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EFFECT OF DICHLOROMETHANE EXTRACT OF *ALSTONIA SCHOLARIS* LEAVES IN DIFFERENT MODELS OF DIABETIC NEPHROPATHY IN RATS

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Keywords:

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ABSTRACT: Aim: The main of the study is to study the Effect of Various Polar & Non-Polar Extracts of *Alstonia scholaris* leaves in Different Models of Diabetic Nephropathy in Rats. **Material & Methods:** Leaves of selected plant was extracted by using various solvents, *i.e.*, Pet ether, Dichloromethane, Ethyl acetate, methanol and water. All the extracts were screened for the presence of various active phytocompounds *i.e.*, alkaloids, terpenoids, tannins, flavonoids and phenolic compounds etc. For the pharmacological studies, OECD guidelines were used for the toxicity studies, and $1/10^{th}$ and $1/5^{th}$ doses used for the entire study. All the extracts were evaluated by different diabetic nephropathy model. After an assessment of activity, various biochemical parameters were measured. **Result:** There was a significantly high level of blood urea nitrogen in diabetic nephropathy animals compared to the normal animals; 28 days treatment regimen showed a significant reduction in elevated urea level. Elevated serum creatinine level was observed in diabetic animals which were significantly reduced by treatment groups; the highest reduction was observed in extracts group treated. Elevated serum creatinine level was observed in diabetic control, nephropathy control and diabetic nephropathy control groups; after the treatment with the drugs, the creatinine level was significantly reduced. Urine protein and albumin excretion were highly increased in the diseased group (DC, NC, and DNC group). After the treatment regimen, the group showed a significant effect on urinary excretion profile. **Conclusion:** The drugs controlling both metabolic and hemodynamic processes can effectively revert the progressive diabetic nephropathy in diabetes patients. Dichloromethane extract ameliorates diabetic nephropathy in both the models better than any other treatment regimen studied.

INTRODUCTION: Diabetic nephropathy is a progressive kidney disease caused by angioplasty of capillaries in the kidney glomerular [1, 2]. Diabetic nephropathy is one of the important microvascular complications of diabetes mellitus and the most common cause of chronic kidney disease and end-stage renal disease in many countries [3, 4].

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The renal disease follows a conventional course from onset of microproteinuria to nephrotic syndrome and finally to renal absence or death; proteinuria develops 14 to 19 years after onset of diabetes, azotemia occurs 4 to 6 years later, and ESRD results in the last year of nephropathy.

Type I diabetes is connected with a higher pervasiveness of renal disease than type II diabetes [5, 6]. Slowing down the sequence of diabetic nephropathy, by modifying the lifestyle is the best way to reduce mortality and maintain a high quality of life [7]. Nephropathy develops in about 20% - 40% of all diabetic patients, with just about 40% of patients with type 1 diabetes and 5 - 15% of patients with

type 2 diabetes, although the frequency is considerably higher in certain ethnic groups [8]. In approaching years, India will see an augment in the number of patients with diabetes and its complication, with 25 -40% of these subjects probable to develop CKD, and burden of the ESRD will rise [9].

In the present study, attempts have been made to downsize the scientific soundness of various polar and non-polar extracts of *Alstonia scholaris* on diabetic nephropathy models.

Plants selected have prominent activity in diabetic condition as well as nephroprotective activity along with antioxidant and antihypertensive potential based on preceding research work in this field. Plants for study were elected on basis of literature; traditional, ancestral, and rural folklore employ.

MATERIALS AND METHODS:

Collection of Plant Materials: Dried leaves of *Alstonia scholaris* were procured from the medicinal garden and campus of Pharmacy College in the month of September, Bhopal, Madhya Pradesh, India.

Authentification of Plants Materials: Dried leaves of *Alstonia scholaris* were authenticated by Prof. Gyanendra Tiwari, Senior Botanist and Scientist, Government College of Horticulture, Mandsaur (M.P.), and voucher specimen for dried leaves of *Alstonia scholaris* were deposited in the herbarium of Department of Pharmacognosy, TIT College of Pharmacy, Bhopal (M.P.).

