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Full Length Research Paper

Anti-diabetic and some haematological effects of ethylacetate and n-butanol fractions of *Indigofera pulchra* extract on alloxan-induced diabetic Wistar rats

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

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

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The current orthodox treatment modalities for diabetes mellitus have many drawbacks including undesirable side-effects and the high cost of long-term treatment. The aim of this study is to determine the anti-diabetic and some haematological effects of ethylacetate and n-butanol fractions of *Indigofera pulchra* extract in alloxan-induced diabetic. Preliminary phytochemical screening and acute toxicity studies of the two fractions of *I. pulchra* were carried out. A dose of 50 mg/kg of ethylacetate and 250 mg/kg n-butanol fractions of the extract was given intraperitoneally daily, for two weeks to the alloxan-induced diabetic Wistar rats. Insulin was also used as a standard anti-diabetic drug and was given intraperitoneally to alloxan-induced diabetic Wistar rats. The fasting blood glucose levels were determined weekly for two weeks. At the end of the two weeks, the animals were sacrificed and blood samples were taken from all the groups for the determination of hematological parameters. The preliminary phytochemical screening of the two fractions *I. pulchra* extract revealed the presence of alkaloids, flavonoids, and saponins. The LD_{50} was 775 and 2,154 mg/kg for ethylacetate and n-butanol fractions of the *I. pulchra* extract respectively. There was a significant ($p<0.05$) reduction in the fasting blood glucose levels of the alloxan-induced diabetic groups after 1 and 2 weeks of treatment with ethylacetate fraction. As regard to the n-butanol, there was a significant ($p<0.05$) reduction in the fasting blood glucose levels of the alloxan-induced diabetic groups after 2 weeks of treatment. Also, in relation to the erythrocyte indices, there was no significant change in the two fractions tested when compared to control. However, there was a significant increase ($p<0.05$) in the white blood cells, neutrophils and eosinophils counts treated with the two fractions after 2 week of treatment when compared with the control. In conclusion, both ethylacetate and n-butanol fractions of *I. pulchra* extract were found to have potent anti-diabetic effects.

Key words: Blood glucose, diabetes mellitus, *Indigofera pulchra*, erythrocyte, leucocytes.

INTRODUCTION

Diabetes mellitus is a condition in which the body does not produce enough, or properly respond to insulin, a hormone produced in the pancreas. Insulin enables cells to absorb glucose in order to turn it to energy. In diabetes mellitus, the body either fails to properly respond to its own insulin, or does not make enough insulin, or both. This causes glucose to accumulate in the blood, often leading to various complications (Tierney et al., 2002; Rother, 2007).The incidence of diabetes mellitus in the human population has reached epidemic proportions worldwide, and it is increasing at a rapid rate. In 2000, there were estimated 150 million cases in the world, and this number is projected to increase to 221 million by 2010. 90% of the present cases are type 2 diabetes, and most of the increase will be in type 2, paralleling the incidence of obesity (Ganong, 2005).

Indigofera pulchra (Wild) family: papilionaceae is an

*Corresponding author. E-mail: yusuftanko@yahoo.com. Tel: +23437054274.

annual non climbing herbs or shrub that can grow up to 1 m tall. It is widely distributed throughout west-Africa (Hepper, 1976). The local name in Hausa is Bakin bunu and in English it is called Indigofera or indigo. In ethnomedicine, the leaves are used to treat infected wound (Hepper, 1976; Burkhill, 1995) while the decoction of the aerial part is used as prophylactic against snake-bite (Sule et al., 2003) and as anti-inflammatory (Abubakar et al., 2007). Previous pharmacological studies on the methanol extract of the aerial part of this plant showed that, it exhibited venom detoxifying activities (Abubakar et al., 2006). Also previously, Tanko et al. (2008) reported that the crude hydro methanolic extract of *I. pulchra* has anti-diabetic effects. The aim of this work was to determine the effects of ethylacetate and n-butanol fractions of *I. pulchra* extract on the blood glucose level and some haematological parameters of alloxan-induced diabetic rats.

