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Methanol leaf extract of *Momordica charantia* protects alloxan-induced nephropathy through modulation of BCL2/ NF-κB signalling pathways in rats

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ABSTRACT

Background & Aim: Nephropathy is one of the major complications of diabetes with oxidative stress as one of the possible mechanisms mediating the event. Natural products with antioxidant property may be a promising therapeutic approach to ameliorate renal damage from diabetic nephropathy hence the renoprotective activity of methanolic leaf extract of *Momordica charantia* (MEMC) was assessed.

Experimental: The effects of MEMC on alloxan-induced nephrotoxicity were examined where toxicity was induced by intraperitoneal administration of alloxan to 50 rats divided into five groups of 10 rats each. MEMC was administered to two groups at the doses of 200 and 400 mg/kg for 28 days; glibenclamide administered to another group of diabetic rats. While another group was left untreated, a group of normal rats received only distilled water. Nephroprotective effect of the extract was studied by assessing its effect on oxidative stress markers, antioxidant defence system, immunohistochemistry, histological and serum urea and creatinine analysis.

Results: Alloxan administration altered renal biomarkers (increased serum urea and creatinine levels), increased renal H_2O_2 malondialdehyde levels, and decreased reduced glutathione, glutathione peroxidase, catalase, and superoxide dismutase. Histological studies showed glomerular degeneration and hypercellularity. However, administration of glibenclamide (4 mg/kg) and MEMC ameliorated the alloxan-induced nephrotoxicity. Immunohistochemical studies revealed lower expressions of BCL2 but greater expressions of NF-κB in the kidney of the toxicant non-treated rats compared with the control, glibenclamide treated and MEMC treated rats.

Recommended applications/industries: MEMC showed renoprotective activity in alloxan-induced nephropathy mediated through its antioxidant and anti-inflammatory activities. This extract could be used in the treatment of acute kidney failure.

1. Introduction

Diabetes mellitus is often associated with decrease functional capacity of the kidney (Jain, 2012), with a prevalence of 20-40% end-stage renal disease reported in diabetic patients (Hakim and Pfluerger, 2010). Several reports have implicated oxidative stress induced by persistent elevation in blood glucose level as a pathogenic mechanism in the development and progression of diabetic nephropathy (Park *et al*., 2001; Kawahito *et al.,* 2009). Hyperglycemia-induced oxidative stress resulting from excessive production of reactive oxygen species increases the expression of transforming growth factor-β (TGF-β), an important mediator of renal interstitial fibrosis, via the modulation of mitogen-activated protein kinase and nuclear receptor peroxisome proliferator-activated receptor-γ (Rueter *et al.,* 2010; Han *et al.,* 2017). Moreover, hyperglycaemia stimulates the production of other inflammatory cytokines such as TNF-α and MCP-1 in the kidney microvasculature, and as a result promotes processes leading to tubular fibrosis, glomerulosclerosis and renal failure (Zyger *et al.,* 2014; Barutta *et al.,* 2015).

In the last few decades, several medicinal plants have been investigated as prospective modulators of human and animal diseases because of their antioxidant, antimutagenic, and anticarcinogenic effects (Vinothini *et al.,* 2009). *Momordica charantia* is a vegetable with widely reported metabolic and hypoglycemic effects (Harinantenaina *et al.,* 2016). Although the mechanism of action is still not clear, pure cucurbutanoid compounds extracted from *M. charantia* were reported to exert hypoglycaemic effects in diabetic mice (Krawinkel and Keding, 2006). This study was designed to assess the protective effects and probable mechanism of action of the extract of *M. charantia* on kidney damage associated with experimental diabetes mellitus.

2. Materials and Methods

2.1. Collection and identification of plant material

Fresh leaves of *M. charantia* were collected from the campus of the University of Ibadan, Nigeria. The leaves were identified and authenticated by a taxonomist at the herbarium in the Department of Botany, University of Ibadan and the Voucher Specimen **(UIH-22563)** was assigned.

