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# Effects of Melatonin and *Azadirachta indica* Administration on serum antioxidant parameters in Streptozotocin induced diabetic Wistar Rats

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**ABSTRACT**

*This study was aimed at evaluating the effects of melatonin and azadirachta indica administration on serum antioxidant parameters in streptozotocin induced diabetes in adult wistar rats. Forty five male Wistar rats were used in the study. The animals were divided into two main groups control group A and diabetic group B. The animals in Group A were sub divided into group 1, 2, 3 and 4 and group B animals subdivided into group 5, 6, 7, 8 and 9 comprises of five rats per group respectively. Group 1 received placebo orally once daily. Group 2 (extract control) received 200mg/kgbw of extract orally daily, Group 3 (melatonin control) received 10mg/kgbw of melatonin intraperitoneally (IP) daily. Group 4 (extract and melatonin control) received 200mg/kg bw of extract orally and 10mg/kgbw of melatonin IP daily. Group 5 (diabetic control) received 10ml/kgbw normal saline daily, group 6 (extract treated) received 200mg/kgbw of extract orally daily, Group 7 (melatonin treated) received 10mg/kgbw of melatonin IP daily, group 8 (extract and melatonin treated group) received 200mk/kgbw of extract orally and 10mk/kgbw of melatonin IP daily while group 9 (metformin treated) received 500mg/kgbw of metformin orally daily. The weight and fasting blood glucose levels were determined at intervals of seven days. At the end of 21 days of treatment, the animals were sacrificed and blood samples collected from all groups and assayed spectrophotometrically for serum concentration of intracellular antioxidants enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). For lipid peroxidation there was a significant reduction ($P<0.05$) in serum MDA for the group treated with extract alone and when combined and high significant value ($P<0.001$) for melatonin treated group only. The study also showed significant increase ($P<0.05$) in serum intracellular antioxidants of the extract and melatonin treatment groups alone for SOD and a highly significant value ($p<0.001$) when extract and melatonin were combined. CAT gave a significant values ($p<0.05$) for melatonin and extract treated groups alone and a high significant value ($p<0.001$) when extract and melatonin were combined. GPx gave significant value ($p<0.05$) of extract treatment alone and highly significant values ($p<0.001$) for melatonin treatment alone and when extract and melatonin were combined.*

**Key words**: Biochemical, Melatonin, *Azadirachta indica*, Streptozotocin, Antioxidant, Wistar rats

*Abbreviations: SOD-superoxide dismutase, CAT- catalase, GPx- glutathione peroxidase, DM- diabetes mellitus, STZ -streptozotocin*

## INTRODUCTION

**Diabetes Mellitus**

Diabetes mellitus (DM) is a metabolic disorder of multiple aetiology, characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both. The effects of diabetes mellitus include long–term damage, dysfunction and failure of various organs [1]. The prevalence of diabetes mellitus varies considerably in different part of the world due to both genetic and environmental factors [1]. Diabetes mellitus is known to have affected Man for many thousands of years. The earliest recorded description of the symptoms was found in the Ebers Papyrus of Egypt which dates back to 1500 BC (Montague, 1983(2). However it was not until the second century AD that Aretaeus of Appadocia named the disease diabetes, the Greek word meaning 'to flow through a siphon'.

The World Health Organization's committee on diabetes mellitus estimated that about 150 million people worldwide suffered from diabetes mellitus in 1995 and predicted that this number will rise to an alarming figure of 300 million by the year 2025 [3].

The search for natural antioxidative agents that will ameliorate the harmful effects associated with hyperglycaemia still continues in spite of considerable progress in the management of diabetes mellitus by synthetic drugs. Many complications of diabetes result due to an increased free radical load [4]. There is therefore an increasing preference for whole plant extract among patients and professionals as these rarely produce side effects, rather they tend to protect the patient from the usual degenerative changes [5].

