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RESEARCH ARTICLE

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Modulatory Effects of Ethylacetate and n-Butanol Fractions of *Indigofera pulchra* on Some Biochemical Parameters in Alloxan -Induced Diabetic Wistar rats

Tanko, Y [1*], M.Y.Fatihu [2], K.Y.Musa [3] and Mabrouk, M.A[4]

[1]Department of Human Physiology, ABU, Zaria. Nigeria
[2]Department of Vet Pathology and Microbiology, ABU, Zaria Nigeria
[3]Department of Pharmacognosy and Drug design, ABU, Zaria Nigeria
[4]Department of Physiology, Faculty of Medicine, Al-Azhar University, Cairo. Egypt

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ABSTRACT

This study was carried out to investigate the effects of ethyl acetate and n-butanol fractions of Indigofera pulchra on liver enzymes and lipid profile in alloxan induced diabetic rats. 20 Wistar rats of both sexes were used for the study. The rats were divided into four groups with five rats in each group. Group 1 and 2 representing negative and positive control respectively. Group 3 and 4 received 50 mg/kg and 250 mg/kg body weights of ethyl acetate and n-butanol respectively. After 14 days of treatment, the animal were anaesthetized and sacrificed to obtain blood by cardiac puncture. Serum was collected and assayed for alanine aminotranferase (ALT) aspartate aminotranferase (AST) alkaline phosphatise(ALP)total protein (TP), albumin (ALB), total cholesterol, triacylglycerol , high density lipoproteins and low density lipoproteins. The result showed that ALT,AST, TP and ALB levels of the treatment groups decrease($P<0.05$) when compared with the ccontrol untreated group(Negative control) . As regards to the lipid profile there was a significant decrease ($P<0.05$) in the triacylglycerol, when compared with the untreated control group. Also in relation to the High density lipoprotein and low density lipoprotein there was a slight increase but no significant. The preliminary phytochemical screening of the two fractions Indigofera pulchra fractions revealed the presence of alkaloids, flavonoids, and saponins. The LD_{50} was 775mg/kg and 2,154 mg/kg for Ethylacetate and n-Butanol fractions respectively.

Key Words: Diabetes ,*Indigofera pulchra* , Liver enzymes, lipid profile,

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INTRODUCTION

The number of people suffering from the disease worldwide is increasing at an alarming rate with a projected 366 million peoples likely to be diabetic by the year 2030 as against 191 million estimated in 2000 [1] . From literature review it has been revealed that 15 - 20% of diabetic patients are suffering from insulin-dependent diabetes mellitus (IDDM) or type-I [2] . The IDDM is noted both in adult and childhood [2,3]. It is characterizedby elevation of both fasting and post-prandial blood sugar levels. Chronic hyperglycemia during diabetes causes glycation of body proteins that in turn leads to secondary complications affecting eyes, kidneys, nerves, and arteries [4]. Lipids and lipoproteins abnormalities are common in diabetes, particularly type 2 [5]. It is very important to lower the levels of lipids in the body. This is done by drug therapy with drugs such as statins[6,7]. This is seen in patients with diabetes with elevated triglyceride concentrations and decreased high-density lipoprotein cholesterol (HDL-C) concentrations [5]. Low-density lipoprotein cholesterol (LDL-C) concentrations may be elevated as well, but this finding is not as

consistent across all patients with diabetes. National guidelines published by the American Diabetes Association [8] and National Heart, Lung, and Blood Institute [9] recommend annual assessment of a fasting lipid profile in patients with diabetes.

