

In vivo and in vitro Antidiabetic and Antioxidant Activity of Spirulina

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ABSTRACT

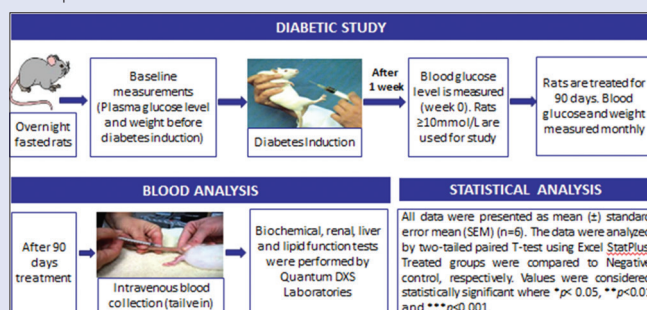
Objective: This study aims to evaluate the effect of spirulina, a biomass produced by cyanobacteria, on the level of plasma glucose, oxidative stress, and other biochemical parameters in diabetes in streptozocin (STZ) 50 mg/kg-induced diabetic-induced rat model. **Materials and Methods:** The *in vitro* antioxidant property of spirulina was assessed by measuring its ability to scavenge free radicals and reactive oxygen species (ROS) such as superoxide anion, nitric oxide, and hydroxyl and lipid peroxy radicals. The inhibition of diabetic link enzymes alpha-glucosidase, alpha-amylase, and dipeptidyl peptidase-4 inhibitor (DPP-IV) were tested *in vitro*. Thirty female Sprague-Dawley rats weighing 150–250 g were divided into five groups: normal, diabetes (negative control), metformin in single dose of 300 mg/kg, spirulina in a single dose of 300 mg/kg and spirulina combined with metformin at dose of 150 mg/kg, and spirulina at dose of 150 mg/kg (spirulina + metformin 300 mg/kg) ($n = 6$). After an acclimation period of 2 weeks, diabetes was induced in the rats through STZ intraperitoneal injection. Spirulina (300 mg/kg) was dissolved in water and was administered orally for 12 weeks, and the rats' that plasma glucose level reached ≥ 11 mmol/L after 12 weeks treatment was selected for the study. After the treatment, the blood and liver were used for the evaluation of antioxidant enzyme activities, lipid, liver, kidney, and hematology profile. **Results:** Spirulina was able to reduce hyperglycemia-induced oxidative stress by reducing plasma glucose levels and scavenging or reducing the production of ROS and free radicals. It was also able to inhibit the activities of the alpha-glucosidase, alpha-amylase, and DPP-IV. With this, it significantly reduced the effect of STZ on the liver and kidney at the organ level and on antioxidant enzymes at the cellular level. **Conclusion:** Spirulina is able to reduce the lipid, liver, and kidney disease markers in STZ-induced rats and therefore is a potential supplement for diabetic patients. The antidiabetic effect of spirulina may be based on the antioxidant effect of the biomass as a whole, or it is based on specific bioactive components present in spirulina.

Key words: Antidiabetic effects, antioxidant, oxidative stress, spirulina

SUMMARY

Spirulina is not cytotoxic to the cells; it possesses *in vitro* and *in vivo* antioxidant and antidiabetic activities and able to moderate the biochemical lipid, liver, and kidney disease markers in streptozocin-induced diabetic rat models. The mechanism of action of spirulina may be through the increase in concentrations of active incretin hormones, glucagon-like peptide-1, and glucose-dependent insulinotropic polypeptide. It also possesses a very

strong *in vitro* and *in vivo* antioxidant property which plays an active role in type 2 diabetes treatment. Antioxidant effect of spirulina maybe because of the presence of significant amounts of phytoconstituents such as chromium, iron phycocyanins, carotenoid, Vitamin E, chlorophyll, flavonoids, saponins, and phenolic compounds which have previously shown antioxidant activity. Based on this finding, spirulina can be a very good potential natural alternative for the treatment and management of diabetes-associated liver and kidney complications.