2.2. Preparation of methanol leaf extract of M. charantia

The leaves were dried at room temperature (27 ± 2^{o}C) and later pulverised to powder. The dried powder samples of *M. charantia* (400 g) were soaked and extracted in 90% methanol (1L) using Soxhlet extractor for 3 days until complete extraction. The extract was filtered through and the filtrate evaporated to dryness under reduced pressure to give an amorphous solid mass with an approximate yield of 12%. The methanol extract was dissolved using 2.5% Tween 80 in normal saline.

2.3. Experimental animals

Fifty healthy male Wistar albino rats (150–200 g) obtained from the Animal house of the Faculty of Veterinary Medicine, University of Ibadan, Nigeria, were used in this study. They were acclimatized to laboratory conditions for two weeks at room temperature (22 ± 2 ºC) under a 12 h light/12 h dark cycle with relative humidity of 55 ± 5% and fed commercial rat cubes (Ladokun and Son Feed, Nigeria Ltd.) and allowed free access to clean fresh water in bottles *ad libitum*. All experimental protocols were following the University of Ibadan Ethics Committee on Research in Animals as well as the National Institute of Health Guide for Care and Use of Laboratory animals (NIH 1985). The Animal Care and Use Research Ethics Committee of the University of Ibadan with UI-ACUREC/App/2015/044 as the approval number approved the study protocol.

2.4. Experimental design

The rats were divided into 5 groups of 10 animals each and the treatment was carried out as follows: Group A (2.5% tween 80 in normal saline), Group B (Alloxan), Group C (Alloxan+ 4 mg/kg glibenclamide), Group D (Alloxan+ 200 mg/kg *M. charantia*), Group E (Alloxan+400 mg/kg *M. Charantia)*. This treatment lasted for 28 consecutive days.

2.5. Preparation of serum and tissues for biochemical assays

Blood samples were collected on days 14 and 28 via the retro-orbital plexus with the aid of clean heparinized capillary tubes into dry clean plain tubes and allowed to coagulate, the blood samples were then centrifuged at 4,000 rpm for 15 min. The serum was

collected and refrigerated at -4^{O}C till the time of analysis. The rats were humanely sacrificed on the 29th day after the termination of the experiment by cervical dislocation and the kidneys were quickly excised, rinsed in normal saline to remove blood, quickly blotted and weighed on digital scale (B303 Mettler-Toledo, Switzerland) and immediately kept in ice to prevent denaturation of biomolecules.

2.6. Biochemical assays

Hydrogen peroxide generation was determined as described by Wolff (1994). Lipid peroxidation (Malondialdehyde, MDA level) was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) according to the methods of Varshney and Kale (1990). Renal non-protein thiol and protein thiol content was determined by the method of Sedlak and Lindsay (1968). Renal GSH (Reduced Glutathione) level was estimated by the method of Jollow *et al.* (1974). The renal glutathione peroxidase (GPx) activity was measured according to Rotruck *et al.* (1973). Glutathione-S-transferase (GST) activity was determined according to Habig *et al.* (1974). The activity profile of SOD in the renal homogenates was determined by the method of Misra and Fridovich (1972). Serum urea and creatinine level were determined using the Randox kits following manufacturer's instructions.

2.7. Immunohistochemistry of renal BCl-2 and NFκB

Immunohistochemistry was done from paraffin sectioned kidney tissues as described by Oyagbemi *et al.* (2015).

2.8. Histopathological Studies

Examination of renal histology was performed according to routine histology techniques as described by Drury *et al.* (1976).

2.9. Statistical analysis

Results were expressed as Mean ± standard deviation (SD) for ten rats in each of the five groups of rats. Statistical analysis was done using the statistical package Graph Pad prism version 5 (Graph Pad software, San Diego CA, USA). The significance of difference in the means was determined using one-way analysis of variance (ANOVA; 95% confidence interval). Tukey post hoc tests were carried out for comparison of all groups with control and comparison of all pairs of groups respectively. Values of $p<0.05$ were considered as significant (Betty and Jonathan, 2003).

3. Results and discussion

The result of this study showed that there was significant increase in renal malondialdehyde (MDA), H_2O_2 levels, myeloperoxidase (MPO) and significant reduction in serum nitric oxide (NO) level while treatment with *M. charantia* extract and glibenclamide caused significant reduction in markers of oxidative stress and improved NO bioavailability (Table 1).

