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Effect of Combination of Hydroalcoholic Seed Extract of *Areca Catechu* With Gliclazide in Alloxan-Induced Diabetic Nephropathy in Wistar Rats

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ABSTRACT
Background and Objective: *To study the renoprotective effect of hydroalcoholic seed extract of Areca catechu (AN) in conjunction with gliclazide in alloxan-induced diabetic nephropathy in male Albino wistar rats.* ***Methods:*** *To induce diabetic nephropathy, a single intraperitoneal injection of 150 mg/kg of alloxan monohydrate was given. Hydroalcoholic seed extract of A. catechu (AN) was administered at three doses i.e., 250mg/kg, p.o., 500mg/kg, p.o., and 1000mg/kg, p.o., and gliclazide (25mg/kg, p.o.) for a period of 28 days as an individual treatment as well as in combination. To evaluate the renal functions, various biochemical tests were carried out, including albumin, serum creatinine, BUN, kidney weight/body weight (%) ratio, and serum glucose. Total brain protein and reduced glutathione were estimated to measure oxidative stress. Lipid profile was evaluated by measuring serum cholesterol, triglycerides, and high-density lipoprotein (HDL).* ***Results:*** *Administration of AN and gliclazide individually as well as in combination significantly attenuated the serum glucose, BUN, serum creatinine, albumin levels, and oxidative stress. In the treated groups, serum lipids such as triglycerides and total cholesterol decreased while HDL increased.* ***Conclusion:*** *The outcomes of our investigation indicate that the glucose-lowering, lipid-lowering, and anti-oxidative properties of the polyphenols found in areca nuts may be responsible for the renoprotective effect of AN both when taken alone* ***and in conjunction with gliclazide.***

Key words: Diabetic nephropathy; *Areca catechu*; Gliclazide; Polyphenols

INTRODUCTION

The microvascular problems linked to diabetes mellitus include diabetic nephropathy (DN). It is characterized by glomerulosclerosis and nephrotic syndrome following a partial loss of the renal function. The main cause of diabetes-related morbidity and mortality is "chronic kidney disease," which is also a key contributor to end-stage renal disease (ESRD). Approximately 20-40% of type I patients with diabetes and less than 20% of patients with type II diabetes develop ESRD. The onset and progression of DN are the primary risk factors for hyperglycemia, hypertension, and genetic predisposition. Oedema, decreased glomerular filtration rate (GFR), arterial blood pressure, and persistently elevated albuminuria are a few clinical characteristics of DN.[1] In order to achieve sufficient blood glucose control and treat concurrent pathologies associated to type II diabetes, combined pharmaceutical therapy is typically used in the management of the condition. Combining a drug with one or more medications or herbal remedies is known as combined therapy. Oral hypoglycemic medication therapy for diabetic kidney disease patients requires to be periodically and carefully monitored because renal function declines below 60ml/min of GFR may result in multiple pharmacokinetic alterations.[2]

One of the most often prescribed medications for diabetes is gliclazide. It is an oral hypoglycemic sulphonylurea of the second generation, which can delay the onset and progression of DN and lower blood sugar levels. However, the fundamental mechanism is still unclear. Multiple studies have shown that gliclazide protects against high glucose levels and advanced glycation end products.[3]

Areca nut is obtained from dried seed of the fruit obtained from *Areca catechu*, which belongs to the family *Arecaceae*. Since prehistoric times, areca nuts have been widely used in Chinese and Indian medicine.[4] The Sanskrit corpus of ayurveda and other works, which include references to the use of areca nut and betel quid (which primarily contains areca nut, lime, and betel leaf) or tambula, provide the complex rationale for the use of areca nut.[5] Polyphenols, which include tannins, flavonoids, and alkaloids, make up majority of the chemical components of areca nut. Plant functional chemicals known as polyphenols have a variety of beneficial bioactivities, one of which is for the management of T2DM.[6] Furthermore, it has been suggested that they can avert chronic diabetes-related problems like neuropathy, retinopathy, nephropathy, and cardiovascular disease.[7]

Hence, the aim of this study was to assess the combined impact of gliclazide and the hydroalcoholic seed extract of *Areca catechu* on diabetic nephropathy.

MATERIALS AND METHODS

Collection of Plant material

The seeds of Areca nut (*A. catechu*) were obtained in Kundapura, Karnataka, in November 2022. Dr. V. Rama Rao, Research Officer (Botany), Central Ayurveda Research Institute, Bangalore, Karnataka, India, carried out the taxonomic identification. A voucher specimen, designated RRCBI-mus114, has been deposited.

Preliminary phytochemical screening

Following an initial screening of the phytochemicals, the following compounds were found: saponins, proteins and amino acids, alkaloids, phytosterols flavonoids, tannins, glycosides, and. cardiac glycosides.[8]

Preparation of hydroalcoholic seed extract of *Areca catechu*

Hot percolation using the Soxhlet method was used to carry out the extraction. A 50:50 ethanol-water mixture was used to extract 200g of areca powder through a continuous hot percolation process with a Soxhlet apparatus[9]. A rotary evaporator was used to evaporate the solvent which produced a concentrated thick mass. After that, a freeze dryer was used to dry it. Before being used, the dried extract was stored in the refrigerator at 4°C. 10%w/w was found to be the percentage yield of the extract.