MATERIALS AND METHODS

Animals

A total of seventy four albino Wistar rats of both sexes between the age of 8 to 12 weeks old and weighing 120 to 250 g 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 *I. pulchra* were collected from the Ahmadu Bello University main campus. It was identified and authenticated at the herbarium unit of Biological Sciences Department, ABU, 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 extracts

The air dried *I. pulchra* leaves under the shade and grounded into a fine powder using mortar and pestle. 500 g of the powdered material was macerated in 70% methanol at room temperature for 48 h. It was then filtered using a filter paper (Whatmann No. 1). The filtrate was then partitioned with ethylacetate to get the ethylacetate fraction which was evaporated to dryness in an oven at 37°C. A greenish-brown residue weighing 6 g was obtained and kept in a sealed container at 4°C in a refrigerator until use. Another 500 g of the powdered material was macerated in 70% methanol at room temperature for 48 h. It was then filtered using a filter paper (Whatman No. 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°C. A brownish residue weighing 20 g was obtained and kept in a sealed container at 4°C in a refrigerator until use.

Phytochemical screening of plant fraction

Preliminary screening of the two extracts were performed for the presence of secondary metabolites, using the following reagents and chemicals: alkaloids - with Mayer's and Dragendorff's reagents (Farnsworth, 1966; Harborne, 1998); flavonoids with the use of Mg and HCl (Silva et al., 1993; Houghton and Raman, 1998); tannins with 1% gelatin and 10% NaCl solutions and saponins with ability to produce suds (Houghton and Raman, 1998).

Acute toxicity (LD_{50}) studies

The LD_{50} determination for each of the fractions was conducted separately using modified method of Lorke (1983). 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 extract/kg body weight intraperitoneally (ip) respectively. The control groups received normal saline. The rats were observed for clinical signs and symptoms of toxicity within 24 h and death within 72 h. Based on the results of phase one for the ethylacetate extract, fifteen fresh rats with three per group were each treated with 600, 1000, 1600 and 2900 mg extract/kg (ip) respectively. The control groups received normal saline. 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 extract, fifteen fresh rats with three per group were each treated with the extract 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, that is 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 to 18 h 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 (Katsuamat et al., 1999). 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 6 h. The rats were then kept for the next 24 h on 5% glucose solution bottles in their cages to prevent hypoglycemia (Dhandapani et al., 2002). The diabetes was assessed in alloxan-induced rats by determining the blood glucose concentration 72 h after injection of alloxan. The rats with blood glucose level above 200 mg/dl were then selected for the study.

Experimental design

After the induction of diabetes mellitus in the rats, the animals were randomly divided into experimental and control groups. All animals were fasted for 16 to 18 h 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 12 to 16 h. 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 rats (received 5 ml/Kg body weight)

Table 1. Showing preliminary phytochemical screening.

S/N	Phytochemical constituents	Ethylacetate fraction	n-Butanol fraction
1	Alkaloids	-	-
2	Flavonoids	+	+
3	Saponins	-	+
4	Tannins	-	+
5	Steroids	-	-

+ = present; - = absent.

Table 2. The percentage mortality of the different doses of ethylacetate fraction of *I. pulchra* extract administered intraperitoneally in Wistar rats during the first phase of the acute toxicity study.

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

Group 2-Diabetic rats were treated with insulin (6 I.U/Kg body weight) (Stanley et al., 2001)
Group 3-Diabetic rats were treated with 50 mg/Kg of ethylacetate fraction
Group 4-Diabetic rats were treated with 250 mg/Kg of n-butanol fraction

Determination of blood glucose levels

Fasting blood glucose levels were determined by using the glucose oxidase method (Trinder, 1969) with ONE TOUCH BASIC® Glucometer (LIFESCAN, Inc 2001 Milpitas, CA 95035, USA) and results were reported as mg/dl (Rheney and Kirk, 2000).

Determination of haematological parameters

After two weeks treatment with the two extracts, blood samples were obtained through cardiac puncture of the rats for the determination of the blood parameters: Red blood cells (RBC), packed cell volume (PCV), hemoglobin concentration (Hb), white blood cell count (WBC) and its differential counts using the method of Dacie and Lewis (1991). The red cell indices were also calculated.

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 (Duncan et al., 1977). 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 *I. pulchra* extracts revealed the presence of flavonoids in the ethylacetate fraction while flavonoids, saponins and tannins were present in the n-butanol fraction as shown in Table 1.

Table 3. The percentage mortality of the different doses of ethylacetate fraction of *I. 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 extract	0/3	0
2	1000 mg/kg extract	0/3	0
3	1600 mg/kg extract	3/3	100
4	2900 mg/kg extract	3/3	100

Acute toxicity study

Table 2 showed there was no mortality at the doses of 10 and 100 mg/kg while there was one mortality at dose of 1000mg/kg. The sign of toxicity were first noticed after 4 to 6 h of extract administration. There was decreased locomotor activity, decreased feed intake, and prostration after 10 h of extract administration. There were deaths recorded in the groups that received 1600 and 2900 mg/kg of the extract as showed in Table 3. 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, that is the geometric mean of the consecutive doses for which 0 and 100% survival rates were recorded in the second

Table 4. The percentage mortality of the different doses of n-butanol fraction of *I. pulchra* extract administered intraperitoneally in Wistar rats during the first phase of the acute toxicity study.