Abbreviations used: DPPH: 2,2-diphenyl-1-picrylhydrazyl; STZ: Streptozocin; OGTT-Oral glucose tolerance test; DPP-IV: Dipeptidyl peptidase-4 inhibitor; DM: Diabetes mellitus; DMEM: Dulbecco's Modified Eagle's Medium; FBS: Fetal Bovine Serum; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; HbA1C: Glycated hemoglobin; AP: Alkaline Phosphatase; AST: Aspartate transaminase; ALT: Alanine aminotransferase; HDL: High density lipoproteins; LDL: Low density lipoproteins; GSH: Glutathione Reductase; LPO: Lipid peroxidation; SOD: Superoxide dismutase; CAT: Catalase; RBC: Red blood cell; WBC: White cell count.

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INTRODUCTION

Spirulina is a microscopic, filamentous, dried biomass of *Arthrospira platensis*, an oxygenic photosynthetic cyanobacterium found in fresh and marine waters worldwide. It can be consumed by humans and animals as a food supplement or as a whole food. Spirulina has been labeled as a superfood because of its richness in proteins, carbohydrates, polyunsaturated fatty acids, sterols and minerals such as calcium,

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chromium, iron, zinc, magnesium, manganese and selenium. It is also a natural source for provitamin A and Vitamin B12, E, C, phenolic acids, linoleic acid, and xanthophylls phytopigments.^[1] The presence of chromium, selenium, phenolic acids, zinc, linoleic acid, and xanthophylls phytopigments may be potential contribution to the antidiabetic effect of spirulina. Potential anti-inflammatory, anticancer, cholesterol-lowering, and antiviral effect of spirulina is gaining attention as a nutraceutical and as a source of a potential pharmaceutical.^[2-6]

Diabetes mellitus (DM) is a metabolic disorder characterized by the presence of chronic hyperglycemia (in untreated patients) either due to insulin resistance or destruction of pancreatic β -cells.^[7] This condition is accompanied by an impairment of carbohydrate, lipid, and protein metabolism that can lead to premature death. Rapid increase in diabetes, especially type 2 diabetes globally has made it an area of concern and research interest.^[7] According to the International Diabetes Federation report by 2030, almost around 552 million will have DM.^[8] In 2014, 422 million adults were living with diabetes compared to 108 million in 1980 across the world. Diabetes caused 1.5 million deaths in 2012. An additional 2.2 million deaths were caused by diseases associated with long-term hyperglycemia such as cardiovascular disease, chronic kidney failure, neuropathies, liver failure, osteoporosis, and lymphatic complications.^[7,9] The incidence of diabetes is expected to increase from 2.8% in 2000 to 4.4% in 2030.^[10] Currently, 90% of the diabetic patients have type 2 diabetes compared to 0.7% with type 1 DM.^[11] Although there are many drugs currently available to treat diabetes, there is a continued need for new therapies which will complement current diabetic therapy and reduce side effects of these drugs and the complications associated with diabetes such as hypertension, dyslipidemia, hypoglycemia, hypokalemia, and lipodystrophy among others. The present study was designed to evaluate the *in vivo* and *in vitro* antidiabetic and antioxidant effects of spirulina.

MATERIALS AND METHODS

Cell culture

An L-6 (Rat, Skeletal muscle) cell (ATCC CRL-1458) was procured from the National Centre for Cell Sciences, Pune, India. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% inactivated fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 mg/ml), and amphotericin B (5 mg/ml) in a humidified atmosphere of 5% CO₂ at 37°C until about 90% confluent. The cells were detached with TPVG solution (0.2% trypsin, 0.02% ethylenediaminetetraacetic acid [EDTA], and 0.05% glucose in phosphate-buffered saline [PBS]). The L-6 cells were grown in 25 cm² culture flasks and all experiments were carried out in 96 well microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India). About 100 μ l of cells (2×10^4 cells) were seeded in the 96 well plates for 24 h.

Determination of cell viability by

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

The monolayer cell culture was trypsinized, and the cell count was adjusted to 2.0×10^4 cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was removed, the cells were washed once with PBS, and 100 μ l of 0.5–2.5 μ g/ml of spirulina was added on to the partial monolayer in microtiter plates. The plates were incubated at 37°C for 3 days in 5% CO₂ atmosphere, and microscopic observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 μ l of

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37°C in 5% CO₂ atmosphere. The supernatant was removed and the cells were washed, and 100 μ l of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage of growth inhibition was calculated using the formula below.^[12]

% Growth Inhibition =

$$100 \left(\frac{\text{Mean of absorbance of Treated Sample}}{\text{Mean of absorbance of control}} \right) \times 100$$

Graphs were formed with X-axis (μ g/ml) and Y-axis (Percentage viability %) and were analyzed using GraphPad Prism Software Version 7.0., (GraphPad Software, San Diego, California).