It has been suggested that increased AST activity in the serum is sensitive marker of liver damage, even if the damage is of subclinical nature [10]). Administration of the two fractions of the plant significantly decreases the levels of serum concentration of transaminase. ALT has the highest concentration in the liver, kidney and skeletal a muscle having the least activity of the enzyme. The AST activity is located in the microsomal and mitochondrial portion of the liver cells as well as the skin, skeletal and cardiac muscles, pancrease and kidney. ALT measurements are more liver specific than AST and its activity is usually greater than AST activity at early or acute hepatocellular disease[11]. AST on the other hand tend to be released more than ALT in chronic liver diseases such as cirrhosis [11] . A normal ALT in the presence of elevated activities of AST and lactate dehydrogenase rules out the hepatic origin of the enzymes while a marked elevation of ALT, however, in the presence of mild to moderate elevation of AST is suggestive of either hepatic disease or disease combined with other conditions[12] .

Indigofera pulchra (Wild) family: papilionaceae is an annual non climbing herbs or shrub that can grow up to 1m tall. It is widely distributed throughout west-Africa [13] . The local name in Hausa is *Bakin bunu* and inEnglish it is called *Indigofera.* In ethnomedicine, the leaves are used to treat infected wound [13,14], while the decoction of the aerial part is used as prophylactic against snake-bite [15]and as anti-inflammatory [16] . Previous pharmacological studies on the methanol extract of the aerial part of this plant showed that it exhibited venom detoxifying activities [17] . Also previous work reported [18] reported that the crude hydro methanolic extract of *Indigofera pulchra* has anti-diabetic effects.

The aim of this research work was to determine the effects of Ethyl acetate and n-Butanol fractions of *Indigofera pulchra* on some biochemical parameters in alloxan-induced diabetic Wistar rats.

MATERIALS AND METHODS

Animals

animals were handled in accordance with international principles guiding the use and handling of experimental animals (United State National Institute for Health, 1985). A total of twenty (20) albino Wistar rats of both sexes between the ages of 8 to 12 weeks old and weighing 120-250grams were used for this study. The animals were housed in the Animal House, Department of Human Physiology, ABU, Zaria. The animals were randomized into experimental and control groups and were kept in polypropylene cages. The animals were maintained on standard animal feeds and drinking water *ad libitum*.

Plant Material

Fresh leaves *Indigofera pulchra* were collected from the Ahmadu Bello University, Zaria main campus. It was identified and authenticated at the herbarium unit of Biological Sciences Department, A.B.U. Zaria .by Mallam A.U.Gallah It was identical with the voucher specimen (No. 6558) previously deposited at the herbarium.

Chemicals and drugs

All chemicals and drugs used were of analytical grade.

Preparation of plant fractions

The air dried *Indigofera pulchra* leaves under the shade and grounded into a fine powder using mortar and pestle. Five hundred grams of the powdered material was macerated in 70% methanol at room temperature for 48 hours. It was then filtered using a filter paper (Whatmann size 1). The filtrate was then partitioned with Ethylacetate to get the Ethylacetate fraction which was evaporated to dryness in an oven at 37 ^{0}C. A greenish-brown residue weighing 6 grams was obtained and kept in a sealed container at 4^0 C in a refrigerator until use. Another five hundred grams of the powdered material was macerated in 70% methanol at room temperature for 48 hours. It was then filtered using a filter paper (Whatmann size 1). The filtrate was then be partitioned with n-Butanol to get an n-Butanol fraction which was evaporated to dryness in an oven at 37^0 C. A brownish residue weighing 20 grams was obtained and kept in a sealed container at 4^0 C in a refrigerator until use.

Phytochemical screening of plant fraction

Preliminary screening of the two fractions were performed for the presence of secondary metabolites using the following reagents and chemicals: alkaloids - with Mayer's and Dragendorff's reagents [19, 20] ; flavonoids with the use of Mg and HCl[21,22] ; tannins with 1% gelatin and 10% NaCl solutions and saponins with ability to produce suds [22]).

Acute toxicity studies (LD_{50})

The LD_{50} determination for each of the fractions was conducted separately using modified method of[23] . For each of the fractions, the evaluation was done in two phases. In phase one, three groups of three rats each, were treated with 10, 100 and 1000 mg/kg body weight of the fractions intraperitoneally (ip) respectively. The control groups received distilled water. The rats were observed for clinical signs and symptoms of toxicity within 24 hours and death within 72 hours.

