Anti-Oxidant Effects of *Silybum marianum* Extracts on Diabetic Wistar Rats

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Abstract

Antioxidants are specialized macro-molecules that neutralize harmful substances; free radicals. These radicals supposedly harm tissues, destroy food items, and damage materials. In living organisms, antioxidants can take the form of enzymes, and may be regularly added to oils, metals, foodstuffs, as well as numerous other materials to mitigate the damaging effect of free radical. Current study was designed to investigate the biochemical changes in antioxidant enzyme activities, following administration of *Silybum marianum* (an ancient medicinal plant of the Carduus marianum family) on Alloxan-Induced, diabetic rats. One hundred and twenty-five (125) rats were procured, made to acclimatize for two weeks, and then randomly grouped into five (5) groups of (n=25). Group 1: Non-Diabetic (Control) rats, Group 2 diabetic untreated rats, while groups 3, 4 and 5 comprised of vitamin-C treated rats (diabetic), Silymarin (extract), and Vitamin C + Silymarin (extract) combined treatment respectively. After four weeks of treatment with test extract, animals were then sacrificed, and blood samples collected and assayed for biochemical (anti-oxidant) enzyme activity. Upon statistical analysis, one way Analysis of variance (ANOVA) showed Catalase (CAT), superoxide dismutase (SOD) and malonaldehyde (MDA) activities to have significantly decreased for extract + vitamin C treated group (Group V) when compared with control (Group I). It was also noted that the use of the combined antioxidants vitamin C and silymarin resulted in a significant reduction in ROS production with decreased SOD and CAT enzyme activities. It is therefore likely that, improvements in antioxidant enzyme activities are a function of extract and/or Vitamin C administration to animals. Thus, Silymarin has antioxidant and regenerative potentials to damaged tissues.

Keywords: *Silybum marianum*, Antioxidant enzymes, Free radicals.

INTRODUCTION

In humans, free radicals are released at specific metabolic situations requiring optimal cellular functioning within the body. These radicals lack electrons, making them electrically charged [1]. To achieve neutrality, these free radicals often snatch electrons from neighboring molecules by way of oxidation, thus, creating newer radicals from their neighbors. By way of continuous instability, newly formed free radicals always consistently search out other molecules and snatch and/or donate electrons in the bid to setting series of reaction that in turn, destroys several other molecules.

Umpteen times, many antioxidant systems themselves act as free radicals that donate electrons for the stabilization and neutralization of the supposedly dangerous effects of free radicals. Some antioxidants act against free radicals by exerting a destructive effect on them prior to the domino effects that may precipitate oxidative damage [2]. During respiration, about 5% of the total oxygen breath delivered to humans is often converted into free radicals. Though the presence of these free radicals may not always bad, studies have suggest that in metabolic processes, these free radicals are special to numerous body functions; including immune responses to injury [3-4]. For diseased or damaged tissues, it is also known that the immune system mobilizes fighting cells damaged sites were free radicals gets released to help combat invaders.

When humans get exposed to environmental pollutants like cigarette smoke, overexposure to sunlight, or smog, their body gradually becomes overwhelmed by excess of free radicals. This then damages major macromolecules, including deoxyribonucleic acid (DNA) [5]. This thus prone the cells to cancer-causing chemicals, called carcinogens, that ultimately results in several diseases that oxidize low-density lipoprotein (LDL) cholesterol.

Antioxidants work by scavenging on the body’s free radicals. For instance, superoxide dismutase (SOD) is an intrinsic body enzyme that transforms free radicals to harmless molecules [6-7].
Dietary antioxidants often supplement the action several of these enzyme systems in the body, with available reports positing that diet high in antioxidants may decrease cardiovascular risk diseases. Inconclusive studies on the health benefits of antioxidants are still ongoing. This has thus implicated research into use of several herbs with antioxidant effects. One of such often alleged herb is *Silybum marianum*.