*Azadirachta indica* of the family- Meliaceae melioideae, is a medium-sized tree that is found throughout the South Asian region, Africa and in Northern Nigeria. Previous studies have reported the beneficial effect of *Azadirachta indica* leaves in the management of diabetes mellitus and the amelioration of the oxidative stress associated with the disease [6]. This was explained by the presence of terpenoids and saponins which have been found to be potentially useful for the treatment of hyperglycaemia. Flavonoid, a known antioxidant is present in *Azadiracta indica* leaves and its extract is currently included in the poly-herbal anti diabetic drugs being subjected to controlled clinical trials in Man

Melatonin has been shown to be a major scavenger of both oxygen and nitrogen based radicals [7]-[8] - [9], including peroxynitrite anion ($ONOO^{-}$) [10]-[11] and [12]. Melatonin may influence diabetes and associated metabolic disturbances not only by regulating insulin secretion, but also by providing protection against reactive oxygen species, since pancreatic β-cells are very susceptible to oxidative stress because they possess only low-antioxidative capacity [12]. In developing countries adequate treatment measures for diabetes mellitus are often unavailable or too expensive hence the need to test for the viability of *Azadirachta indica* ethanolic leaves extract and melatonin a known potent antioxidant as alternatives to conventional antidiabetic drugs. However, Effects of Melatonin and *Azadirachta indica* Administration on serum antioxidant parameters in diabetic subjects are yet to be reported.Thus, our aim is to evaluate the effects of melatonin and *Azadirachta indica* administration on antioxidant parameters in streptozotocin induced diabetes in adult Wistar rats.

## MATERIALS AND METHODS

Fifty three young adult male Wistar rats, weighing approximately 140g each were obtained from the Faculty of Pharmaceutical Sciences of Ahmadu Bello University, Zaria. They were kept in plastic cages and allowed to acclimatize for 2 weeks in the Faculty of Pharmaceutical Sciences Animal house before the experiment, and maintained under laboratory conditions of temperature, humidity and light. They were allowed free access to water and standard pellet diet obtained from Grand Cereals Ltd, Jos Plateau State. The animals were divided into nine groups of five animals each.

**Acquisition and extraction of plant material**

Leaves of fresh *Azadirachta indica* were harvested from Ahmadu Bello University Faculty of Medicine Zaria in the month of April 2012 and authenticated at the Department of Biological Sciences, Ahmadu Bello University Zaria, with a voucher specimen number 900151. The Fresh leaves of *A. indica* were air dried, minced and powdered using laboratory mortar. 1000g of the powdered leaves was extracted in 1.5 liters of 80% ethanol using a soxhlet extractor.

This was filtered using a Whatman filter paper (24mm). The filtrate was dried in a laboratory water bath set at 67°C and total yield of 46.8g was obtained per 1000g of the powdered leaves.

**Chemicals**

Melatonin M5250-1G (Sigma Aldrich USA), Streptozocin SP0130 (Sigma Aldrich, USA). CAT, Catalase Assay kit, (North West life Science Specialties LLC). SOD, Superoxide dismutase Assay kit (North West life Science Specialties LLC). Gluthathione Peroxidase Assay kit (GPx), (North West life Science Specialties LLC). MDA, Malondialdehyde, (North West life Science Specialties LLC).

**Diabetes induction**

A baseline blood glucose levels was taken for all the control and test animals before grouping them. This was done to ensure that the animals were all normoglycaemic. Thirty three Wistar rats were randomly selected and were given a single dose of intra peritoneal injection of streptozotocin, (STZ) (Sigma, Aldrich, USA), at 55mg/kg body weight in citrate buffer (0.1M, pH 4.5). The solution (STZ in citrate buffer) was used within 5 minutes to induce chemical diabetes in the wistar rats after overnight fasting of twelve hours. Blood samples were collected at 72 hours after STZ treatment from the dorsal vein of the tail and the blood glucose levels detected using a One Touch Ultra 2 Glucometer, (Lifescan, CA, USA). Streptozotocin treated adult Wistar rats with fasting blood glucose level at 11mmol/L and above was considered diabetic. Twenty eight Wistar rats in this group were found to be chemically diabetic giving an 84 % diabetic induction. These animals were further grouped into five groups of five Wistar rats each (Group 5, 6, 7, 8, 9) called the diabetic group while group 1, 2, 3, and 4 were the normal control groups with five rats per group.

**Experimental protocol**

Twenty five (25) diabetic animals and twenty (20) normoglycaemic were randomly selected and divided into nine groups of five (5) animals each as follows:

Group 1: Normal control + normal saline orally
Groups 2: Normal + *A.Indica* (200mg/kg bw) orally
Group 3: Normal + Melatonin (10 mg/kg bw) IP
Group 4: Normal + Melatonin (10 mg/kg b w) IP + *A. Indica* (200 mg/kg b w) orally
Group 5: Diabetic control + normal saline orally
Group 6: Diabetic + *A. Indica* 200 mg/kg b w orally
Group 7: Diabetic + Melatonin (10 mg/kg b w) IP
Group 8: Diabetic + *A. Indica* 200 mg/kg b w orally + Melatonin (10 mg/kg b w) IP
Group 9: Diabetic + Metformin (500 mg/kg b w) orally

The extract and drug were administered once daily for a period of three (3) weeks respectively

Streptozotocin was selected to chemically induce diabetes since it potentially destroys the β-cells of the Pancreas to produce diabetes signified by sustained hyperglycaemia above 200mg/dl, (11mmol/L), [13].