Extraction Method by Soxhlet Apparatus: Around 500 gms dried leaves of *Alstonia scholaris* were coarsely powdered, weighed, and filled in Soxhlet apparatus for extraction. First, the powdered drug was defatted with petroleum ether (60 °C - 80 °C); the Defatted drug was then dried and again filled in soxhlet apparatus for extraction with dichloromethane, ethyl acetate, methanol and finally water as a solvent. The extraction was carried out for a period of 72 h. The extract obtained was dried in a vacuum to remove excess solvent. Extracts were weighed to obtain % yield, and phytochemical screening were performed for the presence of various active phytocompounds, *i.e.*, alkaloids, terpenoids, tannins, flavonoids, phenolic compounds, and glycosides, *etc.* [10]

Pharmacological Studies:

Procurement and Selection of Animals: All the animals were procured from the central animal house of B. R. Nahata College of Pharmacy, Mandsaur. Animals were maintained under the standard climatic condition and supplied by Water ad libitum specified by CPCSEA Committee. All the protocols were approved by the Institutional Animal Ethical Committee of the college (Protocol No- 006/PhD/2017/IAEC/BRNCP/Mandsaur). Litters of Wistar albino rats of age 2 days were used for the chronic diabetic model, whereas healthy Wistar albino rats of either sex weighing between 200–250 gm were used for the diabetic nephropathy model.

Acute Toxicity Studies: The acute toxicity studies for all the extracts were carried out in adult Wistar albino rats by OECD (Organization for Economic Co-operation and Development) guideline no. 425 [11].

Neonatal Diabetic Model:

Streptozotocin (STZ) Induced Diabetes: Neonatal diabetic model serves as a chronic diabetes complication model resembling human prolonged diabetic complication. In this model, the 2 day's old litters were isolated from their mothers. Pups were weighed, and streptozotocin (60 mg/kg in 0.1M citrate buffer pH 4.5) was injected by I.P. route. The pups were again placed with their respective mothers. Animals are having fasting blood glucose levels (BGL) more than 250 mg/kg were selected for future screening. Animals were treated with various extracts for 28 days [12].

Renal Ischemia and Reperfusion Model in Diabetic Rat: Renal ischemia and reperfusion in diabetic rats serve as an end phase renal disease model resembling human end period renal disease in diabetic people. Rats are having BGL of more than 250 mg/dl were used for future study. Diabetic rats should be given daily subcutaneous injections of long-acting insulin (2–4 U/rat, Human Mixtard, Abbott India Ltd., Mumbai, India) to uphold blood glucose levels in a pleasing assortment. A degeneration period of 2 weeks was given to rats before surgery. The animals were divided into the following groups,' *i.e.*, Normal control, diabetic control, various extracts treated in a dose of 200 and 400 mg/kg [13].

Induction of Ischemia: The animals were anesthetized by an intraperitoneal injection of ketamine (80 mg/kg; Neon Lab Ltd., Mumbai). During the process, the animals were placed on a thermo pad, which kept the body temperature at 37.5 °C. An incision to some extent deviated from midline towards the left side was made, and the left renal artery was situated and dissected free from its nearby structures.

After a recovery period of 10 min, renal ischemia was evoked by clamping the left renal artery for 30 min. Subsequently, the abdomen was sutured, and the animals were returned to their cages. Animals were watched for 1 h post-surgery for the mending. The animals were divided into the following *groups, i.e.*, normal control, diabetic control, nephropathy control, diabetic nephropathy control, and various extracts treated groups in a dose of 200 and 400 mg/kg [14].

Sample Collection: Blood and urine were collected on 0^{th}, 7^{th}, 14^{th} and 28^{th} day and organs were unruffled on 28th day of the beginning of the experiment. Urine was collected with the help of metabolic cages in the graduated tubes, and volume was deliberate. Collected urine was used to assess the following biochemical parameters like glucose, protein, urea nitrogen, and creatinine level [15].