Chemicals and reagents

Alloxan monohydrate (Loba Chemie Pvt. Ltd. Maharashtra, India), Gliclazide (Gift sample from Bal Pharma, Bengaluru, Karnataka, India), Tween 80 (Spectrum PVT. Ltd. Mumbai, India), Glucose test kit (ERBA Diagnostics Mannheim), Blood urea nitrogen kit (ERBA Diagnostics Mannheim), Creatinine kit (ERBA Diagnostics Mannheim), Albumin kit (Span Diagnostics), Cholesterol test kit (ERBA Diagnostics Mannheim), Triglyceride kit (ERBA Diagnostics Mannheim), High density lipoprotein test kit (Span Diagnostics), Thiopental sodium injection I.P. (Neon Laboratories Limited).

EXPERIMENTAL ANIMAL STUDY

The male Albino wistar rats (200-250g) were kept at room temperature, in adequately ventilated animal house under 12hrs light-dark cycle. Prior to conducting this experiment, Institutional Animal Ethics Committee (IAEC) approval was obtained. The animals were housed in an animal house in standard condition for one week before being used, as directed by the "Committee for the Control and Supervision of Experiments on Animals" (CCSEA). During this period, the Albino wistar rats were provided with unrestricted access to standard rat chow and water.

ACUTE ORAL TOXICITY

The study conducted by Bhandare A M et al. examined the acute oral toxicity of AN. According to OECD standards 423, it was carried out on healthy adult female albino Swiss mice and albino Wistar rats that were not pregnant. The mice and rats were orally administered with 2000mg/kg. During the 14-day observation, the mice and rats receiving the 2000mg/kg dose did not cause mortality, exhibit any toxicity, or exhibit any changes in their general behaviour or other physiological activities[10]. Hence, $1/2^{th}$, $1/4^{th}$ and $1/8^{th}$ doses were selected.

Induction of Experimental Diabetic Nephropathy

The fasting blood sugar was determined in Albino wistar rats after overnight fast with free access to water. A single intraperitoneal injection of newly prepared Alloxan monohydrate (150mg/kg dissolved in normal saline) was used to induce diabetic nephropathy[11]. The animals were then left aside for 4 hours before receiving a 10% glucose solution for 24 hours. By estimating the blood glucose level on the fifth day, the diabetes was confirmed. For the study, rats with blood glucose levels >250mg/dl were selected.

TREATMENT

The animals were segregated into 6 groups (n=6). The treatment for the respective groups were carried out for 28 days.

Group 1- 1% Tween 80 in Normal control animals

Group 2- Alloxan (150mg/kg, i.p.,) in normal saline (0.9% w/v NaCl)

Group 3- Gliclazide (25mg/kg, p.o.,)+ Hydroalcoholic seed extract of *A. catechu* (1000mg/kg, p.o.,) in normal animals

Group 4- Gliclazide (25mg/kg, p.o.,)+ Hydroalcoholic seed extract of *A. catechu* (250mg/kg, p.o.,) in diabetic animals

Group 5- Gliclazide (25mg/kg p.o.,)+ Hydroalcoholic seed extract of *A. catechu* (500mg/kg, p.o.,) in diabetic animals

Group 6- Gliclazide (25mg/kg, p.o.,)+ Hydroalcoholic seed extract of *A. catechu* (1000mg/kg, p.o.,) extract in diabetic animals

Sample Collection

At the end of the trial, blood samples were taken by retro-orbital sinus using thiopental sodium anesthesia (40 mg/kg, i.p.), and serum was separated. The samples that were obtained were stored between 4°C and 6°C until the estimations were finished. Biochemical parameters estimated-

1. Serum glucose [12]
2. Blood urea nitrogen (BUN)[15]
3. Serum creatinine[13]
4. Serum albumin[1]
5. Serum total cholesterol[13]
6. Serum high density lipoprotein[1]
7. Serum triglycerides[13]
8. Glycated haemoglobin content[14]
9. Kidney weight/ body weight (%)[1]
10. Reduced glutathione[1]
11. Total brain protein[1]

All the parameters were assessed on the 28th day.

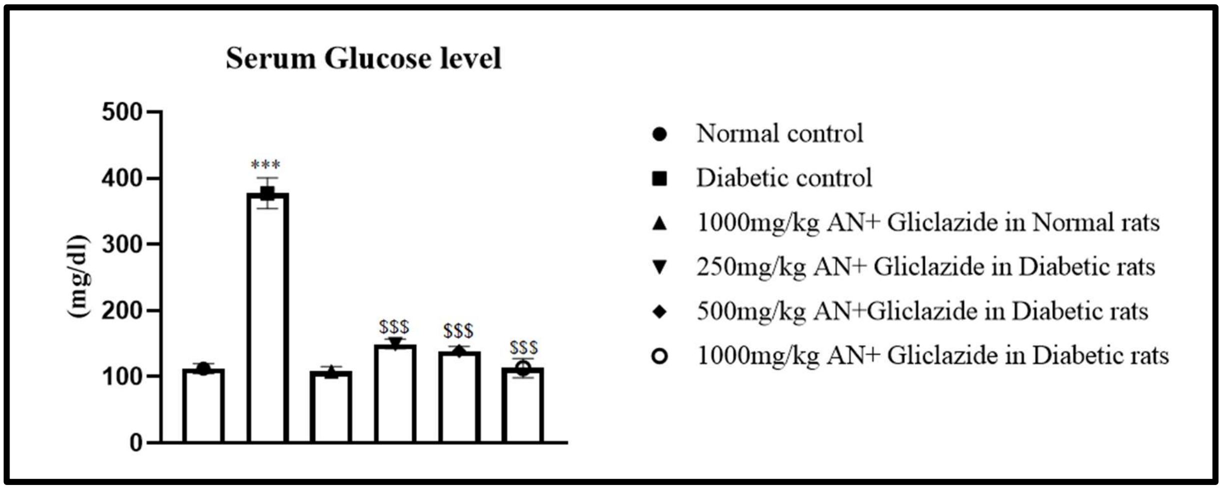
STATISTICAL ANALYSIS

The findings are presented as mean ± S.E.M. To ascertain the statistical significance, one-way analysis of variance (ANOVA) was employed and Tukey's Post hoc test was then performed. Statistics were deemed significant if $p<0.05$.