Based on the results of phase one for the Ethylacetate fraction, twelve fresh rats were divided into 3 groups of 3 rats each and were treated with 600, 1000,1600 and 2900 mg /kg fraction (ip*)*. Clinical signs and symptoms of toxic effects and mortality were then observed for seven days.

Also based on the results of phase one for the n-Butanol fraction, nine fresh rats were divided into 3 groups of 3 rats each and were treated were treated with the fraction at the doses of 1600, 2900 and 5000 mg/kg (ip*)* respectively. Clinical signs and symptoms of toxic effects and mortality were then observed for seven days.

The LD_{50} were then calculated as the square root of the product of the lowest lethal dose and highest non-lethal dose i.e. the geometric mean of the consecutive doses for which 0 and 100% survival rates were recorded in the second phase.

Induction of experimental diabetes mellitus

The animals were fasted for 16–18 hours with free access to water prior to the induction of diabetes. Induction of diabetes was carried out by single intraperitoneal injection of Alloxan monohydrate (Sigma St Louis, M.O., USA) dissolved in $0.9\%^{v/v}$ cold normal saline solution at a dose of 150 mg/kg body weight[24]). Since alloxan is capable of producing fatal hypoglycemia as a result of massive pancreatic insulin release, rats were treated with 20 % glucose solution intraperitoneally after 6h. The rats were then kept for the next 24h on 5 % glucose solution bottles in their cages to prevent hypoglycemia [25] . The diabetes was assessed in alloxan-induced rats by determining the blood glucose concentration 72 hours after injection of alloxan. The rats with blood glucose level above 200mg/dl were then selected for the study.

Experimental design

After the induction of diabetes mellitus in the Wistar rats, the animals were randomly divided into experimental and control groups. All animals were fasted for 16-18 hours before treatment. Fasting blood glucose levels of each group was determined weekly for the two weeks. All the animals were sacrificed at the end of the two weeks after fasting them for 16-18 hours. The rats were anaesthetized at the time of sacrifice by being placed in sealed cotton wool soaked chloroform inhalation jar. Blood was collected via cardiac puncture from each animal for determination of haematological parameters. The Wistar rats were subdivided as follows;

Group 1 - Diabetic control Wistar rats (Received 5ml/Kg body weight)
Group 2 Diabetic Wistar rats were treated with Insulin 6 I.U/Kg body Weight[26]
Group 3-Diabetic Wistar rats were treated with 50mg/Kg of Ethyl acetate fraction
Group 4-Diabetic Wistar rats were treated with 250mg/Kg of n-Butanol fraction

Determination of blood glucose levels

Fasting blood glucose levels were determined by using the glucose oxidase method[27] with ONE TOUCH BASIC® Glucometer (LIFESCAN, Inc 2001 Milpitas, CA 95035, USA) and results were reported as mg/dl[28].

Determination of Liver enzymes

The serum enzymes Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP) were determined spectrophotometrically, using enzymatic colometric assay kits according to the laboratory

procedures of Randox Laboratories Limited kits, United kingdom, using method of[29] . Serum total protein and albumin were assayed by the methods described of [30,31] respectively.

Determination of Lipid profle

The method of [32] was adopted. Total cholesterol, HDL-C, triglycerides, albumin, total protein, alkaline phosphatase, alanine and aspartate aminotransferases were determined in the serum using assay kits from Randox using Automated Bayer chemistry analyzer Express plus system 2004 (Bayer Healthcare, LLC, 9664, Fast 4 system Tarrytown, NY, 10591-5097, USA). LDL-C was calculated by using the formula of [33] .

Statistical analysis

All the data are expressed as mean ± SEM. Statistical comparisons were performed by one way analysis of variance (ANOVA) followed by Duncan's multiple range tests [34]. The results were considered statistically significant if the p values were 0.05 or less.

