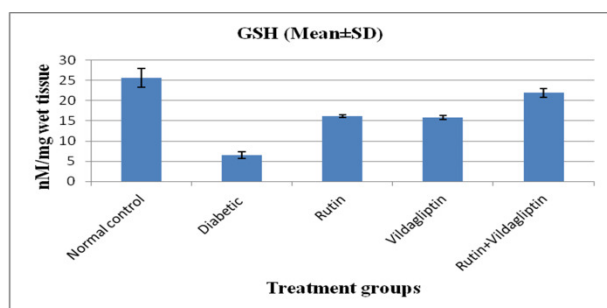


**Figure-2**
**Plasma GSH level (mM/wet gm Tissue)**

**Catalase:** The CAT level in normal control group was 11.433±0.827 U/mg protein which was significantly decreased to 5.766±0.987 U/mg protein in plasma of diabetic groups. After treatment with rutin, vildagliptin and combination CAT was elevated by 7.993±0.757 U/mg protein, 6.875±0.419 U/mg protein and 9.928±0.612 U/mg protein respectively (table 1, figure 3).
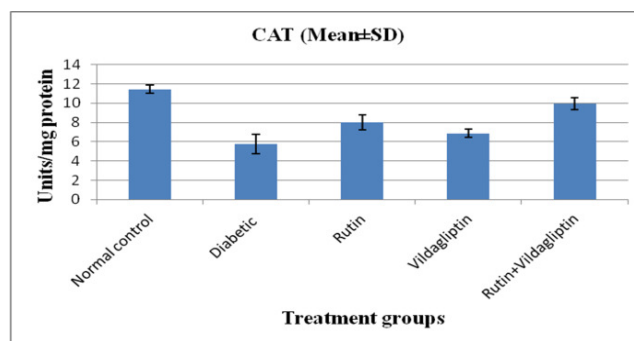


**Figure-3**
**Plasma CAT level (units/mg protein)**

**Superoxide dismutase:** The plasma SOD level of normal control group was 78.485±1.805 U/mg protein that was found decreased to 11.546±1.921 U/mg protein in diabetic mice. After treatment with rutin, vildagliptin and combination it was found significantly increased to 69.311±2.852 U/mg protein, 56.814±1.724 U/mg protein and 72.704±0.822 U/mg protein respectively (table 1, figure 4).
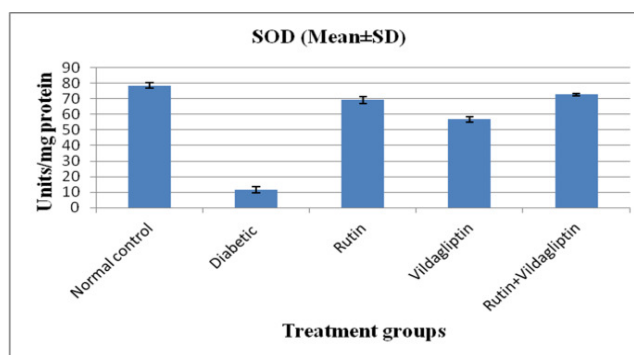