Antioxidant assays

2,2-diphenyl-1-picrylhydrazyl radical scavenging activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was done according to the scavenging assay described Jananie *et al.*, 2011.^[13] Briefly, 1 mL of spirulina at concentrations of 20–100 μ g/mL was added to a container containing 1 mL of DPPH (0.05M) and incubated for 30 min in the dark. The absorbance was measured at 517 nm against blank. About 1 mL of absolute ethanol added to 1 mL of DPPH against blank was used as negative control, while Butylated hydroxytoluene (BHT) was used as positive control. The equation below was used to determine the percentage of radical scavenging activity of each compound:

$$\% \text{ inhibition} = \left(\frac{[\text{OD control} - \text{OD sample}]}{\text{OD Control}} \right) \times 100$$

(OD control: Absorbance of control, OD sample: Absorbance of sample).

Nitric oxide scavenging activity

Nitric oxide scavenging assay was performed according to the method described by Adithya *et al.*, 2013.^[14] The reaction mixture was made up of 2 mL of 10 mM sodium nitroprusside in 0.5 mL of PBS (pH-7.4). 0.5 mL of spirulina at the concentration ranges of 20–100 μ g/mL was added to the reaction mixture, shaken and incubated for 2½ h at room temperature. 0.5 mL was taken from the mixture and mixed in 1 mL of 0.33% sulfanilic acid in different test tube and allowed to stand at room temperature for 5 min. Followed by addition of 1 mL of 0.1% naphthalene diamine chloride and incubation at room temperature for 30 min. The absorbance was measured at 540 nm. Percent inhibition was calculated as above.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging assay was performed according to the method described by Adithya *et al.*, 2013,^[14] 60 μ L of 1 mM ferrous chloride, 90 μ L of 0.2M phosphate buffer (pH 7.8), 150 μ L of 0.17M hydrogen peroxide, and spirulina at concentration ranges of 20–100 μ g/mL were mixed together. The mixtures were shaken and incubated at room temperature for 5 min. The absorbance was measured at 560 nm and % inhibition was calculated as above.

Lipid peroxidation activity

Lipid peroxidation (LPO) activity was done by thiobarbituric acid (TBA)-reactive species. Egg yolk homogenate was used as a lipid-rich medium. 0.5 mL of 10% v/v of egg yolk homogenate and 1 mL of spirulina at concentration ranges of 20–100 μ g/mL were mixed together. About 1 mL of distilled water and 0.005 ml of FeSO₄ (0.07M) were added to the mixture to induce LPO and incubated for 30 min.

Then, 1.5 mL of 20% acetic acid and 1.5 mL of 0.8% (w/v) TBA in 1.1% SDS and 0.5 mL 20% trichloroacetic acid (TCA) were added, and the resulting mixture was vortexed and heated at 95°C for 60 min. Another set of samples were mixed in a similar manner but incubated without TBA. After cooling, 5.0 mL of butanol was added to each tube and centrifuged at 5000 rpm for 10 min. The absorbance of organic layer was measured at 532 nm, and % inhibition was calculated as above Adithya *et al.*, 2013.^[14]

Alpha-glucosidase enzyme inhibition activity

Alpha-glucosidase activity was performed according to the method described by Manikandan *et al.*, 2013.^[15] 1 mL of 2% w/v of sucrose solution was added as substrate in 0.2M of Tris buffer (pH 8.0). 1 mL of spirulina at concentration ranges of 20–100 µg/mL was added to the reaction mixture and incubated for 5 min at room temperature. 1 mL of alpha-glucosidase enzyme (10 U/mL) (Sigma, USA) was added into the reaction mixture for initiation of the reaction, followed by incubation for 40 min at 35°C. The reaction was terminated by the addition of 2 mL of 6N HCL. The intensity of the color was measured at 540 nm. The following equation was used to determine the percentage of inhibiting activity of each compound:

$$\% \text{ inhibition} = \left(\frac{[\text{OD control} - \text{OD sample}]}{\text{OD Control}} \right) \times 100$$

(OD control: Absorbance of control, OD sample: Absorbance of sample)

Alpha-amylase enzyme inhibition activity

Alpha-amylase enzyme (Sigma, USA) was performed according to the method described by Riyanti *et al.*, 2016.^[16] The reaction mixture contained 1 mL of spirulina at concentration ranges of 20–100 µg/mL and 1 mL of alpha-amylase solution. The mixture was preincubated for 30 min and then 1 mL of starch solution was added to reaction and incubated at 37°C for 10 min. The reaction mixture was stopped by addition 1 mL of DNS solution (12.0 g of sodium potassium tartrate tetrahydrate in 8 ml of 2M NaOH and 96 mM of 3,5 dinitrosalicylic acid solution), and the mixture was boiled for 5 min. The negative control was prepared without sample and without alpha-amylase enzyme solution and acarbose was used as positive control. The absorbance was measured at 540 nm.