Assessment of Biochemical Parameters:

Estimation of Blood Glucose Level (BGL): Tails of rats were sterilized with spirit, and a small cut was made on the tail with a fresh blade, and blood glucose level was checked by glucometer (Easy Gluco, Morepen Laboratories Ltd. New Delhi). Excess blood on the tail was wiped out with absorbent cotton.

Estimation of Urea Nitrogen: Blood urea nitrogen (BUN) and urinary urea nitrogen (UUN), was estimated using glutamate dehydrogenase (GLDH) - urease method [16].

Estimation of Creatinine: Plasma & urine creatinine were estimated using Modified Jaffe's Reaction. The sample (serum/urine) was diluted with distilled water (1:50 v/v). 100 µl of samples were mixed with 500 µl of picric acid and 500 µl buffer reagents available commercially (Creatinine Kit, Crest Biosystems, Goa). The absorbance of Orange colored complex formed was calculated photo-metrically at 520 nm. 100 µl of standard Creatinine (Crest Biosystems, Goa) was assorted with reagent as greater than, and the experiment was repeated.

Initial absorbance (A1) was measured 20 seconds after mixing, and final absorbance (A2) was deliberate 80 seconds after mixing using an autoanalyzer (Star Track 22 Plus) [16].

Estimation of Urine Protein: Urine protein was anticipated using Pyrogallole Red – End Point Method. 10 µl of urine collected was mixed with 1000 µl of dye reagent (mixture of pyrogallol red dye and ammonium molybdate in glycine buffer, pH 2.2) accessible commercially (Micro Protein Estimation Kit, Transasia Bio-Medicals Ltd., Baddi, H.P.). The samples were incubated at 37 ºC for 10 min. The absorbance of standard and test against blank was calculated at 600 nm using autoanalyzer within 60 min [16].

Estimation of Urinary Albumin Excretion: Urinary albumin excretion was estimated using bromocresol green (BCG) colorimetry test. 5 µl of urine was mixed with 1000 µl of BCG reagent available commercially (Albumin, BCG colori-metric test, Lab Care Diagnostic (India) Pvt. Ltd.).

Samples were incubated for 5 min at room temperature, and absorbance was measured spectrophotometrically at 578 nm against a blank. The same procedure was repeated for a standard solution (4 gm/dl) (Lab Care Diagnostic (India) Pvt. Ltd.) [16].

Statistical Analysis: The data were expressed as mean ± SEM. The data were analyzed by one-way analysis of variance (ANOVA) followed by "Dunnett's test" P value less than 0.05 was considered as statistically significant with the help of Graph pad Prism 5.04 trial version.

RESULTS:

Toxicity Profile: All the extracts were found to be non-toxic when subjected to acute toxicity study at a dose of 2000 mg/kg as per OECD guidelines. No characteristic behavioral, autonomic, and neuro-logical effects were observed. Hence, $1/10^{th}$ and $1/5^{th}$ *i.e.*, 200 mg/kg and 400 mg/kg were selected as a therapeutic dose of extracts for the entire study.

Pharmacological Study:

Neonatal Diabetic Model: All the extracts showed significant activity (data not shown here) in this model. However, dichloromethane extract showed highly significant activity as compared to all other extracts. Animals showed a slow progression in growth (body weight) compared to the normal animals of the same age. Treatment groups showed a significant increase in body weight **Table 1**.

TABLE 1: CHANGES IN PHYSIOLOGICAL PARAMETERS DURING STUDY PERIOD

S. no.	Group	Parameters			
		Bodyweight (gm)	Food intake (gm)	Water intake (ml)	Urinary output (ml)
1	NC	210.5 ± 6.11	32.38 ± 1.22	32.17 ± 2.11	10.33 ± 1.31
2	DC	146.2 ± 3.27	44.83 ± 3.45	107.80 ± 2.04	38.17 ± 2.19
3	DCM 200	196.4 ± 4.14***	37.31 ± 2.41**	72.66 ± 2.23***	20.41 ± 2.33**
4	DCM 400	210.0 ± 4.21***	30.82 ± 1.72***	49.21 ± 1.56***	16.31 ± 2.14***