RESULTS

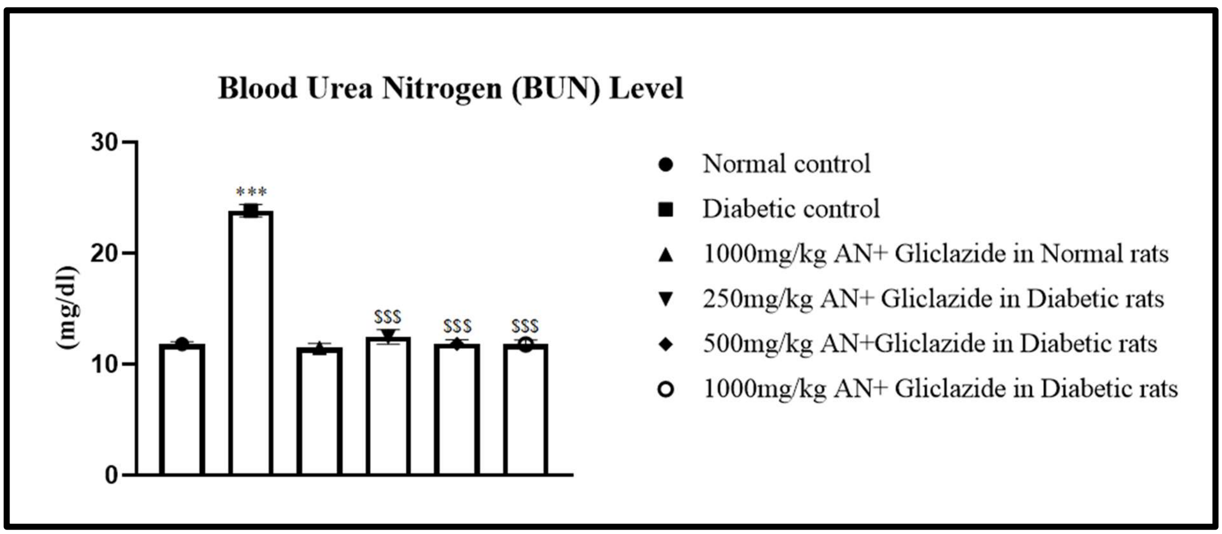
Rats with diabetic nephropathy were chosen based on blood glucose levels greater than 250mg/dl on the fifth day following an intraperitoneal injection of 150mg/kg of alloxan monohydrate. All the treated groups showed decrease in serum glucose, BUN, creatinine, albumin, serum total cholesterol, serum triglyceride, HbA1C, reduced glutathione, and Total brain protein, whereas, an increase in HDL levels were observed.

Fig No. 1- Effect of Areca nut and Gliclazide on Serum Glucose level



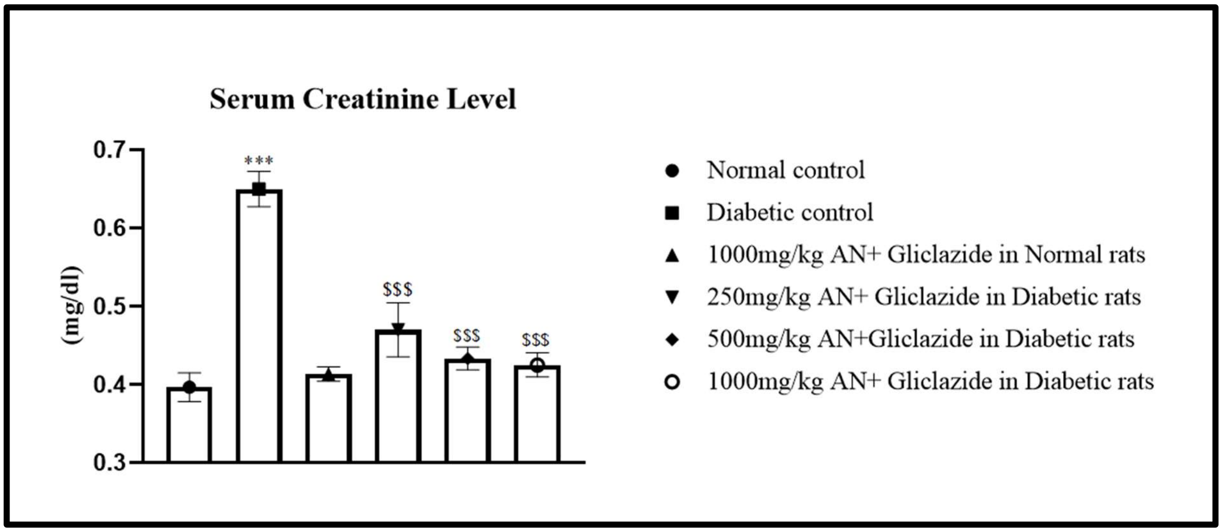
Values expressed as mean ± SEM, n = 6, ANOVA with Tukey's post-hoc test; ***p<0.001 is the difference between the normal control and the diabetic control, and $$$p<0.001 is the difference between the two.