Table 1. Renal markers of oxidative stress and inflammation

	Group A	Group B	Group C	Group D	Group E
Kidney					
H_2O_2	23.89 ± 1.42	28.70 ± 1.42*	23.18 ± 1.89†	23.77 ± 1.43†	23.18 ± 1.04†
MDA	5.83 ± 0.98	8.03 ± 1.04*	5.87 ± 0.58†	5.50 ± 1.02†	5.25 ± 0.73†
NO	3.62 ± 0.21	2.82 ± 0.39*	3.59 ± 0.32†	3.53 ± 0.26†	3.49 ± 0.43†
MPO	40.61 ± 3.40	51.03 ± 5.29*	44.73 ± 4.19†	43.37 ± 4.19†	42.40 ± 2.53†

Data are expressed as mean ± SD (n = 10). Significance was determined by one-way ANOVA followed by turkey post hoc tests. A p value of < 0.05 was considered significant. All treated groups were compared with Group A and Group B. *Significant increase compared with group A (control); †Significant decrease compared with group B ($P < 0.05$). H2O2: hydrogen peroxide (µmole/min/mg protein); MDA: malondialdehyde, (µmol formed MDA/mg protein), NO: Nitric oxide µmol nitrite/mg protein; MPO: myeloperoxidase (µmol/min/mg protein). Group A, normal non-diabetic and non-treated control animals; group B, diabetic non-treated animals control; group C, diabetic animals treated with glibenclamide; group D, diabetic animals treated with MEMC 200 mgkg-1); group E, diabetic animals treated with MEMC 400 mgkg-1; SD, standard deviation.

Persistent hyperglycemia and increased oxidative stress are important modulators in the development of secondary diabetic complications such as nephrotoxicity (Kim *et al.*, 2009). Oxidative stress produces damage to kidney tissue, especially the nephrons by disrupting the function and structure of the

glomerulus and interstitial tubule (Farmer and Davoine, 2007). The markers of oxidative stress employed in this study are hydrogen peroxide (H_2O_2) and Malonaldehyde (MDA), a lipid peroxidation product. MDA is a highly unstable aldehyde that induces oxidative stress by forming covalent protein adducts collectively referred to as advanced lipoxidation end-products (Xu *et al.,* 2009). Hydrogen peroxide is a signalling molecule in the regulation of a wide variety of biological processes (Veal *et al.,* 2007). From our study, alloxan administration induced oxidative stress in the renal tissue, but the extract of *M. charantia* mitigated oxidative stress in the kidney by inhibiting the lipid peroxidation process (indicated by reduced formation of MDA) and reducing the generation of H_2O_2 significantly.

The results in Table 2 shows that there was significant reduction in renal non-protein thiol (NPSH), protein thiol (PSH) and reduced glutathione (GSH) in diabetic untreated rats. However, treatment with *M. charantia* extract and glibenclamide significantly improved the non-enzymic antioxidant deference system. Also, the administration of alloxan was characterized by the significant depletion of non-enzymatic antioxidants in the renal tissues, which are primarily composed of thiols and reduced glutathione (GSH). GSH is a major intracellular redox buffer that participates in the cellular defense against oxidative stress by scavenging free radicals and reactive oxygen intermediates (Shertzer *et al.,* 1990). In this study, the significant reduction of the protein and nonprotein thiols (PSH and NPSH) and GSH levels in the renal tissues following alloxan administration was an indication of oxidative stress. However, the administration of *M. charantia* increased the level of the non-enzymic antioxidants in the renal tissue, thus showing its ameliorative effects on renal injury.

Table 2. Renal non-enzymatic antioxidants

	Group A	Group B	Group C	Group D	Group E
NPSH	56.17± 2.22	53.58 ± 2.56†	55.75 ±3.45	56.55 ± 0.69*	56.87 ± 3.96*
PSH	56.18 ± 3.36	47.17± 1.05†	53.20±3.39*	55.28± 3.93*	56.47± 1.75*
GSH	91.05 ± 2.32	84.74± 2.76†	92.23±2.48*	91.17 ±3.25*	91.57 ±3.23*

Data are expressed as mean ± SD (n = 10). Significance was determined by one-way ANOVA followed by turkey post hoc tests. A P value of < 0.05 was considered significant. All treated groups were compared with Group A and Group B.
†Significant decrease compared with group A (control); *Significant increase compared with group B (P < 0.05). NPT and PT: Non-Protein thiol and protein thiol, respectively (µmol /mg protein), GSH: reduced glutathione (µmol /mg protein). Group A, normal non-diabetic and non-treated control animals; group B, diabetic non-treated animals control; group C, diabetic animals treated with glibenclamide; group D, diabetic animals treated with MEMC 200 mgkg-1); group E, diabetic animals treated with MEMC 400 mgkg-1; SD, standard deviation.