N=6; where δ p < 0.001 *vs.* NC; ***p < 0.001, ** p < 0.01 *vs.* DC; Value expressed in mean ± SEM

Diabetic animals showed an increase in food and water demand, which was significantly reduced by the extracts **Table 2**. During the experimental period, the fasting blood glucose level in diabetic animals was higher than those of normal animals. However, the fasting blood glucose level was significantly reduced in dichloromethane treated groups. There was a significantly high level of blood urea nitrogen in diabetic nephropathy animals as compared to the normal animals 28 days treatment regimen showed a significant reduction in elevated urea level. Elevated serum creatinine level was observed in diabetic animals which were significantly reduced by treatment groups; the highest reduction was observed in Dichloro-methane extract group treated.

TABLE 2: CHANGES IN BLOOD GLUCOSE LEVEL (mg/dl) DURING STUDY PERIOD

S. no.	Group	Blood Glucose Level (Days)			
		0	7	14	28
1	NC	97.3 ± 2.11	98.3 ± 3.22	97.2 ± 2.42	97.6 ± 3.12
2	DC	312.5 ± 3.23	357.4 ± 5.22	363.5 ± 4.19	346.2 ± 3.12
3	DCM 200	315.3 ± 4.33	298.8 ± 4.19**	230.8 ± 3.31***	170.5 ± 3.11***
4	DCM 400	320.5 ± 5.20	262.4 ± 2.11***	210.2 ± 3.22***	150.3 ± 3.48***

N=6; where δ p < 0.001 *vs.* NC; * p < 0.05, ** p < 0.01, ***p < 0.001, ns non significant; *vs.* diabetic control (DC); Value expressed in mean ± SEM

TABLE 3: CHANGES IN BLOOD UREA NITROGEN (mg/dl) DURING STUDY PERIOD

S. no.	Group	Days			
		0	7	14	28
1	NC	15.22 ± 3.32	14.41 ± 2.31	15.32 ± 2.08	16.22 ± 1.68
2	DC	33.12 ± 2.11	34.35 ± 1.33	33.26 ± 3.71	34.11 ± 2.70
3	DCM 200	35.83 ± 3.23	30.00 ± 2.52	26.50 ± 2.43**	27.22 ± 2.42**
4	DCM 400	34.17 ± 2.26	24.31 ± 2.12*	19.21 ± 2.11***	12.42 ± 3.18***

N=6; where δ p < 0.001 *vs.* NC; * p < 0.05, ** p < 0.01, ***p < 0.001, ns non significant; *vs.* diabetic control (DC); Value expressed in mean ± SEM

TABLE 4: CHANGES IN SERUM CREATININE LEVEL (mg/dl) DURING STUDY PERIOD

S. no.	Group	Days			
		0	7	14	28
1	NC	0.36 ± 0.028	0.35 ± 0.011	0.33 ± 0.008	0.35 ± 0.004
2	DC	1.03 ± 0.019	1.12 ± 0.019	1.14 ± 0.014	1.20 ± 0.021
3	DCM 200	1.06 ± 0.024	0.98 ± 0.018	0.82 ± 0.029*	0.70 ± 0.032**
4	DCM 400	1.07 ± 0.018	0.84 ± 0.009*	0.70 ± 0.014**	0.38 ± 0.015***

N=6; where δ p < 0.001 *vs.* NC; * p < 0.05, ** p < 0.01, ***p < 0.001, ns non significant; *vs.* diabetic control (DC); Value expressed in mean ± SEM

Urinary protein, albumin excretion were highly increased in diabetic nephropathy animals, and glucose was also found in the urine of diabetic animals GFR was also increased to many folds in

diabetic animals. After the treatment for 28 days, all groups showed highly significant results on urinary protein excretion **Table 5**.

