Annals of Biological Sciences
ISSN: 2348-1927

RESEARCH ARTICLE

Annals of Biological Sciences
2014, 2 (2):58-65

Effects of N-Butanol And Ethylacetate Fractions of *Indigofera Pulchra* on Serum Lipid Peroxidation and Anti-Oxidant Enzymes on Normoglycaemic and Alloxan-Induced Diabetic Wistar Rats

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ABSTRACT

This study was carried out to investigate the effects of ethylacetate and n-butanol fractions of Indigofera pulchra on lipid peroxidation and antioxidant enzymes on normoglycaemic and alloxan induced diabetic rats. Diabetes was induced by a single intraperitoneal injection of alloxan dissolved in 0.9%v/v cold normal saline solution at a dose of 150 mg/kg body weight, after which the rats were randomly divided into four groups for diabetic study and four groups for the normoglyceamic study . After 14 days of treatment, the animal were anaesthetized and sacrificed to obtained blood by cardiac puncture. Serum was collected and assayed for lipid peroxidation, superoxide dismutase and Gluththione peroxide (MDA, SOD, GPx) As regards to the normoglyceamic groups there was no significant change in the levels of malonaldehyde, supraoxide dismutase and gluthathione peroxidise when compared with untreated control group. Although in the diabetic treated groups with the ethyl acetate and n-butanol fractions there was a significant decrease in the level s of malonaldehyde when compared with the control diabetic untreated group. In relations to the antioxidant biomarkers (SOD and GPx) there were no significant change, but there was an increase in the levels when compared with the control. 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.

Keywords: Diabetes , *Indigofera pulchra* , Lipid peroxidation, antioxidant enzymes.

INTRODUCTION

Free radicals are chemical species that have an unpaired delocalized electron in an outer orbital [1,2] , hence they are molecules with an “open” or half bond [3] and are capable of independent existence no matter how brief [4] The unpaired electron confers on these molecules a strong propensity to reacts with target molecules, by withdrawing one electron from target molecules to complete their own orbital[5,4] and thereby stabilizing themselves. Thus, FR causes the molecule whose electron had been withdrawn to become non-functional and even become a FR itself [4] . The resulting FR can in turn react with another molecule until the chain of reaction is terminated either by random collusion of two or three FRs to form a molecule with stable bond or by one of the cellular defence mechanisms [6 ,3, 7] . Biological systems have evolved endogenous defence mechanisms to help protect against FR-induced cell damage [8]. These are called antioxidants.. The antioxidants neutralize FR by

donating an electron to stabilize the FR[9]. The antioxidant molecules "internalize" the loss of an electron through resonance between carbon bonds. The antioxidant systems can prevent oxidative damage by [10,11]

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[12] (Hepper, 1976). The local name in Hausa is *Bakin bunu* and in English it is called *Indigofera.* In ethnomedicine, the leaves are used to treat infected wound [12,13], while the decoction of the aerial part is used as prophylactic against snake-bite [14] and as anti-inflammatory [15]. Previous pharmacological studies on the methanol extract of the aerial part of this plant showed that it exhibited venom detoxifying activities [16] . Also previous work by [17] reported that the crude hydromethanolic extract of *Indigofera pulchra* has anti-diabetic effects.

The aim of this research work was to determine the effects of n-butanol and ethylacetate fractions of *Indigofera pulchra* on serum lipid peroxidation and anti-oxidant enzymes on normoglycaemic and alloxan-induced diabetic Wistar rats.

MATERIALS AND METHODS

Animals

The 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 (40) 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

Superoxide dismutase activity was analyzed using NWLSS^{TM} NWK-SODO2 Superoxide dismutase activity assay kit (NorthWest Life Science Specialties, Vancouver, WA 98662) as stated by the manufacturer. Glutathione peroxidase was determined using the NWK-GPx01 glutathione assay kits as calibrated by the manufacturers; 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 [18, 19] ; flavonoids with the use of Mg and HCl[20,21] ; tannins with 1% gelatin and 10% NaCl solutions and saponins with ability to produce suds [21]).

Acute toxicity studies (LD_{50})

The LD_{50} determination for each of the fractions was conducted separately using modified method of [22]. 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 [23]). 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 [24]. 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 considered diabetic, while blood glucose levels 70 mg/dl and below were considered normoglycaemic.

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 and the serum was assayed for lipid peroxidation and antioxidant enzymes.