The activities of renal glutathione peroxidase (GPx), glutathione-S-transferase (GST) and superoxide dismutase fell significantly in diabetic untreated rats when compared to the control and the groups that received *M. charantia* extract and glibenclamide, respectively. The administration of *M. charantia* extract and glibenclamide caused restoration of the activities of the antioxidant enzymes (Table 3).

Table 3. Renal enzymic antioxidants

	Group A	Group B	Group C	Group D	Group E
GPx	198.16 ± 5.92	186.91 ± 4.55†	198.38 ± 1.75*	196.13 ± 1.66*	196.47 ± 3.91*
SOD	11.33 ± 1.43	8.57 ± 1.20†	12.22 ± 0.77*	11.78 ± 0.94*	11.63 ± 0.67*
GST	0.43 ± 0.09	0.39 ± 0.08†	0.40 ± 0.08*	0.43 ± 0.09*	0.46 ± 0.08*

Data are expressed as mean ± SD (n = 10). Significance was determined by one-way ANOVA followed by turkey post hoc tests. A P value of < 0.05 was considered significant. All treated groups were compared with Group A and Group B.
†Significant decrease compared with group A (control); *Significant increase compared with group B (P < 0.05). GPx: glutathione peroxidase, (µmol/mg protein); SOD: superoxide dismutase, U/µg protein; GST: Glutathione-S-transferase (mmol 1-chloro-2, 4-dinitrobenzene-GSH complex formed/min/mg protein). Group A, normal non-diabetic and non-treated control animals; group B, diabetic non-treated animals control; group C, diabetic animals treated with glibenclamide; group D, diabetic animals treated with MEMC 200 $mgkg^{-1}$); group E, diabetic animals treated with MEMC 400 $mgkg^{-1}$; SD, standard deviation.

The result of our study also showed that alloxan-treated rats exhibited decreased renal antioxidant enzymes activities such as glutathione peroxidase (GPx) and superoxide dismutase (SOD). Low

glutathione peroxide level in the renal tissue has been reported in patients with type 2 diabetes (Senthilkumar *et al.,* 2006). However, administration of *M. charantia* extract to the diabetic rats increased the level of these antioxidant enzymes. The significant increase in the activities of these enzymes in *M. charantia*-treated diabetic rats suggests the ameliorative effect of *M. charantia* against oxidative stress generated by free radicals in the diabetic rat kidney. The observed amelioration of oxidative stress by the extract in improving the antioxidant defense system might be ascribed to the free radical scavenging/antioxidant properties of the phytochemical constituents' present in *M. charantia*. Furthermore, the observed significant increase in the serum level of urea and creatinine, in this study, in the untreated diabetic rats compared to the rats treated with the extract of *M. charantia* suggests a protection of the plant extract against acute kidney dysfunction and renal damage. Since alloxan administration can increase serum urea by various mechanisms, including enhancement of protein catabolism, conversion of ammonia to urea (Hooper *et al.,* 1998), inhibition of amino acids incorporation in proteins and elevation of endogenous oxygen species levels (Tamay-Cach *et al.,* 2016), it is possible that the protective effect of *M. charantia* against renal damage, observed in this study, may involve an interference with any of the aforementioned mechanisms. Interestingly, the extract of *M. charantia*, at the two doses tested appears to confer better protection in this respect, than glibenclamide. The findings in our study agree with previous reports on the antioxidant effects of the *M. charantia* (Singh *et al*., 2004; Thenmozhi and Subramanian, 2011). The administration of alloxan induced oxidative stress by significantly ($P<0.05$) increasing blood glucose level in the group two animals; while administration of MEMC (200 mg/kg and 400 mg/kg) and glibenclamide (4 mg/kg) significantly ($P<0.05$) reduced the level of the oxidative stress blood glucose (Figure 1).