TABLE 5: CHANGES IN BIOCHEMICAL PROFILE OF URINE DURING STUDY PERIOD

S. no.	Group	Urinary protein (mg/24 h)	Urine albumin excretion (mg/24 h)
1	NC	19.12 ± 2.33	3.31 ± 2.14
2	DC	135.24 ± 2.72	12.31 ± 2.52
3	DCM 200	74.12 ± 2.35***	7.43 ± 1.18*
4	DCM 400	52.31 ± 2.55***	4.21 ± 1.01***

Renal Ischemia & Reperfusion in Diabetic Rats: Body weight of diseased animals was highly decreased, *i.e.*, almost 100 gm reduction was observed in rats, which were laterally improved by drug treatment biggest improvement was seen in dichloromethane extract-treated. Food intake, water intake, and urinary output were increased to many folds in diabetic and diabetic nephropathy groups which were controlled by treatment groups. Treatment groups showed a significant reduction in blood glucose levels.

TABLE 6: CHANGES IN BLOOD GLUCOSE LEVEL (mg/DL) DURING STUDY PERIOD

S. no.	Group	Days			
		0	7	14	28
1	NC	98.2 ± 2.23	97.2 ± 3.22	98.8 ± 3.21	98.1 ± 3.45
2	DC	95.8 ± 3.22	270.3 ± 3.54	298.3 ± 3.51	310.8 ± 3.73
3	NeC	96.5 ± 2.22	99.3 ± 2.39	98.3 ± 2.22	97.4 ± 4.48
4	DNC	99.3 ± 2.44	280.2 ± 7.22	298.3 ± 3.54	325.5± 4.31
5	DCM 200	97.7 ± 3.21	261.2 ± 3.22***	248.2 ± 4.51***	210.3 ± 2.57***
6	DCM 400	101.3 ± 4.41	223.2 ± 2.46***	197.2 ± 2.51***	160.4 ± 5.22***

TABLE 7: CHANGES IN BLOOD UREA NITROGEN (mg/DL) DURING STUDY PERIOD

S. no.	Group	Days			
		0	7	14	28
1	NC	17.23 ± 2.31	14.34 ± 1.31	15.17 ± 2.22	17.67 ± 3.67
2	DC	16.31 ± 3.44	34.27 ± 1.29	35.67 ± 2.25	36.27 ± 2.71
3	NeC	16.52 ± 4.31	32.83 ± 1.25	34.32 ± 4.67	35.42 ± 2.49
4	DNC	14.55 ± 3.26	32.42 ± 1.67	33.43 ± 1.36	34.17 ± 2.52
5	DCM 200	17.34 ± 3.44	29.53 ± 4.51	24.42 ± 4.62**	22.12 ± 1.15***
6	DCM 400	18.37 ± 2.48	25.62 ± 3.43***	20.31 ± 3.77***	17.24 ± 3.41***

TABLE 8: CHANGES IN SERUM CREATININE LEVEL (mg/DL) DURING STUDY PERIOD

S. no.	Group	Days			
		0	7	14	28
1	NC	0.34 ± 0.013	0.35 ± 0.015	0.36 ± 0.014	0.35 ± 0.013
2	DC	0.33 ± 0.015	1.12 ± 0.021	1.18 ± 0.012	1.18 ± 0.017
3	NeC	0.34 ± 0.011	1.08 ± 0.022	1.19 ± 0.041	1.18 ± 0.011
4	DNC	0.31± 0.016	1.15 ± 0.024	1.22 ± 0.043	1.26 ± 0.015
5	DCM 200	0.32 ± 0.012	0.97 ± 0.014**	0.61 ± 0.036***	0.51 ± 0.038***
6	DCM 400	0.36 ± 0.014	0.82 ± 0.012***	0.53 ± 0.017***	0.33 ± 0.016***

There were a noteworthy high level of serum blood urea nitrogen in the diabetic control group, nephropathy control group, and diabetic nephropathy group as compared to the normal animals; 28 days treatment regimen showed significant lessening in elevated urea level. Elevated serum creatinine level was observed in diabetic control, nephropathy control, and diabetic nephropathy control groups; after the treatment with the drugs, the creatinine level was significantly concentrated.