The alloxan-induced diabetic rats were randomly group into five with 5 rats in each group
Group 1 - Diabetic control Wistar rats received 5ml/Kg *b.w*
Group 2 Diabetic Wistar rats were treated with Insulin 6 I.U/Kg *b.w* [25]
Group 3-Diabetic Wistar rats were treated with 50mg/Kg of Ethyl acetate fraction *b.w*
Group 4-Diabetic Wistar rats were treated with 250mg/Kg of n-Butanol fraction *b.w*

The normoglycemic Wistar rats were also randomly grouped into five with five rats (n=5) in each group as follows:
Group 5 – Normoglycaemic control Wistar rats (Received 5ml/Kg *b.w*)
Group 6 -Normoglycaemic Wistar rats were treated with Insulin 6.I.U/Kg *b.w* [25]
Group 7-Diabetic Wistar rats were treated with 50mg/Kg of Ethyl acetate fraction *b.w*
Group 8-Diabetic Wistar rats were treated with 250mg/Kg of n-Butanol fraction *b.w*

Determination of blood glucose levels

Fasting blood glucose levels were determined by using the glucose oxidase method of [26] with ONE TOUCH BASIC® Glucometer (LIFESCAN, Inc 2001 Milpitas, CA 95035, USA) and results were reported as mg/dl [27].

Evaluation of Lipid Peroxidation

The level of thiobarbituric acid reactive substance, malondialdehyde (MDA) as an index of lipid peroxidation was evaluated on the plasma using the method of Draper and Hadley [28]. The principle of the method was based on spectrophotometric measurement of the colour developed during reaction of thiobarbituric acid (TBA) with MDA. The MDA concentration in each sample was calculated by the absorbance coefficient of MDA-TBA complex $1.56\times10^{5}cm^{-1}M^{-1}$ and expressed as nmol mg^{-1} [29].

Evaluation of Serum Superoxide Dismutase (SOD) Activity
Superoxide dismutase activity was analysed using NWLSS^{TM} NWK-SODO2 Superoxide dismutase activity assay kit (NorthWest Life Science Specialties, Vancouver, WA 98662) as stated by the manufacturer. This is based on the method of [30] with modifications to increase robustness and reliability. Ultrospec Plus Spectrophotometer Model number 4054 at wavelength of 540nm was used.

Evaluation of Serum Glutathione Peroxidase (GPx)
Glutathione peroxidase was determined using the NWK-GPx01 glutathione assay kits as calibrated by the manufacturers; this test was an adaptation of the method of [31] . In this reaction glutathione peroxidase catalyses the reduction of hydrogen peroxidase (H_2O_2), oxidizing reduced glutathione (GSH) to form oxidized glutathione (GSSG). Oxidized glutathione is then reduced by glutathione reductase and β-nicotinamide adenine dinucleotide phosphate (NADPH) forming $NADP^+$ (resulting in decreased absorbance at 340nm) and recycling the reduced glutathione. The decrease in absorbance at 340nm is directly proportional to the GPx concentration.

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 [32]. 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* 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* 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* 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* 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.

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Table 6: Effects of n-butanol and ethylacetate fractions of *Indigofera pulchra* on Serum Lipid peroxidation and Anti-oxidant enzymes (Mean ± SEM) on alloxan-induced diabetic Wistar rats (n=5) after 14 days treatments.

Groups	Malonaldehyde (nmol/mL)	Superoxide dismutase (Units/mL)	GlutathionePeroxidase (Units/L)
Control Distilled water	2.39±0.15	1.70±0.19	35.6±0.97
Insulin(6.I.U/kg)	2.06±0.20[a]	1.70±0.12[ns]	37.2±1.06[ns]
Diabetic +250 mg/kg of n-Butanol fraction	1.83±0.18[a]	1.48±0.15[ns]	43.6±3.22[ns]
Diabetic +50 mg/kg of Ethylacetate fraction	2.00±0.41[a]	1.48±0.11[ns]	40.0±3.79[ns]

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 serum malonaldehyde, superoxide dismutase and glutathione peroxidase on alloxan induced diabetes Wistar rats after 14 days of treatment. However there was no significant change in all the tested parameters when compared to the control group.

Table 7: Effects of n-butanol and ethylacetate fractions of *Indigofera pulchra* on Lipid peroxidation and Anti-oxidant exzymes (Mean ± SEM) on normoglycemic Wistar rats (n=5) after 14 days of treatments.

Groups	Malonaldehyde (nmol/mL)	Superoxide dismutase (Units/mL)	Glutathione Peroxidase (Units/L)
Control Distilled water	74.8±4.75	1.66±0.14	37.6±3.74
Insulin(6.i.u/kg)	69.2±5.34[ns]	1.68±0.16[ns]	37.6±0.93[ns]
Normal +250mg/kg of n-Butanol fraction	73.0±6.41[ns]	1.56±0.16[ns]	44.0±2.94[ns]
Normal+50mg/kg of Ethylacetate fraction	64.2±5.11[ns]	1.48±0.17[ns]	33.6±1.83[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 serum malonaldehyde, superoxide dismutase and glutathione peroxidase on normoglycemic Wistar rats after 14 days of treatment. However there was no significant change in all the tested parameters when compared to the control group.