*Silybum marianum* (aka Milk thistle), is one of the oldest medicinal plant of early histories that taxonomically belongs to the Carduus marianum family [8-9]. Its exploration dates back to centuries where it functioned in the treatment of several diseases, including those of the gallbladder. *S. marianum* is known to be hepatoprotective; especially in situations of snake bites and insect stings, mushroom poisoning as well as alcohol abuse [3-5].

Generally, fruits and vegetable nutrients are known to prevent body damage due to Reactive Oxygen Species (ROS). Phytochemicals of natural occurring antioxidants have been reported to have great potentials in free-radical scavenging [10]; especially with flavonoids posing powerful antioxidant and protective roles in free radicals mediated ailments. Recently, attentions have also been drawn on the potential use of flavonoids-based drugs cubing possible tissue damages due to free radicals [11]. Silymarin has also been shown with some antioxidant effects in stress managements, induced as a result of defient disorders in metabolic processes, including Diabetes and cardiovascular ailments [12].

Evidence based reports are available that predicts the mechanisms of action of silymarin. In most cases, silymarin is thought of as a potent incrementor of regenerative tendencies of damaged liver cells through improvements of DNA and RNA synthesis [8]. Theoretically function is possibly accomplished through alteration of membranes, prevention of subsequent entrance of the xenobiotics into cells, and Scavenging of free radicals that may result from damages to tissues [9-10].

Antioxidant studies in recent times have also implicated silymarin as potent in reducing complications due to DM [11]. However, clinical reports on this effect is exclusively inconclusive, even though there appears to be limited clinical trials on antioxidants in DM managements, few of these trials however focused vitamins E and C usage in this instance.

**Aim of Study**

Current study determined the Antioxidant properties of Silymarin on alloxan-induced, diabetic wistar rats. Study also determined the efficacy of Silymarin to conventional antioxidant vitamins, examining in any case, the changes in antioxidant levels of stomach, pancreatic and duodenal tissues in Diabetic wistar rats with Silymarin and vitamin C.

**MATERIALS AND METHODS**

**Resources and Sources**

| Materials       | Source                                                                 |
|-----------------|------------------------------------------------------------------------|
| Silymarin       | Sigma-Aldrich Chemicals Company (St. Louis, Mo, USA)                   |
| Vitamin C       | Emzor Pharmaceuticals, Lagos, Nigeria                                  |
| Assay kits      | Randox Laboratories Limited, United Kingdom and Sigma-Aldrich Chemicals Company (St. Louis, Mo, USA) |
| Distilled water | Physiology Laboratory, Delta State University Abraka Nigeria           |
| Wistar rats     | Animal unit of the College of Medicine, Ambrose Alli University Ekpoma in Edo State of Nigeria. |

**Study Design**

One hundred and twenty-five (125) rats weigh 130 - 180 grams between 6 to 8 weeks old were procured. Next, animals were given two weeks of acclimatization, thereafter, grouped into five (n=5); Group 1: Non-Diabetic (Control) rats, Group 2 diabetic untreated rats, while groups 3, 4 and 5 comprised of vitamin-C treated rats (diabetic), Silymarin (extract), and Vitamin C + Silymarin (extract) combined treatment respectively at daily doses of 100mg/kg body weight and 30mg/kg body weight for Vitamin C and Silymarin respectively. After 4 weeks of treatment, animals were then sacrificed, and blood sample collected and assayed for biochemical [anti-oxidant] enzyme activity.

**Procedure**

**Ethical Clearance**

Prior to commencement of actual experiment and animal procurement, ethical clearance was first sought from the Bio-research and ethics committee of the Faculty of basic medical Sciences, Delta State, University, Abraka, Delta State.

**Determination of Weights**

Next, Animals were cautiously weighed by placing each of them on electronic weighing scales, with their weights read (in grams). This procedure was repeated weekly, just before and after treatment with test substances.