Table 4. Serum markers of renal damage

Parameters	Group A	Group B	Group C	Group D	Group E
Urea (mg/dl)	19.08±1.23	28.87±0.47*	18.93±1.96†	18.08±1.31†	18.42±0.27†
Creatinine (mg/dl)	0.65±0.22	1.80±0.26*	0.91±0.29†	0.71±0.23†	0.66±0.24†

Data are expressed as mean ± SD (n = 10). Significance was determined by one-way ANOVA followed by turkey post hoc tests. A P value of <0.05 was considered significant. All treated groups were compared with Group A and Group B. *Significant increase compared with group A (control); †Significant decrease compared with group B ($P < 0.05$). Group A, normal non-diabetic and non-treated control animals; group B, diabetic non-treated animals control; group C, diabetic animals treated with glibenclamide; group D, diabetic animals treated with MEMC 200 $mgkg^{-1}$); group E, diabetic animals treated with MEMC 400 $mgkg^{-1}$; SD, standard deviation.

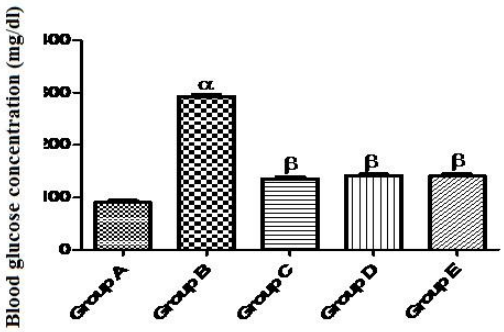

Figure 1. Blood glucose level

α shows significant increase when compared with normal control; while β shows significant reduction when compared with diabetic control ($P<0.05$). Group A, normal non-diabetic and non-treated control animals; group B, diabetic non-treated animals' control; group C, diabetic animals treated with glibenclamide; group D, diabetic animals treated with MEMC (200 mg/kg); group E, diabetic animals treated with MEMC (400 mg/kg).

The histopathology of the kidney revealed the following: the control group showed normal histoarchitecture of the kidney with no visible lesions; the glomeruli and tubules have normal appearance. In contrast, kidney sections from the diabetic non-treated group showed severe distortion of the histoarchitecture of the kidney with extensive destruction of glomeruli (glomerular degeneration characterised by glomerular hypercellularity) and tubular structures. There was congestion and areas of necrosis and infiltration of inflammatory cells. The kidney section of the glibenclamide treated group showed mild distortion of the histoarchitecture of the kidney; there was tubular desquamation and mild infiltration by inflammatory cells. On the other hand, the kidney section of the MEMC treated group revealed mild distortion of the renal architecture as evidenced by the less severe tubular and glomerular damages with mild infiltration by inflammatory cells, this showed that treatment with

the extract resulted in marked attenuation of the histopathological abnormalities that were seen in the kidney of the diabetic rats (Figure 2).

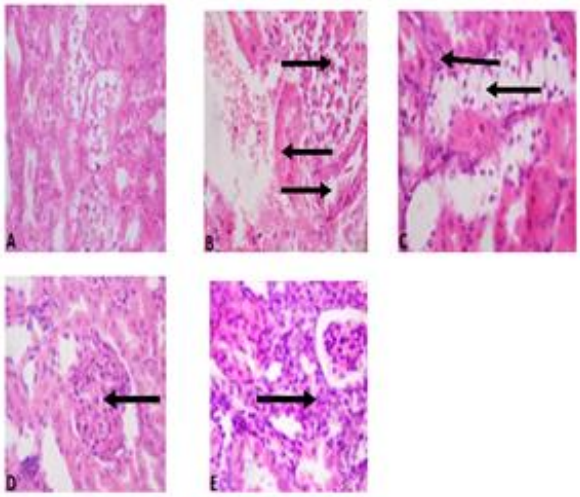

Fig. 2. Photomicroghraph of kidney. A (NADT) on visible lesion. B (DNT) congestion, infiltration of inflammatory cells. C (DTG) infiltration of inflammatory celld, tubular desquamation. D (MEMC200) hypercellularity of mesangial cells. E (MEMC400) infiltration of inflammatory cells. (x 400, H and E)

Immunohistochemistry revealed higher expressions of renal NF-kB and lower expressions of renal BCL2 antibodies in alloxan untreated rats compared to the control, while higher expression of renal BCL2 and lowers expressions of renal NF-kB were observed in the MEMC and glibenclamide treated rats (Figures 3 and 4).