DISCUSSION: Plants were used for the treatment of disease from time immemorial. Glycosides, alkaloids, terpenoids, flavonoids, tannins, *etc.*, are frequently implicated as phytoconstituents having antidiabetic activity [17]. In a nearby study, neonatal diabetic model and renal ischemia & reperfusion model were used. Neonatal diabetic model exhibit type II diabetes mellitus facial appearance such as impaired glucose tolerance and severe glycemia [18].

A major deficiency in conventional animal models of diabetic nephropathy is the absence of kidney failure. To overcome this, the renal ischemia and reperfusion model was used. 2 weeks after the induction of diabetes (STZ treatment), animals were subjected to 30 min of ischemia, and then the wounds were sutured. In this model, STZ caused

features resembling human diabetes, whereas ischemia caused symptoms resembling nephropathy and also increased oxidative burden on the body [19]. Excessive loss in weight circumstances with Polydipsia, Polyuria, and Polyphagia is commonly pragmatic in diabetic nephropathy; recent studies showed that enhancement in the above physio-logical condition characteristic to the control of the progression of diabetic nephropathy. In the present study, dichloromethane extract attenuated the physiological effect supporting their promising role in controlling the succession of diabetic nephro-pathy. Hyperglycemia causes activation of polyol pathway, non-enzymatic glycation, glucose autoxidation, and de novo synthesis of diaglycerol most important to protein kinase C and phospholipases A2 activation [20]. In the present study fasting blood glucose of diabetic animals was condensed by treating animals with dichloro-methane extract attributing to anti-hyperglycemic activity.

Previous researchers have instated on controlling the lipid levels as it plays a crucial role in developing glomerulosclerosis, endothelial cell damage, and stimulating mesangial cell proliferation [21]. Although hyperlipidemia is not a consideration to be a primary cause of renal injury in humans, several studies put it to somebody that hyperlipidemia is a risk factor for the progression of established renal disease and optional that hypercholesterolemia leads to increased activity of the Renin-Angiotensin System and elevates angiotensin II leads to increased TGF-β [22]. In the present study, Streptozotocin causes an increase in LDL, triglycerides, and total cholesterol whereas HDL was decreased; after the treatment with extracts, the elevated levels of LDL, triglycerides, and total cholesterol was brought down, and the good cholesterol (HDL) level was elevated [23].

Increment in urinary albumin, blood and urinary nitrogen urea, creatinine, urinary protein, and glucose excretion was usually observed in diabetic nephropathy indicating progressive damage to glomerular and tubular cells consequential in decline in GFR [23]. Treatment groups showed a decrease in urea and creatinine level and decreased urinary excretion suggesting the beneficial effect of drugs. Glucosuria and albuminuria are cardinal features of diabetic nephropathy [23]. Normally albumin cannot diffuse across the glomerular, whereas glucose diffuse from side to side the glomerulus; diffusion of glucose doesn't cause Glucosuria due to tubular reabsorption [23]. None of the treatment groups was able to eliminate the damage fully, suggesting that the treatment regimen can only prevent but doesn't cure the circumstances, suggesting that altered glomerular activity can't relapse totally.

The specific mechanism of action cannot be reached as there is unfocused pathophysiology of diabetic nephropathy. Selected extracts and isolated compounds studied showed a promising effect in controlling diabetic nephropathy. The data showed that dichloromethane extract revolutionizes diabetic nephropathy significantly in both the models better than any other treatment schedule studied. Thus the result of this study could make available efficacious and cost-successful treatment for diabetic nephropathy without any side effects.

CONCLUSION: The onset of diabetic nephro-pathy is often quiet, and progression is very sluggish, so it is difficult to diagnose in a straight line or reach the stage to confirm the disease. Diabetic kidney disease is characterized by structural and functional changes. Metabolic, hemodynamic, and genetic processes supply to the expansion of diabetic nephropathy.

The drugs controlling both metabolic and hemodynamic process can efficiently revert the progressive diabetic nephropathy in diabetes patients. The specific mechanism of the exploit cannot be reached as there is a diverted patho-physiology of diabetic nephropathy. Dichloromethane extract ameliorates diabetic nephropathy in cooperation with the models better than any other behavior regimen studied.

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