**Inducing Diabetes Mellitus**

With the aid of a syringe, 140mg/kg body weight of Alloxan monohydrate was given to DM group (rats) intraperitoneally. The rats were then ascertained to be diabetic just immediately, using a glucose monitoring device [13]. 140mg/kg body weight of a single alloxan monohydrate injection was given intraperitoneally [14].

**DM Confirmation**

Serum glucose level by standards (in Wistar rat) ranges from 50 to 135 mg/100ml [15]. DM was then affirmed after 48 hours of Alloxan injection on rats. Blood glucose levels above 200mg/dl were taken as diabetic. This process was carried out weekly for reason of sustainability throughout the experimentation. A 12 days rest period was also allowed for rats prior to commencement. During this period, animals had access to standard food and water *ad libitum* [15].

**Administration of Vitamin C and Silymarin**

With the oral intubation (gavage), 30 mg/kg (of Silymarin) per body weight was administered once daily to rats *ad libitum*, while also providing them with clean food and water. Also, 100 mg/kg body weight of Vitamin C was give once daily throughout the period of experimentation with *ad libitum* provision of food and water [16-17].

**Blood Samples Collection**

At the end of four (4) weeks of treatment with test substance(s), animals were euthanized by cervical dislocation after inrapertoneally administration of 30 mg/kg of thiopentone sodium [18]. At the point of dissection, animals’ abdomen was dissected, and blood sample obtained by cardiac puncture. Stomach duodenum and pancreas were also removed and homogenized after weighing. Thereafter, it was then kept in phosphate buffer for a period of 10 minutes and centrifuged with 4000 rpm. Supernatant was then cooled at 4°C in the refrigerator.
Determination of superoxide dismutase (SOD) activity

In principle, SOD is known to inhibit the self-oxidation of Epinephrine by the generation of SOD radical (O₂). By this, a mixture of 0.2ml of obtained samples was reacted with 2.5ml of 0.05mM of carbonate buffer at a pH of 10.2. Next, a reference cuvette of 2.5ml buffer, 0.2ml of water and 0.3 ml of the epinephrine (substrate) was then added, following which increased absorbance was observed at 480nm per 30seconds for 150seconds. Thereafter, Percentage SOD activity inhibition was measured as the supernatant volume that yields 50% reaction inhibition, and expressed in µg/mg.

Measuring Catalase (CAT) Activity

In principle, determination of CAT activity is made possible because Catalase converts hydrogen peroxide (H₂O₂) to molecular oxygen and water as given;

$$2\text{H}_2\text{O}_2 \text{ Catalase} \rightarrow 2\text{H}_2\text{O} + \text{O}_2$$

To achieve this, about 0.1ml of homogenates (supernatant) was first pipetted into a 1.9ml of 500mM phosphate buffer containing cuvette whose pH was 7.0. Next, 1.0ml of freshly prepared hydrogen peroxide (H₂O₂) was added to the mixture. Catalase activity was then measured with the use of spectrophotometer at an absorbance of 240 nm. This absorbance decrease was then recorded (µg/mg) in 15 seconds for every 3 min.

Measurement of Malondialdehyde (MDA) activity

Lipid peroxidation reactions are known to changes with MDA levels in response to oxidative stress. Thus, in principle, spectrophotometric measurement of color absorbance rate at 535nm forms MDA and Thiobarbituric acid, (TBA). In each sample, MDA formed was then computed by TBARS level. First, about 0.1ml of sampled homogenate was treated with 2ml of TBA reagent of 0.37% TBA, HCL 0.25N and 15% TCA. Next, the mixture was then fumed for 30 min at a temperature of 95 °C. It was thereafter ice-cooled for 5 min and centrifuged for 10 min at 10000×g, supernatant absorbance was then obtained and modified for unspecific turbidity (at 532 nm). MDA concentration was then obtained from extinction coefficient in nmols/ml at a measurement of 155 mM-1cm-1 [18].