**Figure-4**
**Plasma SOD level (units/mg protein)**

**Protein and albumin:** The level of protein (figure 5) and albumin (figure 6) of normal control group was 7.263±0.746 g/dl and 3.926±0.616 g/dl respectively that was decreased in diabetic mice to 3.058±0.416 g/dl and 1.882±0.332 g/dl respectively. After treatment with rutin, vildagliptin and combination significant increase was in level of protein was found i.e. 5.918±0.338 g/dl, 3.828±0.334 g/dl and 7.097±0.733 g/dl while those of albumin was 2.765±0.262 g/dl, 2.015±0.382 g/dl and 3.076±0.409 g/dl respectively (table 2).
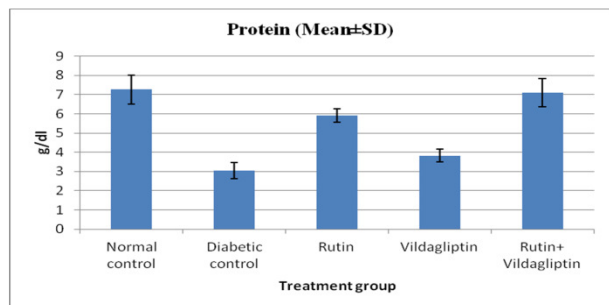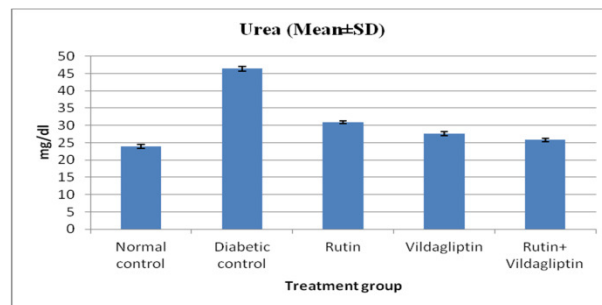
**Figure-5**
**Plasma level of Protein**



**Figure-8**
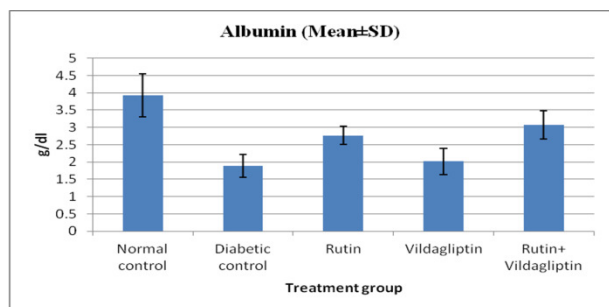**Plasma level of urea**

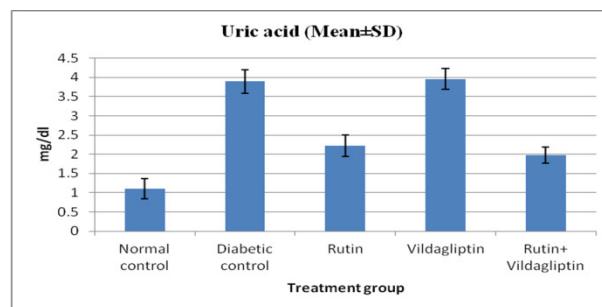

**Figure-6**
**Plasma level of albumin**



**Figure-9**
**Plasma level of uric acid**

**Creatinine and blood urea nitrogen:** The level of creatinine (figure 7), urea (figure 8) and uric acid (figure 9) in normal control group was 0.952±0.291 mg/dl, 23.915±0.527 mg/dl and 1.108±0.266 mg/dl respectively but it was increased in diabetic group i.e. 2.748±0.327 mg/dl, 46.355±0.615 mg/dl and 3.893±0.312 mg/dl respectively. Treatment of diabetic mice with rutin, vildagliptin and combination reduced the level of creatinine, urea and uric acid in plasma. Rutin reduced creatinine, urea and uric acid level to 2.195±0.163 mg/dl, 30.918±0.403 mg/dl and 2.228±0.277 mg/dl respectively while vildagliptin reduced their level to 1.723±0.184 mg/dl, 27.526±0.579 mg/dl and 3.958±0.272 mg/dl respectively. More significant reduction in level of creatinine, urea and uric acid was seen with the combination of rutin and vildagliptin i.e. 1.041±0.217 mg/dl, 25.770±0.473 mg/dl and 1.983±0.208 mg/dl respectively (table 2).