RESULTS

Preliminary phytochemical Screening

Preliminary phytochemical screening of the two fractions of *Indigofera pulchra* extracts revealed the presence or absence of the secondary metabolites as shown in Table 1 below.

Table 1 : Preliminary phytochemical screening of Ethylacetate and n-Butanol fractions of *Indigofera pulchra.*

S/no	Phytochemical Constituent	Ethylacetate Fraction	n-Butanol Fraction
1	Alkaloids	-	-
2	Flavonoids	+	+
3	Saponins	-	+
4	Tannins	-	+
5	Steroids	-	-

+ = present; - = absent

Acute Toxicity Studies

Table 2: The Percentage Mortality of the Different Doses of Ethylacetate Fraction of *Indigofera pulchra* Extract Administered Intraperitoneally in Wistar Rats during the First Phase of the Acute Toxicity Study.

Group(n=3)	Treatment	Mortality	% Mortality
1	Control	0/3	0
2	10 mg/kg fraction	0/3	0
3	100 mg/kg fraction	0/3	0
4	1000 mg/kg fraction	1/3	33.3

The sign of toxicity were first noticed after 4-6 hours of fraction administration. There was decreased locomotor activity, decreased feed intake, and prostration after 10 hours of extract administration.

Table 3: The Percentage Mortality of the Different Doses of Ethylacetate Fraction of *Indigofera pulchra* Extract Administered Intraperitoneally in Wistar Rats during the Second Phase of the Acute Toxicity Study.

Group(n=3)	Treatment	Mortality	% Mortality
1	600 mg/kg fraction	0/3	0
2	1000 mg/kg fraction	0/3	0
3	1600 mg/kg fraction	3/3	100
4	2900 mg/kg fraction	3/3	100

There were deaths recorded in the groups that received 1600mg/kg and 2900mg/kg of the extract as showed in Table 3 above. The percentage mortality in each group was 100 %. The LD_{50} were then calculated as the square root of the product of the lowest lethal dose and highest non-lethal dose i.e. the geometric mean of the consecutive doses for which 0 and 100% survival rates were recorded in the second phase. The LD_{50} of the Ethylacetate fraction was thus; $\sqrt{1000 \times 1600} = 774.6$ mg/kg. The median lethal dose (LD_{50}) in rats was calculated to be 7.75 mg/kg body weight.

Table 4: The Percentage Mortality of the Different Doses of n-Butanol Fraction of *Indigofera pulchra* Extract Administered Intraperitoneally in Wistar Rats during the First Phase of the Acute Toxicity Study.

Group(n=3)	Treatment	Mortality	% Mortality
1	Control	0/3	0.00
2	10 mg/kg fraction	0/3	0.00
3	100 mg/kg fraction	0/3	0.00
4	1000 mg/kg fraction	0/3	0.00

The sign of toxicity were first noticed after 8-10 hours of fraction administration. There was decreased locomotor activity, decreased feed intake, and prostration after 14 hours of fraction administration.

Table 5: The Percentage Mortality of the Different Doses of n-Butanol Fraction of *Indigofera pulchra* Extract Administered Intraperitoneally in Wistar Rats during the Second Phase of the Acute Toxicity Study.

Group(n=3)	Treatment	Mortality	% Mortality
2	1600 mg/kg fraction	0/3	0.00
3	2900mg/kg fraction	2/3	66.6
4	5000mg/kg fraction	3/3	100

The LD_{50} were then calculated as the square root of the product of the lowest lethal dose and highest non-lethal dose i.e. the geometric mean of the consecutive doses for which 0 and 100% survival rates were recorded in the second phase. For the n-butanol fractions there was two mortality at the doses of 2900 mg/kg and three mortality at the dose of 5000 mg/kg. The percentage mortality was 66.6% mortality for the dose of 2900mg/kg and 100% for the dose of 5000mg/kg as shown in Table 5 above. The LD_{50} of was thus; $\sqrt{1600 \times 2900} = 2.154$ mg/kg.body weight.