Dipeptidyl peptidase IV activity

Dipeptidyl peptidase IV activity (DPP-IV) activity was performed in 96 micro well plates. A preincubation volume 200 µl contained 50 µl of DPP IV enzyme (500 µg/ml) (MERCK Millipore, USA) and 25 µl of 20–100 µg/mL concentration of spirulina material/standard. This mixture was incubated at 37°C for 5 min, followed by addition of 100 µl of Rebaudioside (substrate). The reaction mixture was incubated for 15 min at 37°C and reaction was terminated with 25 µl of 25% glacial acetic acid. Absorbance was measured at 405 nm. % inhibition was calculated as above Riyanti *et al.*, 2016.^[16]

Preparation of animals and study design

Healthy adult female Sprague-Dawley rats weighing 150–250 g were purchased from the Institute of Medical Research Kuala Lumpur, Malaysia and left for 2 weeks to acclimatize prior to the commencement of the experiment. The rats were housed in a standard polypropylene cages maintained under standard laboratory conditions and were fed with standard rat pellet and water. The rats were divided into five groups with six rats per cage. Group I – Normal rats were not induced with streptozocin (STZ) 50 mg/kg, but treated with saline. Group II – Negative control rats induced with STZ but treated with saline. Group III – Positive control rats induced with STZ treated with Metformin at 300 mg/kg, Group IV – Test group 1 – rats induced with STZ treated with Spirulina

at 300 mg/kg, and Group V – Test group 2 – rats induced with STZ treated with Metformin + Spirulina at 300 mg/kg. To be able to account for the effect of spirulina at different stages of hormonal changes, female Sprague-Dawley rats were preferred in this experiment. The dose of spirulina and metformin used were adopted from La Fontaine *et al.*, 2016,^[17] and Devesh *et al.*, 2012.^[18]

Biological assay

Oral glucose tolerance test for normal rats

Normal rats were tested for oral glucose tolerance test. Rats were divided into four groups of six rats per group as follows: Group I – Normal rats treated with saline, Group II – Positive control rats treated with Metformin at 300 mg/kg, Group III – Test group 1 – rats treated with spirulina at 300 mg/kg, and Group IV – Test group 2 – rats treated with Metformin + Spirulina at 300 mg/kg. After 60 min of drug administration, the rats were orally treated with 2 g/kg of glucose. The blood glucose level was estimated at 0-, 30-, 60-, 90-, and 120-min interval Chaimum-Aom *et al.*, 2017.^[19]

Diabetes induction

The rats were fasted overnight prior to STZ diabetic induction, and the induction of diabetes was performed through a single intraperitoneal injection (50 mg/kg) of STZ prepared in cold citrate buffer (0.05 mol/L sodium citrate, pH 4.5).^[20] The rats were left for 7 days to develop hyperglycemia, after which the plasma glucose was re-evaluated. Plasma glucose levels above 11 mmol/L were considered as diabetic Ekeuku *et al.*, 2015.^[20] The induction was successful as there was no mortality recorded during the experiment. Each drug was administered via oral gauge once a day for 12 weeks according to the experimental grouping shown above. The weight and plasma glucose level of the rats were measured every 4 weeks for 12 weeks. The procedures adopted for this research are in accordance with the standards set forth in the eighth edition of Guide for the Care and Use of Laboratory Animals and was approved by the ethical body of Universiti Kebangsaan Malaysia Animal Ethical Committee with approval number: UCSI/2016/PATRICK/23-N OV/801-JAN.-2017-DEC.-2018.