The extract (200mg/kgbw) [13] was administered by orogastric intubation once daily for three weeks while melatonin (10mg/kgbw) was administered intraperitoneally once daily for three weeks in all treated groups while metformin was the standard drug (500mg/kgbw). The experimental animals were weighed weekly, at the start and during the experiment. On day 23, the animals were humanely sacrificed using chloroform.

**Biochemical studies**

Blood was collected at sacrifice through cardiac puncture into plain sterilized centrifuged bottles and allowed to clot. The clotted blood samples were centrifuged and the serum harvested for analysis to quantify the serum enzymes SOD, CAT, GPx and serum MDA levels.

**Detection of oxidative stress markers**

**Malondialdehyde assay (MDA)**

Malondialdehyde was assayed using North West life Science Specialties LLC, assay kit according to manufacturer's instructions.

**Assay Preparation**

Spectrophotometer was set at Wavelength of 514nm with a Spectral bandwidth: ≤ 2 nm, Resolution: ≤ 1 nm and Heat block or water bath preheated to 60°C.

**Reagent Preparation:**

**TBA Reagent (2-Thiobarbituric Acid)**

10.5 mL deionized water was added to the TBA bottle. Magnetic stir bar was inserted and mixed until TBA had dissolved for 10 minutes approximately. This was stored at room temperature.

**Other Reagents**

The Acid Reagent, (Butylated hydroxytoluene, 2, 6-di-tert-butyl-4-methylphenol, BHT, CAS 128-37-0) Reagent, Assay Buffer and Calibrators were supplied ready-to-use.

**Assay Protocol:**

10 µL BHT Reagent was added to micro-centrifuge vials. 250 µL Calibrator (sample) was then added to the vials with further addition of 250 µL Acid Reagent to vials. 250 µL TBA Reagent was later added to the vials. They were vortex vigorously (5-count). These were incubated for 60 minutes at 60°C and centrifuged at 10,000 x*g* for 3 minutes. The reaction mixtures were then transferred to cuvettes and spectras recorded from 400-700 nm.

**Glutathione Peroxidase Assay**

Glutathione Peroxidase was assayed using North West life Science Specialties LLC, assay kit according to manufacturer's instructions.

**Assay Preparation**

Plate Reader Setup was set as recommended at a Wavelength of 340 nm, duration of 5 minutes interval with a 30 seconds data reduction and linear regression temperature of 25 ºC.

**NADPH, (β-Nicotinamide adenine dinucleotide phosphate) Diluent**

This reagent was supplied ready to use.

**NADPH Reagents**

The entire contents of one NADPH diluents were added to one NADPH Reagent bottle and the caps on the vials secured. This was mixed briefly by inverting the bottle. The Working *NADPH* solution were kept on ice to prolong the useful life of the reagent during the procedure.

**$H_2O_2$ Reagent**

3% hydrogen peroxide diluted as supplied 1:100 i.e, .02 mL 3% $H_2O_2$ to 2 mL Assay Buffer was marked as Diltution 1, Dilution 1 was further diluted 3:100 (e.g. 0.3 mL Dilution 1 to 10 mL Assay Buffer), this was marked as Working $H_2O_2$.

**Assay Protocol: Standard Procedure for Microplate Assay**

All reagents were brought to room temperature and the microplates were removed from plastic bag. 50 µL of diluted sample and controls were added to wells. 50 µL of Working NADPH was added to each well. 50 µL of Working $H_2O_2$ were further added to each well and observed for 1 minute, $A_{340}$ for 5 minutes with a recording interval of every 30 seconds. Glutathione perioxidase, (GPx) activities were calculated from the net rate.

**Superoxide Dismutase Activity Assay**

Superoxide Dismutase activity was assayed using North West life Science Specialties LLC, assay kit according to manufacturer's instructions.