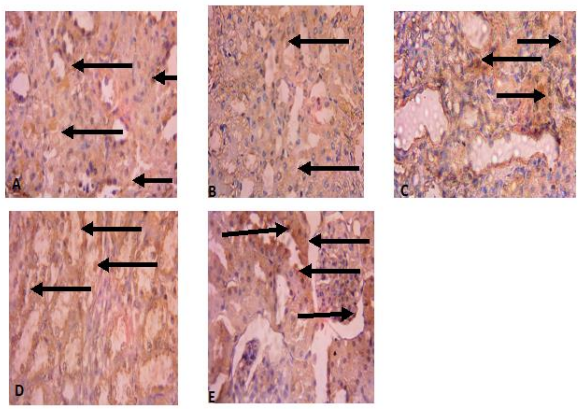

Fig. 3. Immunohistochemical staining for renal BCl2. A ontrol (NDNT) shows high expression of BCl2, B (DNT) showes lower expression of BCl2 than control, C (DTG) showes overexpression of BCl2 similar to control, D (DTMC200) shows high expression of BCl2 similar to control, E (DTMC400) shows higher expression of BCl2 similar to control. The slides were counterstained with high definition Haematoxylin and viewed x 400.

From the immunohistochemical studies, alloxan administration caused decreased expressions of BCL2, which is an anti-apoptotic protein thus implying a promotion of apoptosis in renal cells by alloxan (Subramanian *et al.*, 2012).

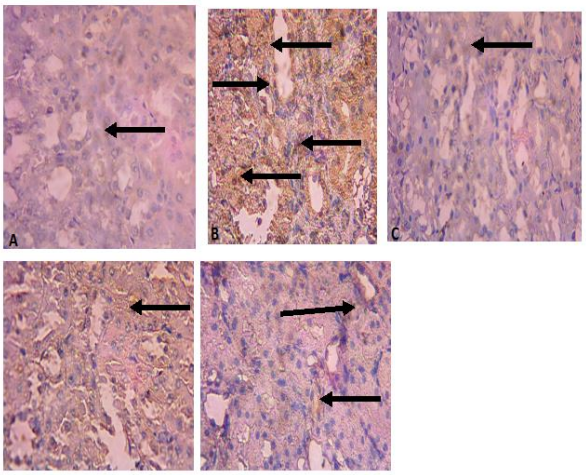

Fig. 4. Immunohistochemical staining for renal NF-KB. A ontrol (NDNT) shows low expression of NF-KB, B (DNT) showes higher expression of NF-KB than control, C (DTG) showes lower expression of NF-KB similar to control, D (MEMC200) shows lower expression of NF-KB similar to control, E (DTMC400) shows lower expression of NF-KB similar to control. The slides were counterstained with high definition Haematoxylin and viewed x 400.

However, the observed increased expression of BCL2 in the *M. charantia* treated rats suggests a potent anti-apoptotic effect for the extract. Also, in this study, there was high expression of NF-κB in the renal tissue of the untreated alloxan-induced diabetic rats, while treatment with *M. charantia* showed low expression of NF-κB, thus suggesting an anti-inflammatory potential of *M. charantia*. Apoptosis is a process of physiological cell death that maintains tissue homeostasis. In excess, however, apoptosis may lead to various pathological processes (Tak and Firestein, 2001). Apoptosis and inflammation have been reported in diabetic nephropathy (Ha *et al.*, 2008). The ability of *M. charantia* to inhibit oxidative damage by boosting renal antioxidant status is a possible mechanism of its anti-apoptotic activity.

4. Conclusion

In conclusion, the results of this study showed that *M. charantia* exhibited nephroprotective effects by mitigating oxidative stress, improving the antioxidant defense system and reversing the redox imbalance in diabetes mellitus associated nephropathy.

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