Fig No. 2- Effect of Areca nut and Gliclazide on Blood Urea Nitrogen level



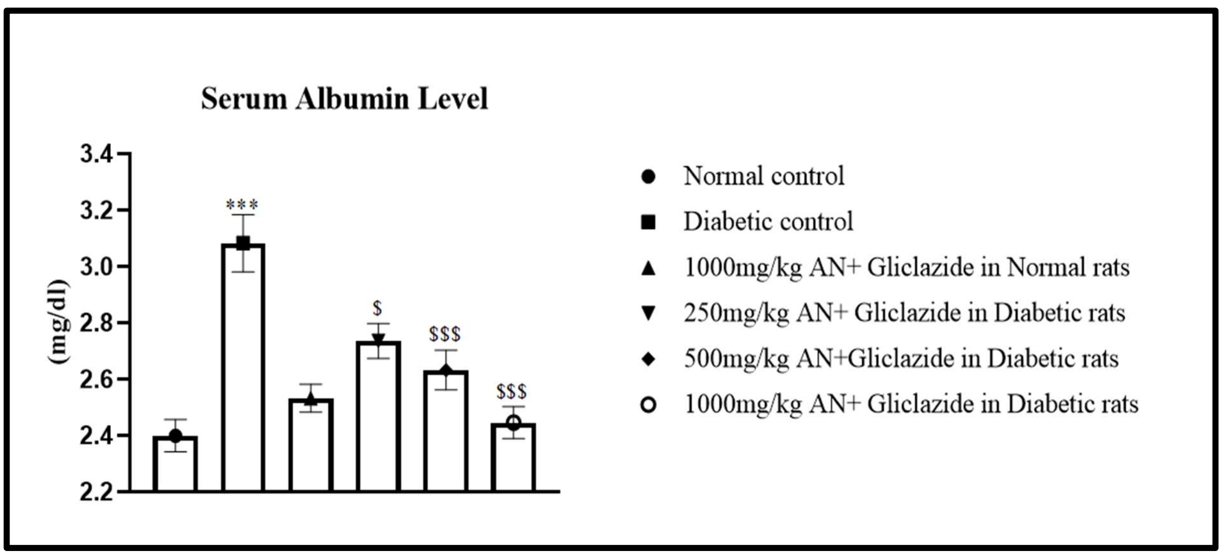
The values were expressed as mean ± SEM, n = 6, ANOVA with Tukey's post-hoc test applied; the comparisons to the control group were $$$p<0.001 and the diabetes control group was ***p<0.001.

Fig No. 3- Effect of Areca nut and Gliclazide on Serum Creatinine level



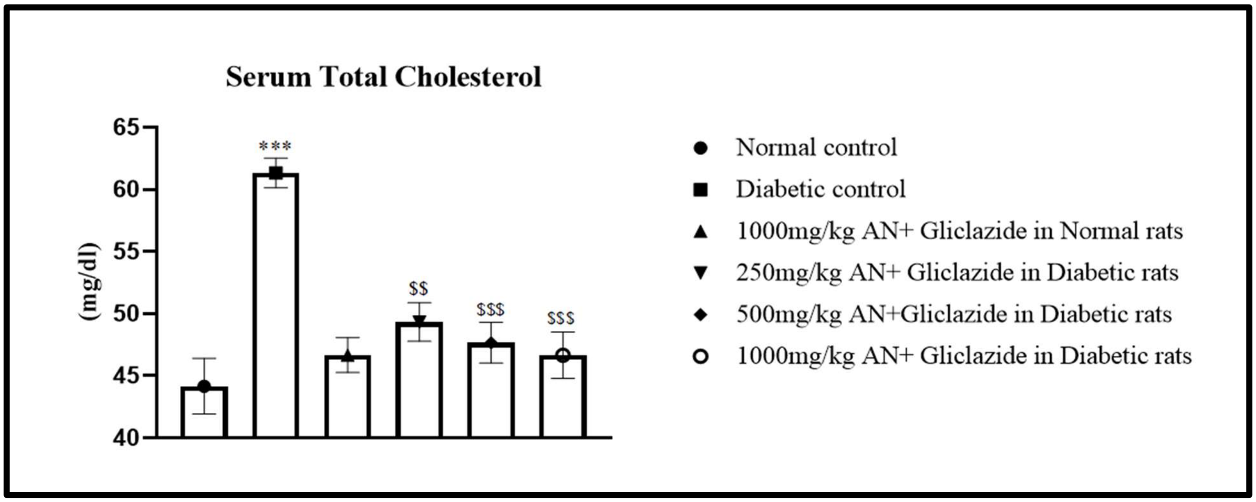
The values were expressed as mean ± SEM, with n = 6. The analysis was done using ANOVA and Tukey's post-hoc test. The results showed that ***p<0.001 was different from the normal control and $^{\$\$\$}p<0.001$ was different from the diabetic control.