**Urine output and urine albumin:** The urine output of normal control group i.e. 9.730±0.630 ml/h was found significantly increased in diabetic mice i.e. 42.52±0.821 ml/h. Treatment with rutin decreased it to 19.27±1.981 ml/h similarly vildagliptin decreased to 24.13±1.206 ml/h while combination of both decreased urine output to 13.01±0.896 ml/h. On the other hand urinary output of albumin was also measured. The level of urine albumin in normal control group was 3.830±0.492 mg/dl that was found increased to 11.25±0.504 mg/dl in diabetic mice. Rutin reduced urine albumin level of diabetic mice to 6.93±0.411 mg/dl while vildagliptin reduced to 8.87±0.372 mg/dl. More efficient reduction was observed in case of treatment with combination i.e. 4.76±0.180 mg/dl (table 3, figure 10).
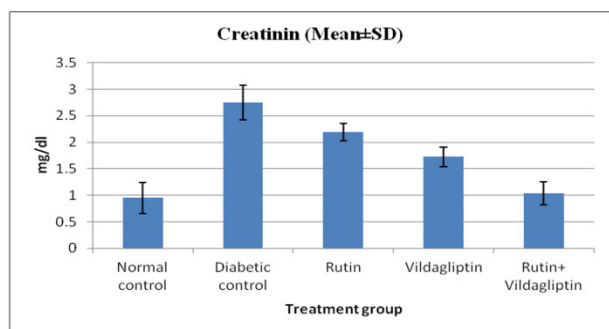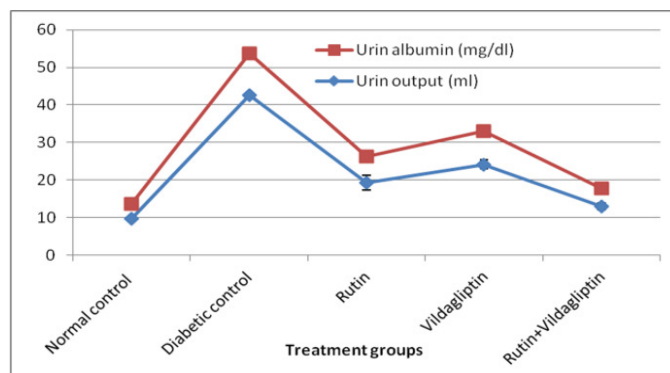


**Figure-7**
**Plasma level of creatinine**



**Figure-10**
**Urin output and level of urine albumin in different group**

**Histopathology study in different groups:** The histological examination of tissues isolated from mice showed variation in normal, diabetic and treated groups. Diabetic nephropathy resulted in degeneration of epithelium, interstitial oedema and necrosis. The combination of rutin and vildagliptin attenuated necrosis, reduced inflammatory cells and improved tubule and glomeruli (figure 11).

**Table-1**
**Observation table of oxidative stress in alloxan induced nephropathy**

| S. No. | Treatment Group | LPO (nM/mg protein) | GSH (nM/mg wet tissue) | CAT (U/mg protein) | SOD (U/mg protein) |
|---|---|---|---|---|---|
| 1 | Normal control | $1.867 \pm 0.263^{\#}$ | $25.586 \pm 2.276^{\#}$ | $11.433 \pm 0.827^{\#}$ | $78.485 \pm 1.805^{\#}$ |
| 2 | Diabetic control | $6.947 \pm 0.396^{*}$ | $6.520 \pm 0.786^{*}$ | $5.766 \pm 0.987^{*}$ | $11.546 \pm 1.921^{*}$ |
| 3 | Rutin | $4.822 \pm 0.378^{*,\#}$ | $16.173 \pm 0.390^{*,\#}$ | $7.993 \pm 0.757^{*,\#}$ | $69.311 \pm 2.852^{*,\#}$ |
| 4 | Vildagliptin | $5.499 \pm 0.386^{*,\#}$ | $15.741 \pm 0.504^{*,\#}$ | $6.875 \pm 0.419^{*,\#}$ | $56.814 \pm 1.724^{*,\#}$ |
| 5 | Rutin+Vildagliptin | $2.165 \pm 0.268^{*,\#}$ | $21.947 \pm 1.089^{*,\#}$ | $9.928 \pm 0.612^{*,\#}$ | $72.704 \pm 0.822^{*,\#}$ |

Values are given as means±standard deviation (mean of six determinations), **\***Mean significant difference in compare to the vehicle group p<0.05, **\*\***Mean no significant difference in compare to the vehicle group p>0.05, **#**Means significant difference in compare to the diabetic control group p<0.05, **##**Means no significant difference in compare to the diabetic control group p>0.05.