**Assay/Instrument Preparation: Spectrophotometer (Cuvette Assay) Setup**

Temperature was set at 25°C. The spectrophotometer Zero absorbance was set at 560 nm using distilled $H_2O$

**Plate Reader (Microplate Assay) Setup**

Microplate Reader temperature was set at room temperature and experiment carried out.

**Reagent Preparation*:***

The kit was allowed to warm up to room temperature completely before use.

**Assay Buffer and Sample Dilution Buffer**
This was supplied ready to use. However, the reagent bottles were shaken and opened to air and repeated four (4) more times before use to saturate with $O_2$. Sample dilution buffer was supplied ready to use.

**Hematoxylin Solution*:***
Reconstituted hematoxylin was used as supplied with 1.2 mL (1200 µL) of $dH_2O$ at room temperature and was used within 6 hours.

**Assay Protocol: Cuvette Assay**
920 µL of Assay Buffer was added to each cuvette for assay. 40 µL of Assay Buffer were added for blank and 40µL of Sample. These were mixed and incubated for two (2) minutes. 40µL haematoxylin reagent was further added to start the auto-oxidation reaction. The assays were mixed quickly and immediately the absorbance recorded at 560 nm every 10 seconds for 5 minutes.

**Microplate Assay**
Sample and standard lay out were recorded. 230 µL of Assay Buffer was added to each well used for testing and 10 µL of Assay Buffer to the blank and 10 µL Sample. These was shaken to mix and incubated for 2 minutes. 10 µL of hematoxylin reagent was further added with a multichannel pipette to begin reaction. The assayed containers were mixed quickly and immediately the absorbance was taken at 560 nm every 10 seconds for 5 minutes.

**Employing a Control**
The control samples were prepared, aliquoted and frozen at –20°C. These Control samples were assayed each time along with other samples and the final results normalized with the control sample.

## RESULTS

**Oxidative stress markers**
The table blow showed a significant increase in superoxide dismutase (SOD), Catalase (CAT), glutathione peroxidase (GPx) due to the administration of melatonin and *Azadirachta indica* extract as compared to low levels obtained in the Diabetic control group (DC). The level of Malonaldehyde (MDA) showed a significant decrease in the treated groups as compared with the Diabetic control group (DC) where there was a significant increase in MDA.

**Table 1 Effects of ethanol leave extract of *Azadirachta indica* and melatonin on serum antioxidant parameters of Malonaldehyde (MDA), Superoxide dismutase (SOD), Catalase (CAT), and Gluthathione Peroxidase (GPx) in normoglycaemic and streptozotocin-induced diabetic wistar rats.**

| GROUPS n=5 | MDA (IU/L) | SOD (IU/L) | CAT(IU/L) | GPx(IU/L) |
|---|---|---|---|---|
| NC | 1.340 ± 0.812$^{a}$ | 1.640 ± 0.0927$^{b}$ | 47.20 ± 4.554$^{b}$ | 50.40 ± 2.926$^{a}$ |
| N+AI | 1.440 ± 0.0927$^{b}$ | 1.740 ± 0.1122$^{c}$ | 63.80 ± 4.769$^{c}$ | 59.40 ± 2.676$^{c}$ |
| N+ML | 1.450 ± 0.0812$^{a}$ | 1.940 ± 0.1536$^{d}$ | 55.40 ± 3.429$^{b}$ | 68.00 ± 1.225$^{d}$ |
| NAI+ML | 1.540 ± 0.0245$^{b}$ | 1.720 ± 0.0583$^{c}$ | 66.60 ± 4.155$^{d}$ | 58.20 ± 2.437$^{c}$ |
| DC | 2.180 ± 0.1655$^{d}$ | 1.380 ± 0.0583$^{a}$ | 38.60 ± 2.112$^{a}$ | 45.80 ± 1.241$^{a}$ |
| D+AI | 1.540 ± 0.0678$^{b}$ | 1.720 ± 0.0663$^{c}$ | 64.80 ± 2.853$^{d}$ | 57.40 ± 2.272$^{c}$ |
| D+ML | 1.520 ± 0.0583$^{b}$ | 1.740 ± 0.1166$^{c}$ | 64.40 ± 3.370$^{d}$ | 65.80 ± 1.393$^{d}$ |
| DAI+ML | 1.560 ± 0.0812$^{b}$ | 1.820 ± 0.1158$^{d}$ | 67.40 ± 2.159$^{d}$ | 64.40 ± 2.421$^{d}$ |
| D+MF | 1.820 ± 0.0860$^{c}$ | 1.520 ± 0.0860$^{b}$ | 44.80 ± 3.826$^{b}$ | 49.00 ± 1.049$^{a}$ |