Table 6: Effects of n-butanol and ethylacetate fractions of *Indigofera pulchra* Serum biochemical changes (Mean ± SEM) on Alloxan-Diabetic Rats (n=5) at 14 days of treatments

Groups	AST (iu/L)	ALT (iu/L)	ALP (iu/L)	Total protein (g/L)	Alb (g/L)
Diabetic control + distilled water	77.6+8.91	65.8+10.5	72.0+8.31	90.0+3.20	70.0+5.54
Diabetic+Insulin 6.i.u/kg	29.8+3.10[a]	34.8+2.72[a]	57.8+5.73[ns]	65.2+1.59[a]	32.6+1.24[a]
250 mg/kg n-Butanol	31.6+3.29[a]	35.2+2.05[a]	56.6+0.81[ns]	66.2+2.24[a]	38.6+2.37[a]
50 mg/kg Ethylacetate	29.8+1.52[a]	34.4+2.18[a]	52.6+2.60[a]	67.8+1.35[a]	34.6+1.50[a]

Values are mean ±SEM; n=5.; Values are statistically significant compared to control at:[a]P<0.05
ns= not significant.

Table 6 above showed the effect of the two fractions of *Indigofera pulchra* on liver enzymes, total ptotein and albumin after 14 day of treatment.However there was a significant decrease ($p<0.05$) in the aspatate amino transferase, alanine amino trasferase,total protein and albumin when compared to control group. As regard to the alkaline phosphatase levels there was no significant change with the n-butanol fraction when compared to control while there was a significant decrease ($p<0.05$) with the ethylacetate fraction when compred to the control group.

Table 7: **Effects of n-butanol and ethylacetate fraction of *Indigofera pulchra* on serum Cholesterols, Triacylglycerols, High density lipoproteins and Low density lipoproteins (Mean + SEM) on alloxan-diabetic rats (n=5) at 14 days of treatments.**

Groups	Total cholesterol (mmol/L)	Triacylglycerols (mmol/L)	High density lipoproteins (mmol/L)	Low density Lipoproteins (mmol/L)
Diabetic+ Distilled water	7.80±0.66	4.90±1.68	1.98±0.10	3.59±1.08
Diabetic+ Insulin(6.i.u/kg)	4.40±0.10[a]	1.54±0.13[a]	1.12±0.10[ns]	2.57±0.14[ns]
Diabetic+250mg/kg of n-Butanol fraction	3.72±0.19[a]	1.38±0.08[a]	1.02±0.08[ns]	2.07±0.17[ns]
Diabetic+50mg/kg of Ethylacetate fraction	3.82±0.09[a]	1.78±0.06[a]	1.08±0.14[ns]	1.92±2.57[ns]

Values are mean ±SEM; n=5.; Values are statistically significant compared to control at:[a]P<0.05; ns= not significant.

Table 7: Above showed the effect of the two fractions of *Indigofera pulchra* on total cholesterol, triglycerides, low density lipoproteins, and high density lipoproteins after 14 days of treatment. However there was a significant decrease ($p< 0.05$) in total cholesterol and triglyceride while there was a slightly increase but not significant when compared with the control.

DISCUSSION

The preliminary phytochemical screening of n-butanol fraction of the plant *Indigofera pulchra* revealed the presences of tannins, saponins, and flavonoids. The sign of toxicity were first noticed after 8-10 hours of extract administration. There was decreased locomotor activity, decreased feed intake, and prostration after 14 hours of extract administration. The median lethal dose (LD_{50}) in rats was calculated to be 2,154 mg/kg body weight. Also the phytochemical screening of the ethylacetate fraction of the plant revealed the presences of alkaloids and flavonoids. The sign of toxicity were first noticed after 4-6 hours of extract administration. There was decreased locomotor activity, decreased feed intake, and prostration after 10 hours of extract administration. The median lethal dose (LD_{50}) in rats was calculated to be 775 mg/kg body weight.