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

Table 5. The percentage mortality of the different doses of n-butanol fraction of *I. pulchra* extract administered intraperitoneally in Wistar rats during the second phase of the acute toxicity study.

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

Table 6. Effects of daily doses of ethylacetate and n-butanol fractions of *I. pulchra* extract on blood glucose levels of diabetic Wistar rats.

Group (n = 5)	Treatment	Fasting blood glucose levels(mg/dl)		
		0 day	7th Day	14th Day
1	Control	440.0 ± 36.4	321.6 ± 35.0	277.8 ± 37.8
2	Insulin (6.I.U/Kg body weight)	429.0 ± 48.5	332.6 ± 41.3ns	229.6 ± 39.1ns
3	n-butanol (250 mg/Kg body weight)	434.6 ± 13.0	265.8 ± 40.19ns	175.8 ± 14.9a
4	Ethylacetate (50 mg/Kg body weight)	428.8 ± 53.7	96.0± 17.9a	112.6 ± 28.1a

Values are mean ± SEM. Values are statistically significant compared to control group at: a $P<0.05$; ns= not significant. Mean fasting blood glucose levels at 0 Day: 440.0-428.8 mg/dl.

phase. The LD_{50} of the ethylacetate fraction was thus; √1000 x 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 showed there was no mortality three doses administered. The sign of toxicity were first noticed after 8 to 10 h of extract administration. There was decreased locomotor activity, decreased feed intake, and prostration after 14 h of extract administration.

The LD_{50} were then calculated as the square root of the product of the lowest lethal dose and highest non-lethal dose, that is 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 2 mortality at the doses of 2900 mg/kg and 3 mortality at the dose of 5000 mg/kg. The percentage mortality was 66.6% mortality for the dose of 2900 mg/kg and 100% for the dose of 5000 mg/kg as shown in Table 5. The LD_{50} of was thus; √1600 x 2900 = 2.154 mg/kg body weight.

Effects of daily doses of ethylacetate and n-butanol fractions of *I. pulchra* extract on blood glucose levels of diabetic Wistar rats

There was a significant decrease ($p<0.05$) in the blood glucose levels of the fractions treated diabetic groups after the 7th and 14th days of treatment, when compared to the control as shown in Table 6. Effects of daily doses of ethylacetate and n-butanol fractions of *I. pulchra* extract on some erythrocytes indices of diabetic Wistar rats. Table 7 showed that, there was no significant change in the erythrocyte indices in all the tested groups, when compared to the control group.

Effects of daily doses of ethylacetate and n-butanol fractions of *I. pulchra* extract on leucocytes of diabetic Wistar rats

As regard to the WBC, there was a significant increase ($p<0.05$) when compared to control in all the treatment

Table 7. Effects of daily doses of ethylacetate and n-butanol fractions of *I. pulchra* extract on some erythrocytes indices of diabetic Wistar rats.

Group(n = 5)	Treatment	RBC × 1012 / L	Hb (gm/dl)	PCV (%)
1	Control normal saline	4.9±0.21	14.5±0.23	43.0±0.55
2	Insulin (6 IU/Kg)	4.7±0.25ns	13.2±0.58ns	39.6±1.74ns
3	Ethyl acetate fraction (50 mg/Kg)	4.32±0.13ns	14.4±0.28ns	43.2±0.80ns
4	n-Butanol fraction (250 mg/kg)	4.98±0.11ns	14.5±0.26ns	43.0±0.74ns

Values are mean ± SEM. Values are statistically significant compared to control group at: aP<0.05; ns= not significant.

Table 8. Effects of daily doses of ethylacetate and n-butanol fractions of *I. pulchra* extract on leucocytes of diabetic Wistar rats.