Fig No. 4- Effect of Areca nut and Gliclazide on Serum Albumin level



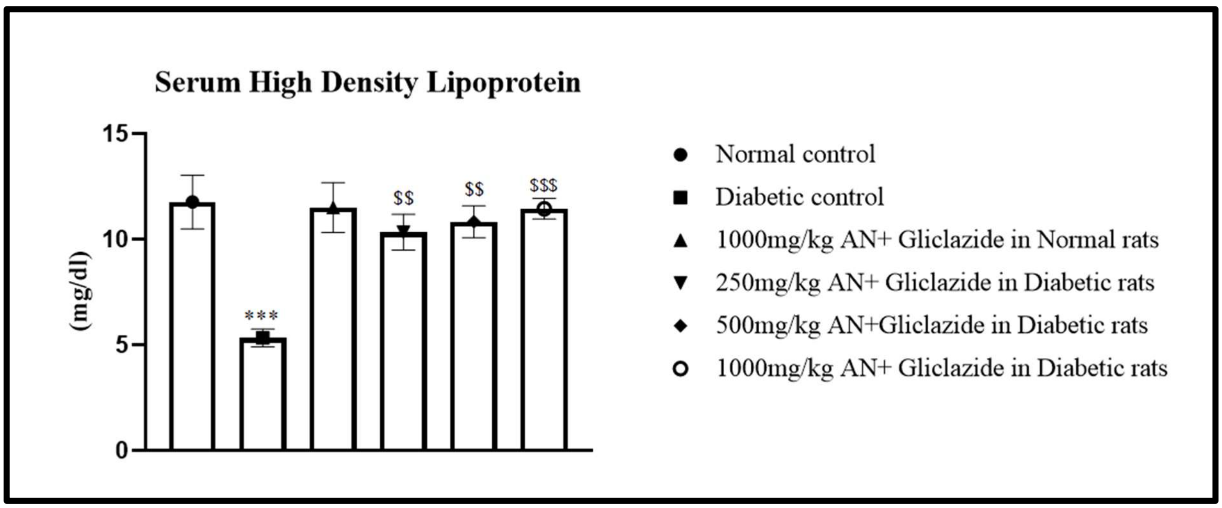
ANOVA with Tukey's post-hoc test was used to analyze the values, which are shown as mean ± SEM, n = 6, ***p<0.001 compared to normal control, $p<0.05 compared to diabetes control, and $^{\$\$\$}p<0.001$ compared to diabetic control.

Fig No. 5- Effect of Areca nut and Gliclazide on Serum Total Cholesterol level



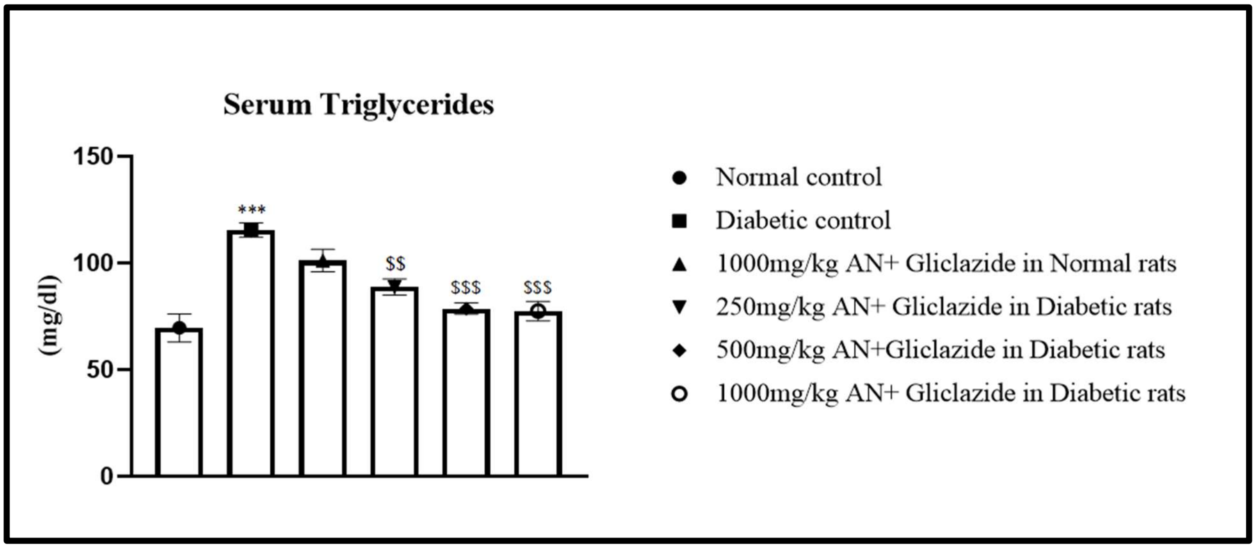
This study used ANOVA with Tukey's post-hoc test and the following values were expressed as mean ± SEM, n = 6, ***p<0.001 compared to normal control, $^{\$\$}$p<0.01 compared to diabetic control, and $^{\$\$\$}$p<0.001 compared to diabetic control.