Analytical Approach

Results obtained from the study were expressed as Mean ± SEM (Standard Error of Mean). With P-value of less than 0.05 (p < 0.05) considered to be statistically significant, a one-way analysis of variance (ANOVA) was used to determine the mean differences for variables between groups.

RESULTS

Figure I (below) shows changes in Catalase (CAT) activity for four weeks of administration of silymarin to various groups, and the responses. As seen, Catalase activity was observed in diabetic tissues as against Vitamin C and Silymarin treated tissues * statistically Significant increase # statistically significant decrease as compared to control group.
Figure IV: Effect of Silymarin and Vitamin C Treatments on Pancreatic catalase Activities

* = statistically Significant increase
# = statistically significant decrease as compared to control group.

Figure V: Effects of Silymarin and Vitamin C Treatments on Gastric SOD Activity

* = statistically Significant increase
# = statistically significant decrease as compared to control group.

Figure VI: Effects of Silymarin and Vitamin C Treatments on Duodenal SOD Activity

* = statistically Significant increase
# = statistically significant decrease as compared to control group.

Figure VII: Effects of Silymarin and Vitamin C Treatments on Pancreatic SOD Activity

* = statistically Significant increase
# = statistically significant decrease as compared to control group.

Figure VIII: Changes in Gastric MDA Activities in Silymarin and Vitamin C Treated Diabetic Rats

*Significant increase of MDA at the 3rd and 4th week.

Figure IX: Changes in Duodenal MDA Activities in Silymarin and Vitamin C Treated Diabetic Rats

*Significant increase of MDA at the 3rd and 4th week.
**DISCUSSION**

This study was engineered to investigate effects of silymarin and/or vitamin C administration on oxidative stress markers. The study specifically examined the antioxidant properties of silymarin on selected tissues of diabetic wistar rats.

Firstly, Diabetes Mellitus (DM) was induced with Alloxan monohydrate, a substance known to the researchers cause DM by depleting pancreatic beta-cells. Treatment of these diabetic rats with vitamin C, silymarin, and a combination of vitamin C and silymarin was observed on selected tissues, including blood (baseline comparison), following period of experimentation. Analysis showed an all statistically significant decrease in blood glucose levels upon comparison with diabetic rats (Figures I).

In another study on Rutin (a known antioxidant), combination treatments with vitamin C for 5-weeks on diabetic rats reportedly lead to a decrease in glucose levels, with accompanying upsurge in insulin concentrations when compared to diabetic control group of rats. However, Rutin’s effects were far apparent on combinational therapy with selected antioxidant vitamins. For current study however, the combined effects of Silymarin and vitamin C co-administration caused a statistically significant increase (ameliorative changes) than single treatment with vitamin C or silymarin only. Also, it is further observed that silymarin and vitamin C co-administration caused a significant amelioration of hyperglycaemia, lipid peroxidation, and increased the activities of serum and antioxidant enzymes across sampled tissues (Figures I-X).

Again from this study, vitamin C and silymarin or a combined treatment with both (groups III - V) showed a statistically significant decrease (lowering effect) of blood sugar, whilst exerting a potent anti-hyperglycemic change as demonstrated by the significant decrease ($P < 0.05$) in blood glucose. The insufficient secretion of insulin due to pancreatic cell destruction may have resulted in oxidative damage by generation of reactive oxygen species (ROS) [21], thus complicating the DM, and over flooding sampled tissues with free radicals [22]. Thus, these free radicals and reactive oxygen species (ROS) may have caused severe destructive to the body [23] from reactivation of fatty acids resulting from the tissue damage.