**Table-2**
**Observation table of kidney function test in alloxan induced nephropathy**

| S. No. | Treatment group | Protein (g/dl) | Albumin (g/dl) | Creatinine (mg/dl) | Urea (mg/dl) | Uric acid (mg/dl) |
|---|---|---|---|---|---|---|
| 1 | Normal control | $7.263 \pm 0.746^{\#}$ | $3.926 \pm 0.616^{\#}$ | $0.952 \pm 0.291^{\#}$ | $23.915 \pm 0.527^{\#}$ | $1.108 \pm 0.266^{\#}$ |
| 2 | Diabetic control | $3.058 \pm 0.416^{*}$ | $1.882 \pm 0.332^{*}$ | $2.748 \pm 0.327^{*}$ | $46.355 \pm 0.615^{*}$ | $3.893 \pm 0.312^{*}$ |
| 3 | Rutin | $5.918 \pm 0.338^{*,\#}$ | $2.765 \pm 0.262^{*,\#\#}$ | $2.195 \pm 0.163^{*,\#}$ | $30.918 \pm 0.403^{*,\#}$ | $2.228 \pm 0.277^{*,\#}$ |
| 4 | Vildagliptin | $3.828 \pm 0.334^{*,\#}$ | $2.015 \pm 0.382^{*,\#}$ | $1.723 \pm 0.184^{*,\#}$ | $27.526 \pm 0.579^{**,\#}$ | $3.958 \pm 0.272^{*,\#}$ |
| 5 | Rutin+Vildagliptin | $7.097 \pm 0.733^{**,\#}$ | $3.076 \pm 0.409^{**,\#}$ | $1.041 \pm 0.217^{**,\#}$ | $25.770 \pm 0.473^{*,\#}$ | $1.983 \pm 0.208^{**,\#}$ |

Values are given as means±standard deviation (mean of six determinations), **\***Mean significant difference in compare to the vehicle group p<0.05, **\*\***Mean no significant difference in compare to the vehicle group p>0.05, **#**Means significant difference in compare to the diabetic control group p<0.05, **##**Means no significant difference in compare to the diabetic control group p>0.05.

**Table-3**
**Urinary analysis parameters of different group**

| S. No. | Treatment group | Urine output (ml/hour) | Urine albumin (mg/dl) |
|---|---|---|---|
| 1 | Normal control | $9.730 \pm 0.630^{\#}$ | $3.830 \pm 0.492^{\#}$ |
| 2 | Diabetic control | $42.52 \pm 0.821^{*}$ | $11.25 \pm 0.504^{*}$ |
| 3 | Rutin | $19.27 \pm 1.981^{*,\#}$ | $6.93 \pm 0.411^{*,\#}$ |
| 4 | Vildagliptin | $24.13 \pm 1.206^{*,\#}$ | $8.87 \pm 0.372^{*,\#}$ |
| 5 | Rutin+Vildagliptin | $13.01 \pm 0.896^{*,\#}$ | $4.76 \pm 0.180^{**,\#}$ |