Fig No. 6- Effect of Areca nut and Gliclazide on Serum High Density Lipoprotein (HDL) level



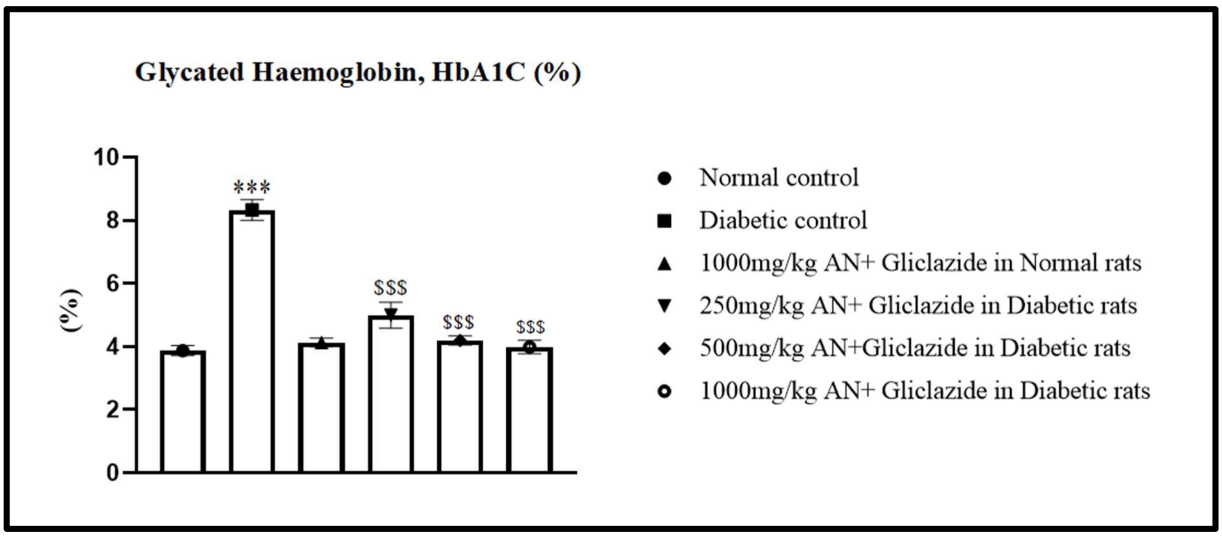
Values were expressed as mean SEM, n = 6, ANOVA, followed by Tukey's post-hoc test, ***p<0.001 when compared to normal control, $^{\$\$}$p<0.01 when compared to diabetes control, and $^{\$\$\$}$p<0.001 when compared to diabetic control.

Fig No. 7- Effect of Areca nut and Gliclazide on Serum Triglyceride level



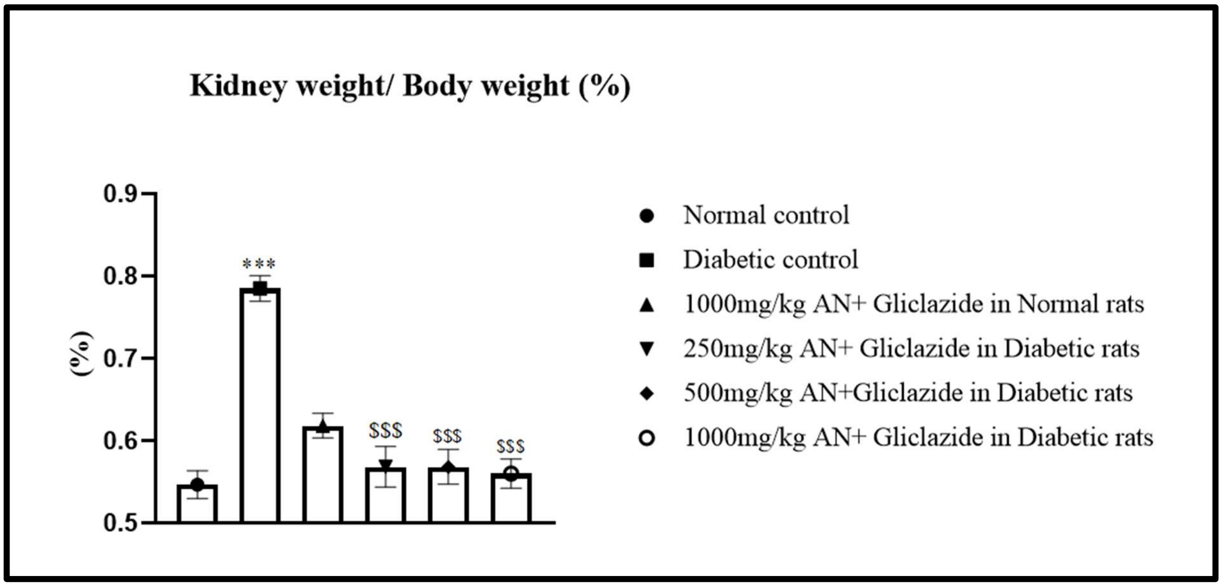
Values are expressed as mean SEM, with n = 6, ANOVA followed by Tukey's post-hoc test, ***p<0.001 when compared to normal control, $^{\$\$}$ p<0.01 when compared to diabetic control, and $^{\$\$\$}$p<0.001 when compared to diabetic control.