In general with liver disease, serum levels of AST and ALT rise and fall at the same time [35] . A mild elevation of AST level has been shown to be associated with liver injury or myocardial infarctions [36] . The higher the activity of AST, the larger the infarctions size [37] . ALT level is known to increase in liver disease and it has been used as a tool for measuring hepatic necrosis, especially in small animals[38] . In a serious liver injury through oxidative stress, currently available drugs have little effect and thus create a demand to develop new drugs. Herbs have attracted a great deal of interest as physiologically functional foods and as a source for the development of drugs because herbal constituents may have stimulating or regenerating effect on hepatocytes and restored the activities of hepatic system through their anti-hepatotoxic, antioxidant and antihyperlipidemic activities [39] .

The results of this study showed liver enzymes alkaline phosphatase, alanine amino-transferase albumin and total protein, in normal rats, there was no significant change when compared to control group. In relation to the diabetic group control there was increase levels of AST, ALP, ALT ,ALB and TP is suggestive of hepatocellular damage which may be accompany with alloxan induced diabetes mellitus in rats. Also after treatment of the two fractions of the plant, there was a significant decrease ($p<0.05$) when compared to the control as showed in table 6. Elevated serum transaminases are generally associated with tissues especially the liver tissues damage. In this study the fractions caused a significant decrease in both AST and ALT it may be inferred, therefore that the changes may be due to the effect of the fractions coupled with some other intrinsic factors. In relation to the ALP the n-butanol fraction of the plant did not cause significant changed in which might also be due to some other intrinsic factors while the ethyl acetate fraction of the plant caused a significant decreased as compared to the diabetic untreated control group. The total protein measurement provides a good assessment of glomerular and tubularnephropathies and also assessment of the synthetic function of the liver [11,12.] Proteinuria is one of the features associated with long term diabetes mellitus. Proteinuria is a common feature of diabetic glomerulosclorosis and there is usually a progression from symptomatic proteinuria to the nephritic syndrome and finally chronic renal failure. In this study total protein was no significant change when administered to the normoglycemic groups two fractions of *Indigofera pulchra* while there was significant decrease of total protein level in diabetic rats treated when compared with the control untreated group.

The serum lipid profile (cholesterol, triacylglycerol and high density liporoteins) are usually elevated in diabetes [40]. The marked hyperlipaemia that characterizes the diabetic state may be regarded as a consequence of uninhibited actions of lypolytic hormones on the fat depot [41]. Diabetes significantly increased the levels of cholesterol and triglycerides in this study. The fractions of *Indigofera pulchra* plant administered to the diabetic rats significantly reduced ($p<0.05$) the concentrations of cholesterol, triglycerides when compared to the control untreated group. Also high density lipoprotein was decrease but not significant when compared with the control untreated group as showed in Table 7. The hypolipidaemic effect of the two fractions can therefore be explained as a consequence of reduction in blood glucose. Diabetes induced hyperlipidemia may be due to low level of serum insulin or due to interference in insulin action. The fractions of the extract resulted significant recovery in the serum levels of lipid profile biosensors that may be explained from above two dimensions. From this result, it may be stated that the plant may exert its remedial efficacy against experimentally induced diabetes by two ways. One way may be the insulinotrophic effects of this fractions, which is proved by other plant parts as investigated by[42] .

CONCLUSION

The results obtained in our study demonstrated that the ethyl acetate and n-butanol fractions of *Indigofera pulchra* at tested doses significantly the decrease the liver enzyme, total protein and albumin levels in diabetic rats. However, it

also decrease the levels of triacylglycerol, cholesterol, low density lipoprotein and slightly increase the level of high density lipoprotein but not significant.

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