Basically, Antioxidants are known actors that inhibit the oxidation of lipids via initiation of oxidative chain reactions that prevent or repair damages done to the cells by oxygen. Their actions are often mediated via numerous mechanisms as reduced peroxidation, free radical-scavenging, potential complexing of pro-oxidant metals and quenching of singlet oxygen. Intrinsically, the body has numerous antioxidant systems that prevent ROS formations, included non enzymatic antioxidants (vitamin C, vitamin D and glutathione), enzymes regenerating the reduced forms of antioxidants, and ROS-scavenging enzymes such as SOD, CAT, GPx and GST [24].

Also from Figure II – IX, the antioxidant properties of Silymarin and/or vitamin C administrations on Catalase and Superoxide dismutase activities of stomach, duodenum and pancreas is shown. Apparently, a statistically significant increase in antioxidant enzyme activities for is seen compared to those of normal tissues. Here, antioxidant enzymes were observed to have increased in activity, maintaining homeostasis as such. Across groups, SOD activity was seen to have significantly increased in diabetic than non-diabetic (control) rats, with higher SOD activity probably due to increased dismutation of superoxide anions due to their increased production [25]. Diabetic rats treated with silymarin and vitamin C combination had similar activity to non-diabetic rats. This suggests an increment in SOD activity as a result of superoxide anions at the very beginning of DM with antioxidant vitamin administration. This further reduced ROS production with resultant reduction in SOD activity. Available reports however posit conflicting result on SOD and Catalase activities in diabetes, implicating increased and decreased concentration in enzyme activity with no visible change.

From current study, antioxidant enzyme activities of diabetic untreated rats are seen to have decreased significantly in accordance with Saravanan et al. report [26] that found low SOD and CAT activity in diabetic rats as against those of control group. Also, DM is seen in free radical production, leading to exhaustion and disruption of cellular functions due to oxidative damage [26]. This antioxidant enzymes activity halt is suggestive of an increase in ROS build up for DM untreated rats, and thus leading to oxidative stress. Also, SOD and CAT activity level due to stress was significantly increased with supplementation of antioxidant vitamins to wistar rats as against those of control.

SOD and CAT increased activity (seen in this study) is likely due to the supplementation of vitamin C and silymarin on rat’s diet. Apparently, this ameliorated the generated ROS toxic effect due to DM induction. Previous reports had suggested antioxidant therapy as adjunct treatment option for DM management. Current study has also demonstrated the possible beneficial effect of antioxidant vitamin C and silymarin co-administration in ameliorating oxidative stress due to DM. MDA, a by-product of lipid peroxidation reaction, is also a known oxidative stress predictor [26], as these products are indicative of ageing, stress, and oxidative damage on living cells.

Also from our findings (Fig VIII and X) was significant increase in serum Malondialdehyde (MDA) concentration of diabetic untreated rats. This increase may be due to destruction of membranes of the red blood corpuscles resulting from oxidative stress, supporting records from other studies that DM causes an increase in lipid peroxidation [24-26]. Also, a significantly MDA levels in untreated diabetic rats (seen from our result) concurs with those of Saravana et al. [26] who noted a significant high level of MDA in the untreated diabetic rats in oxidative stress. However, current study observed significantly increased; relatively stable MDA level in the early stress period, suggestive that antioxidants may be potent in ameliorating the damaging effect of oxidative stress. This finding concurs with those of Okon et al. [27], which implicates the extent of lipid peroxidation.

**CONCLUSION**

From current study, two of the three antioxidant enzymes analyzed; i.e. catalase (CAT) and superoxide dismutase (SOD) showed a significant increase in various tissues as observed in Fig II - IX respectively, indicative of oxidative stress in diabetic rats compared to their controls. Within the same tissues however, malondialdehyde
(MDA) levels remained relatively unchanged in the first two weeks, but was observed to increase through the third and fourth weeks; indicative of chronic oxidative stress. However the levels of these parameters were greatly reversed in response to treatment of these rats using vitamin C, silymarin and a combination of both. It was also noted that the use of the combined antioxidants vitamin C and silymarin resulted in a significant reduction in the production of ROS with resulting decrease in SOD and CAT activity.

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