*Values are expressed as mean ±SD; Values with diffe rent superscripts in a column are significantly different ($p < 0.05$); NC = Normal control + normal saline orally; N+AI= Normal + Azadirachta indica (200 mg/kg bw); N+ML = Normal + Melatonin (10 mg/kg bw); NAI+ML= Normal + Azadirachta indica (200 mg/kg bw) + Melatonin (10 mg/kg b w); DC = Diabetic control+normal saline; D+AI=Diabetic+Azadirachta indica (200 mg/kg b w); D+ML=Diabetic +Melatonin (10 mg/kg b w); DAI+ML=Diabetic+Azadirachta indica(200 mg/kg b w)+Melatonin (10 mg/kg b w); D+MF=Diabetic + Metformin (500 mg/kg b w)*

## DISCUSSION

**Lipid Peroxidation (MDA) Studies**
Increased oxidative stress and impaired antioxidant defense have been widely accepted and suggested as participant in the development and progression of diabetes and their complications [14]-[15]. Streptozotocin is a classical chemical diabetogenic agent which is used in β cells destruction and type 1 diabetes, due to its selective cytotoxic

effects on pancreatic β-cells. Taken together, streptozotocin-induced diabetes increases oxidative stress through generation of free radical [16], increase levels of lipid peroxidation, decreased levels of superoxide dismutase, catalase and gluthathione peroxidase [17], as well as DNA single strand break

.

Malondialhyde is produced by peroxidative decomposition of poly unsaturated lipid. It is used as a marker of free radicals in tissue damage and oxidative stress. The level of malondialdehyde (MDA) significantly increased in the animals of diabetic control group which was statistically significant when compared with the control group and the *Azadirachta indica*, melatonin and melatonin and *Azadiracta indica* treated groups. In the treated groups, melatonin group showed the least values followed by *Azdirachta indica* while the combined group gives similar results. The normal control for extract and melatonin gave similar results to the treated diabetic group, except for metformin treated diabetic group. The results obtained were similar to those obtained by [15] which showed that treatment with melatonin decreases and normalised the MDA levels and increased and normalised GPx levels in alloxan induced diabetic rats when compared with untreated rats.

The restoration of antioxidant status is an important parameter to evaluate the effect of anti-diabetic compounds. In diabetes, the persistence of hyperglycaemia has been reported to cause increased production of free radicals. Under normal physiological condition there is a critical balance in the generation of oxygen free radicals and its antioxidant defense systems used by organisms to deactivate and protect themselves against free radical's toxicity [18]. Impairment in the oxidant/antioxidant equilibrium in favour of the former provokes a situation of oxidative stress which is known to be a component of molecular and cellular tissue damage mechanisms in a wide spectrum of human diseases, and contributes substantially to the pathogenesis of diabetic complications [19[-[20].

**Serum Antioxidant Studies (SOD, CAT, GPx)**

Result from the study for SOD, CAT and GPx from the *Azadirachta indica* and melatonin treated diabetic groups resulted in significant increase in serum levels in the enzymes in diabetic treated rats. SOD levels increased significantly in melatonin and extract combined treated group, melatonin treatment alone and *Azadirachta indica* extract treatment alone when compared with diabetic control group which showed decreased enzymes values. A similar result for the normal control treatment groups was obtained when compared with the diabetic treated groups. The results showed that melatonin and *Azadirachta indica* ethanolic extract have antioxidant potentials with normalization of these elevated liver enzymes when used therapeutically. When the extract was combined, the highest values in the elevations of SOD, CAT and GPx in the serums of the treated rats showed that they complemented each other and potentiated removal of free radicals better. SOD destroys free radicals superoxide by converting it to hydrogen peroxide that is further decomposed by CAT and GPx, thereby improving antioxidant levels in the serum and detoxifying generated free radicals released by STZ induced hyperglycaemia in the diabetic rats.

## CONCLUSION

It can be concluded that the administration of *Azadirachta indica* ethanolic leaves extract and melatonin separately and when combined, showed increased levels of serum antioxidants (SOD, GPX and CAT) and a decrease in the levels of malondialdehyde (MDA) as evidenced in the study. Ethanolic extract of *A. indica* leaves and melatonin possesses therapeutic effects in STZ induced diabetes in adult Wistar rats.

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