Fig No. 8- Effect of Areca nut and Gliclazide on Glycated Haemoglobin level



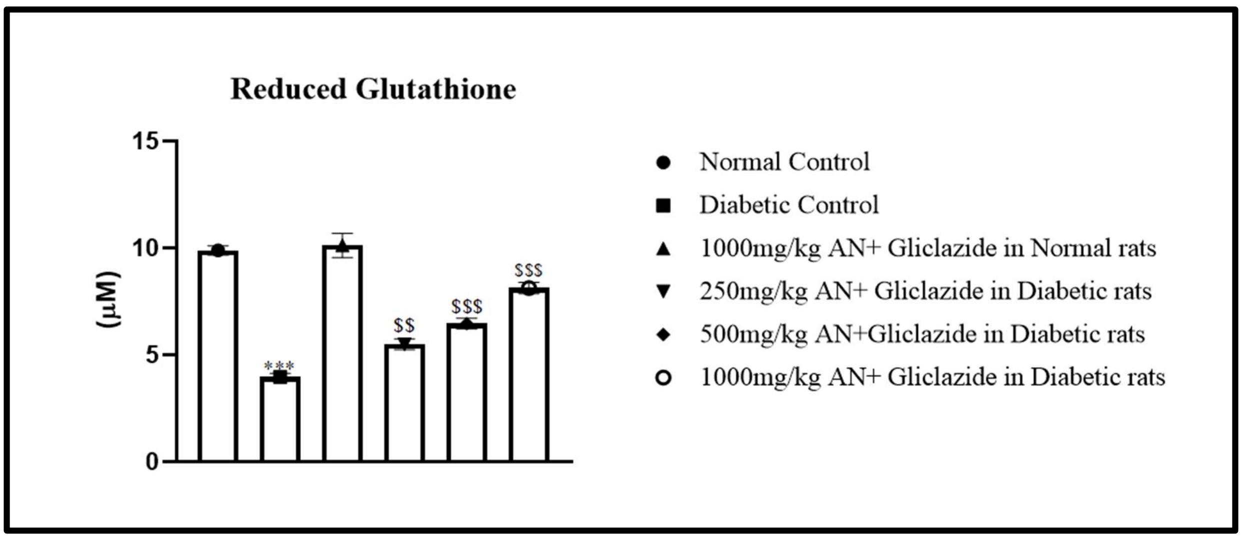
The values were expressed as mean ± SEM, n = 6, ANOVA with Tukey's post-hoc test applied; the comparisons to the control group were $$$p<0.001 and the diabetes control group was ***p<0.001.

Fig No. 9- Effect of Areca nut and Gliclazide on Kidney weight/Body weight (%)



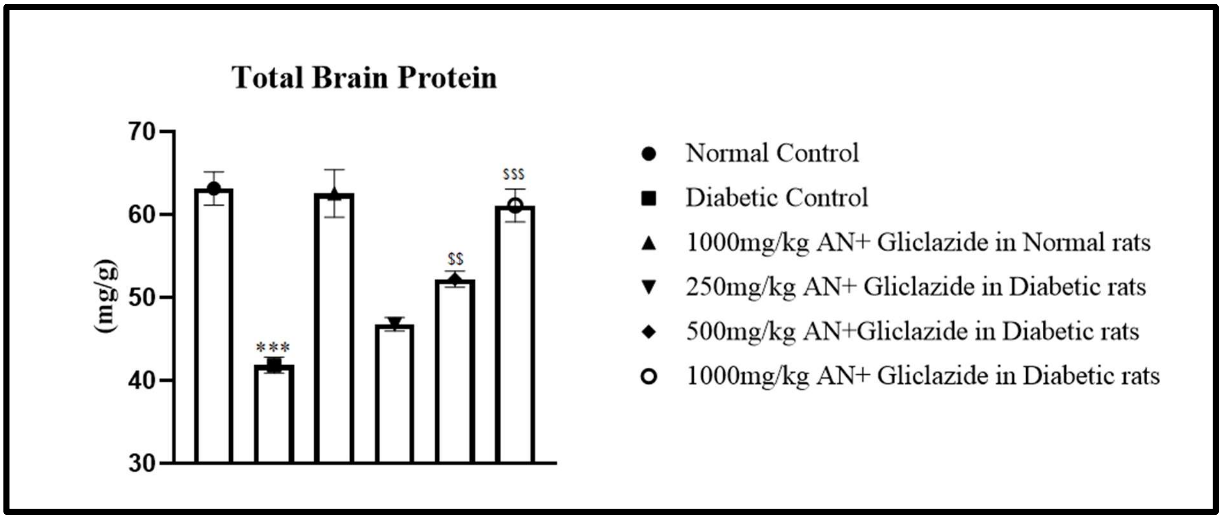
The values were expressed as mean ± SEM, with n = 6. The analysis was done using ANOVA and Tukey's post-hoc test. The results showed that ***p<0.001 was different from the normal control and $$$p<0.001 was different from the diabetic control.

Fig No. 10- Effect of Areca nut and Gliclazide on Reduced Glutathione (µM)



Values are expressed as mean SEM, n = 6, ANOVA followed by Tukey's post-hoc test, ***p<0.001 when compared to normal control, and $$p<0.01 when compared to diabetes control, respectively.

Fig No. 11- Effect of Areca nut and Gliclazide on Total Brain Protein



Values expressed as mean SEM, n = 6, ANOVA with Tukey's post-hoc test, and ***p0.001 when compared to normal control In comparison to the diabetic control, $^{\$\$}p<0.01$ and $^{\$\$\$}p<0.001$ respectively.

DISCUSSION

In the present study, diabetes-induced group had significantly higher serum glucose levels than the normal control group. This occurs as a result of a partial insulin deficiency, which causes abnormal carbohydrate metabolism.[12] In contrast to the diabetic control group, normal rats given 1000mg/kg of AN and 25 mg/kg of gliclazide did not exhibit any statistically significant differences. The other groups in which different doses of AN was administered along with 25mg/kg of gliclazide treated in diabetic rats showed dose dependent decrease in serum glucose level which was significant. This could be due to the presence of polyphenols which has a potential hypoglycemic activity. Studies conducted by Chempakam B (1993), Anthikat et al, Amudhan and Begum (2008), indicated the anti-diabetic property of areca nut due to the presence of alkaloids, mainly, arecoline.[16, 17, 18] The possible mechanism explained by them was its inhibitory action on α-glycosidase enzymes and ability to increase the insulin levels.[16, 17, 19]