Values are given as means±standard deviation (mean of six determinations) *Mean significant difference in compare to the vehicle group p<0.05, **Mean no significant difference in compare to the vehicle group p>0.05,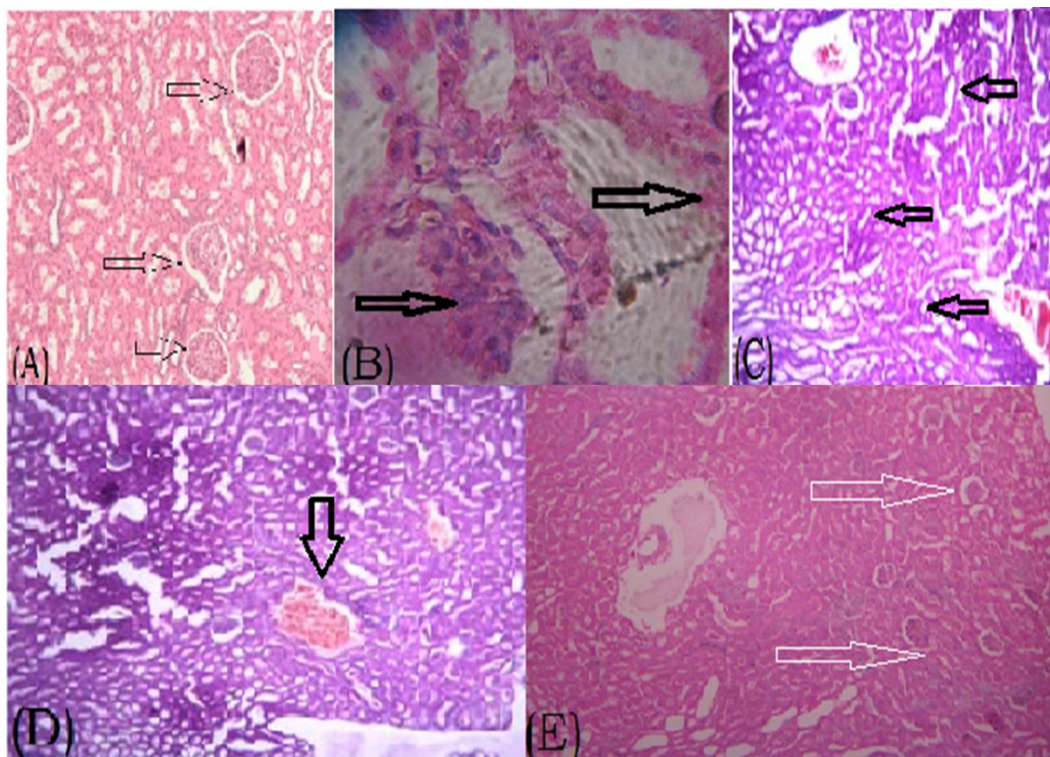 **#**Means significant difference in compare to the diabetic group p<0.05, **##**Means no significant difference in compare to the diabetic group p>0.05.

**Figure-11**

**Histological sections of kidney of (A) normal control mice with normal tubular brush-borders (B) diabetic group showing swelling of tuft and degeneration and desquamation of capsular epithelium marked concentration of erythrocytes in tubular cap, tubular brush-borders loss, interstitial oedema, glomerular hypercellularity, necrosis of epithelium and inflammatory cells infiltration (C) rutin treated group with improved tubule and glomeruli no evidence of interstitial odema (D) vildagliptin treated group with improved tubule and glomeruli, and (E) rutin and vildagliptin combination treated group (hematoxylin and Eosin, 400 X) with attenuated necrosis, reduced inflammatory cell and improved tubule and glomeruli.**

**Discussion:** Rutin and vildagliptin are two important drugs and are useful on account of their antioxidant and antidiabetic potential. Diabetic nephropathy induced by alloxan injection results an enhanced blood glucose level and oxidative damage to kidney tissues. As a result of oxidative stress sudden increase in level of antioxidant enzyme lipid peroxidase and decrease in the level of superoxide dismutase, catalase and reduced glutathione was also observed. Plasma protein and albumin was found decreased on the other hand creatinine and blood urea nitrogen was increased. Urine output and urine albumin level was increased as compared to that measured from normal control group. Signs of severe damage to kidney tissues were seen when examined under light microscope after the animals were sacrificed. The biochemical parameters measured after treatment with rutin, vildagliptin and the combination were correlated with those obtained from untreated normal animals. It was observed that the combination of rutin and vildagliptin brought the biochemical parameters more close to normal control better than rutin and vildagliptin alone. At the same time the histological studies revealed that alloxan induced damage to the glomerular and tubular structure was also ameliorated with combination therapy.

## Conclusion

The proposition of our research was made to hit upon an alternative treatment therapy for diabetes induced nephropathy. The combination of rutin and vildagliptin provides the advanced results of treating diabetic nephropathy than either of the two drugs alone. All the biochemical parameters in our results advocate the combination as one of the most constructive combination therapy against diabetes.

## Acknowledgement

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