DISCUSSION

The preliminary phytochemical screening of n-butanol fraction of the hydromethanolic extract of *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 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 *Indigofera pulchra* 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.

Increased oxidative stress has been proposed to be major causes of hyperglycemiainduced diabetes complication. Hyperglycemia stimulates reactive oxygen spacies (ROS) formation and MDA can react and damage DNA bases, it is reported to be mutagenic in bacterial and mammalian cells and carcinogenic in rats [33] .

In diabetes, hypoinsulinaemia also increases the activity of the enzyme, fatty acyl Coenzyme A oxidase, which initiates beta-oxidation of fatty acids, resulting in lipid peroxidation [34].Increased lipid peroxidation impairs membrane functions by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptors [34]. Its products (lipid radical and lipid peroxides) are harmful to the cells in the body and are associated with atherosclerosis and brain damage [34] . The increase in the level of lipid peroxides in plasma generally is thought to be the consequence of increased production of and liberation into the circulation of tissue lipid peroxides due to pathological changes [35]. Lipid peroxidation (MDA) is widely used as an indicator to reflect oxidative stress and cell membrane damage[36].

Thiobarbituric acid reacting substance (TBARS) or malondialdehyde is a major product of lipid peroxidation, thus an index of measuring the degree of lipid peroxidation. MDA is know marker of lipid peroxidation [37] . There was no significant change in the normoglycaemic treated groups when compared with the control group as showed in table 7. Also in relation to the diabetic groups administered the fractions of the plant, there was a significant decrease ($p<0.05$) in the MDA level when compared with the diabetic untreated group as showed in table 6. This might suggests that the extract exert a stimulation of membrane lipid peroxidation. While the non significant increase in MDA level of the serum in the control group may imply leakage of end product of lipid peroxidation (MDA) into the serum. It has been reported that membrane lipid peroxidation results in the loss of polyunsaturated fatty acids, decreased membrane fluidity, and severe structural changes leading to loss of enzymes and receptor activity [38]. Direct free radical damage to membrane proteins may occur as a result of lipid peroxidation leading to their activation [39]. Thus, the loss of enzyme activities from tissues and sometimes the elevated activities of tissue enzymes as observed with the enzymes studied may be attributed to membrane lipid peroxidation and direct free radical damage to membrane proteins.

[40] reported a decrease in MDA concentration and an increase in activities of SOD and CAT in streptozocin – induced diabetes rats, pretreated with resveratrol, and concluded that, the observed effect was due to the anti-oxidant effect of the drug. The decrease concentration observed might be connected to the secondary metabolite in the plant which might has some anti-oxidant properties which exert the scavenging effect on the generated free radicals and thereby sparing the cells

Superoxide dismutase (SOD) is an antioxidant enzyme which scavenges free-radicals. It protects oxygen-metabolizing cells against harmful effects of free-radicals [41]. There was no significant change the serum lipid peroxidation as compared to the control group but there was a slight increased in the level of SOD which was not significant. The slight increase in SOD activity obtained may indicate the free radical generating potential of the plant. This consequently triggered increased synthesis of SOD to mop the free radicals produced. Also the increased SOD activity may be due to the effects of some metal ions like zinc, copper and manganese. Zinc, copper or manganese plays significant roles in SOD activity as cofactors for SOD isoenzymes [42]. These metals might be present ethylacetate and n-butanol fractions of *Indigofera pulchra* extract.

Superoxide dismutase (SOD) is an antioxidant enzyme which mops up free-radicals. It protects oxygen-metabolizing cells against harmful effects of free-radicals [41]. The slightly increase but not significant in SOD activity obtained at a doses tested may indicate that the free radical generating potential of the fractions which consequently triggered increased synthesis of SOD to mop the free radicals produced. Also as regards to GSH-Px was considered biologically essential in the reduction of hydrogen peroxide. In the present study, there was a slightly decreased in the GPx the antioxidant enzyme when compared to control but the decreased is not significant. As GSH-Px is an important antioxidant molecule, its depletion leads to the increase of oxidative stress. Our result is consistent with the result of [43], who reported an increase in antioxidant enzymes such as SOD, and GSH-Px in diabetes mellitus which gives an evidence of increased reactive oxygen species production .Increased oxidative stress as measured by the index of lipid peroxidation has been shown to be increased in both insulin-dependent (IDDM), and non-insulin dependent (NIDDM) diabetes mellitus [44].

CONCLUSION

The two fractions of *Indigofera pulchra* fractions have showed a significant decrease in the serum level of lipid peroxidation and a decrease in the levels of antioxidant enzymes but not significant when compared with the control group in the diabetic. Also in relation to the normoglyceamic groups there were no significant changes in the lipid peroxidation and antioxidant enzymes.

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