Group(n = 5)	Treatment given	WBC × 109 /L	Neutrophils (%)	Eosinophils (%)	Basophils (%)	Lymphocytes (%)	Monocytes (%)
1	Control normal saline	8.2±0.22	28.6±1.80	32.0±0.94	4.6±0.50	3.20±0.20	1.0±0..00
2	Insulin (6 IU/Kg)	16.3±0.64a	56.2±2.31a	49.2±1.15a	4.8±0.37ns	3.80±0.58ns	0.60±0.24ns
3	Ethyl acetate (50 mg/Kg)	9.0±0.18ns	32.8±0.92a	60.2±0.81a	4.8±0.58ns	3.20±0.48ns	1.00±0.00ns
4	n-Butanol (250mg/kg)	14.7±0.44a	39.8±1.01a	51.4±1.16a	6.0±0.84ns	3.40±0.51ns	0.80±0.20ns

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

groups. Also, there was a significant increase (p<0.05) in the neutrophils and eosinophils count, in all the treatment groups when compared to control. As regard to the basophils, lymphocytes and monocytes, there was no significant change in all the treatment groups when compared to the control group (Table 8).

DISCUSSION

Alloxan monohydrate is known to induce diabetes by partial destruction of pancreatic beta cells of islet of langerhan (Cakici et al., 1994; Abdel-Barry et al., 1997). This results in depletion of insulin levels and hyperglycemia leading to type 1 diabetes mellitus. The alloxan- treated rats, therefore, appear to represent a good laboratory model for IDDM, experimental diabetes state, with residual or remnant insulin production by the pancreatic beta -cells. There is possibility for the survival of a few beta-cells and this has been proved by several workers who observed antihyperglycemic activity with oral hypoglycemic agents like glibenclamide, tolbutamide etc. in alloxan-induced diabetic rats (Sheeja and Augusti, 1993; Kumud and Mathew, 1995; Subramoniam et al.,1996) The diabetic state of alloxan- treated diabetic rats is therefore, not the same as that obtained by total pancreatectomy, as daily administration of insulin is not required for the survival in alloxan-treated diabetic animals.

Medicinal plant extracts have been valuable anti-diabetic agents and may involve one or more active components responsible for blood glucose reduction (Marles and Farnsworth, 1995; Grover et al., 2002). Flavonoids of different plant origin showed a promising anti-diabetic activity, as demonstrated in diabetic animal models (Zarzuelo et al., 1996; Nojima et al., 1998; Kim et al., 2004). Saponins are glycosides of triterpenes, steroids or alkaloids. Previous researchers have demonstrated the hypoglycemic activity of triterpenoid glycosides (Reher et al., 1991; Kako et al., 1997). Our results revealed that, Wistar rats treated for two weeks with both ethylacetate and n-butanol fractions of *I. pulchra* extract resulted in a significant reduction (p<0.05) in the fasting blood glucose levels of diabetics, when compared to control group. However, the diabetic group that received insulin had a significant reduction < 0.05) in the fasting blood glucose levels after one week of treatment only. The preliminary screening of the fractions revealed the presence of flavonoids in ethylacetate fraction and flavonoids and tannins in the n-butanol fraction which may be responsible for the (p<0.05) in the

fasting blood glucose levels after one week of treatment only. The preliminary screening of the fractions revealed the presence of flavonoids in ethylacetate fraction and flavonoids and tannins in the n-butanol fraction which may be responsible for the observed anti-diabetic effects of these fractions by possible stimulating insulin release from the pancreatic beta cells.

Reactive oxygen species has also been implicated in the mechanism of red cells damage (Rao et al., 2003). During diabetes the excess glucose present in blood reacts with haemoglobin to form glycosylated haemoglobin. So the total haemoglobin level is decreased in alloxan diabetic rats (Sheela and Augusti, 1992). The results of the red cell indices of this study revealed non-statistically significance in the groups that received n-butanol and ethylacetate fractions of *I. pulchra* extract. The increase in total leucocytes, eosinophil and neutrophils by the n-butanol fraction of *I. pulchra* extract may indicate an anti-infective effect, but not a boost in the immune system as exhibited by some other plant extracts reported earlier by some researchers (Yakubu et al., 2007). The extract *I. pulchra* extract might have potent anticomplement activity against the classical pathway of complement system. This might be due to the secondary metabolites presents in the extract, especially flavonoids which was reported by Min et al. (2001) that medicinal plants with terpenoids / flavonoids has anticompliment activity against the classical pathway of compliment system.

In conclusion, both ethylacetate and n-butanol fractions of *I. pulchra* extract were found to have potent anti-diabetic effects. Both fractions may also have an anti-infective effect, as they were found to have significantly increased the total leucocytes, eosinophil and neutrophils counts. The ethylacetate and n-butanol fractions has no significant effect on erythrocyte.

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