The diabetic control group had higher serum BUN and creatinine levels than the normal control group. Due to abnormal glucose regulation, alloxan-induced diabetes causes significant renal damage. This includes increased tissue levels of glucose and glycosylated proteins, hemodynamic alterations in the tissues of the kidney, and an increase in oxidative stress. Such an increase in diabetes could be caused by damage or loss of glomeruli.[20] Normal rats treated with 1000mg/kg of AN was non-significant in contrast to normal control group. Whereas, rats with diabetes, treated with various doses of AN along with 25mg/kg of gliclazide was found to significantly decrease the serum BUN and serum creatinine. The effect that has been observed may be explained by the existence of polyphenols, which have been shown to have beneficial impacts on serum glucose levels, glucose regulation, tissue levels of glycosylated proteins, renal tissue hemodynamic changes, and oxidative stress reduction. It has been demonstrated that polyphenols such as epicatechin, epigallocatechin, and catechin effectively lower serum BUN and serum creatinine.[21, 22]

The diabetic control group had a significantly increased level of serum albumin in comparison to the normal control group. Abnormalities in the glomerular endothelial barrier lead to both excessive filtration and renal albumin degradation and reabsorption, which in turn causes an increase in serum albumin levels. Serum albumin levels considerably rose in normal rats given 1000mg/kg of AN in contrary to the normal control group. In contrast, diabetic rats treated with three doses of AN along with 25mg/kg of gliclazide showed significant alleviation in serum albumin levels. Reduced oxidative stress, reduced apoptosis, and alleviation of renal fibrosis could be the mechanisms by which polyphenols lower serum albumin levels.[21]

Diabetes mellitus is characterized by insufficient insulin secretion or action, which causes abnormalities in the metabolism of fats and proteins, raising serum levels of lipoprotein and lipids and contributing significantly to the development of severe and premature atherosclerosis. The primary cause of these abnormal lipid levels is the unrestricted activity of lipolytic hormones present on the fat depots as a result of reduced insulin action. Triglycerides are hydrolysed by lipoprotein lipase, an enzyme that is normally activated by insulin. Hypertriglyceridemia results from insulin deficiency, which prevents lipoprotein lipase from being activated in diabetics. Moreover, hypercholesterolemia is associated to this.[23] When correlated to the normal control group, normal rats given 1000mg/kg of AN did not exhibit a statistically significant difference in serum triglyceride and total cholesterol levels. Whereas, diabetic rats administered with 3 doses of AN along with 25mg/kg of gliclazide was found to significantly alleviate the serum total cholesterol and serum triglyceride levels. This implies that, respective treatment can prevent or be helpful in reducing the complications of lipid profile by its protective activity over pancreatic cells leading to insulin secretion to activate lipoprotein lipase.[24] Similarly, the concentration of HDL will be reduced due to inactivation of lipoprotein lipase in case of diabetic condition. Similarly, diabetic control rats had significantly lower HDL concentrations than normal control rats. A non-significant difference in serum HDL levels of normal rats administered with 1000mg/kg of AN was observed in contrary to normal control rats. Whereas, diabetic rats administered with 3 doses of AN along with 25mg/kg of gliclazide was found to significantly increase the serum HDL level.

Renal hypertrophy resulting from the accumulation of glycogen granules in the kidney's distal tubules is a significant morphological alteration related to diabetic nephropathy. In diabetic nephropathy, kidney weight/body weight (%) is said to indicate renal hypertrophy.[1] Compared to the rats in the normal control group, the kidney weight/body weight (%) of the diabetic control group was substantially higher. The difference between the normal rats treated with 1000mg/kg of AN was non-significant in contrast to normal control rats. The kidney weight/body weight (%) of diabetic rats treated with three doses of AN and 25 mg/kg of gliclazide, however, was substantially decreased.

Prolonged exposure to high blood glucose concentrations causes an upsurge in the non-enzymatic and auto-oxidative glycosylation of proteins. There was a substantial increase in the glycosylated hemoglobin level (HbA1C) between diabetic control rats and normal control rats. There were no discernible changes between the normal control group and the normal rats treated with 1000 mg/kg of AN. There was a significant alleviation of HbA1C in the rats of the treatment groups compared to diabetic control group. This could be the result of a reduced exposure to elevated blood glucose levels.[25]

Reduced glutathione and total brain protein decrease are the main factors influencing oxidative stress in diabetic nephropathy. In normal control group, there was an alleviation in the levels of total brain protein and reduced glutathione, comparatively. The reduced glutathione was significantly elevated in diabetic rats which were administered with 250mg/kg, 500mg/kg, and 1000mg/kg of AN along with 25mg/kg gliclazide in contrast to diabetic control group. The total brain protein level was non-significant in diabetic rats which were administered with 250mg/kg of hydroalcoholic extract of *A. catechu.* Whereas, it was increased significantly in diabetic rats treated with 500mg/kg and 1000mg/kg of AN along with 25mg/kg gliclazide in contrast to diabetic control group. Polyphenols is a phytochemical which is previously established for its antioxidant activity. Hence, polyphenols are responsible for reducing the oxidative stress.[24, 26]

CONCLUSION

It is possible to draw the conclusion that arecanut combined with gliclazide could be an option for therapy for diabetic nephropathy based on the study's results. Its possible anti-diabetic, anti-oxidant, hypolipidemic, and renoprotective properties may be responsible for this protective effect.

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CONFLICT OF INTEREST

None

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