Determination of hematology, biochemical, and lipid profile

The rats were anesthetized with xylazil, zoletil, and ketamine (1:2:1) after the 12 weeks treatment, and plasma blood was obtained intravenously from the tail vein and sent to a commercial pathology laboratory for the respective biochemical analysis. The method used in anesthetizing the rats was a standard method adopted from our previous study, Ekeuku *et al.*, 2015.^[20]

In vivo antioxidant assay

Preparation of tissue samples

The rats were sacrificed by cervical dislocation under anesthesia, and the liver and pancreas were harvested. The liver was washed, excised, and homogenized in 10 ml of 0.15 M Tris buffer (pH 7) per 2 g of tissue and 6 ml of 0.15 M phosphate buffer (pH 7.4) per 1.8 g of tissue. The tris buffer homogenate was centrifuged for 30 min at 3000 rpm at 40°C, while the phosphate-buffered homogenate was centrifuged for 1 h at 3000 rpm at a temperature at 40°C.^[20] The supernatant was collected and stored at 40°C. The pancreas was dehydrated in 10% of neutral-buffered saline.

Glutathione reductase

1 ml of tissue sample was added to an empty test tube, followed by 0.5 ml of Ellman's reagent (19.8 mg DTNB/0.1% sodium nitrate). Finally, 3 ml of 0.2 M phosphate buffer (pH 8) was added to the mixture. The mixture was thoroughly mixed and after 5 min, absorbance was read at 412 nm.^[20]

Lipid peroxidation

LPO was measured according to Ekeuku *et al.*, 2015.^[20] About 2 ml of 10% TCA, 1 ml of 0.9% saline (w/v), and 1 ml of Tris-buffered sample were mixed in a 15 ml centrifuge tube. The samples were centrifuged at 3000 rpm and temperature at 25°C for 10 min. 2 ml supernatant was transferred into a new test tube containing 0.5 ml of 1% of TBA. The tubes were incubated in a 95°C water bath for 1 h. The test tubes were removed from the water bath, and absorbance was read at 532 nm.

Catalase

1 ml of 0.01 M phosphate buffer (pH 8), 0.1 ml of phosphate-buffered liver sample, and 0.4 ml of 2 M hydrogen peroxide (H₂O₂) were added into a beaker. After 3 min, 2 ml of dichromate-acetic acid (which contained of 5% potassium dichromate and acetic acid in the ratio of 1:3) was added. The mixture was boiled for 10 min and absorbance was measured at 570 nm.^[20]

Superoxide dismutase

1 ml of 50 mM sodium carbonate, 0.4 ml of 25M NBT and 0.2 ml of 0.1 mM EDTA, and 0.5 ml of Tris tissue sample were mixed. To the mixture, 0.4 ml of 1 mM hydroxylamine hydrochloride was added and after 2 min absorbance was read at 560 nm.^[20]

Statistical analysis

All data were presented as mean (\pm) standard deviation using SPSS Statistics Version 7.0 (IBM Corp, Chicago, USA). The data were statistically analyzed by two-way ANOVA, followed by Dunnet's test. Values were considered statistically significant when * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

RESULTS

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test

The cell viability at the mitochondrial level has been reported to depend on the drug concentration on the cells.^[21] Concentrations assessed in this experiment were between 0.5 and 2.5 $\mu\text{g/ml}$ with SM mixed at a 1:1 ratio. The results are represented in graph Figure 1.

In vitro antioxidant activity

2,2-diphenyl-1-picrylhydrazyl antioxidant activity

Spirulina showed a very strong inhibition of DPPH free radical generation; the effect was more significant than the positive control BHT.

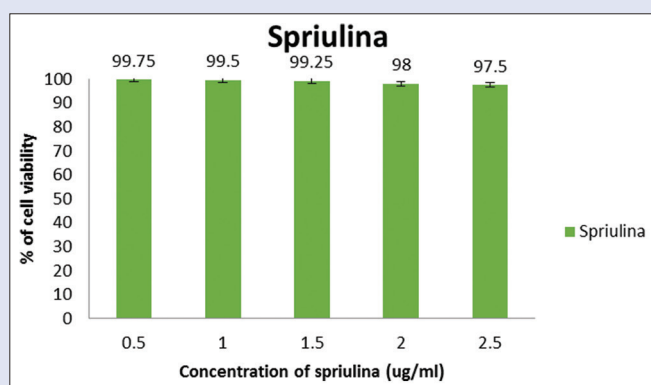


Figure 1: Cell viability of L6-skeletal muscle cells: Effect of spirulina on cell viability of the L6 skeletal muscle cells was evaluated based on the drug concentration ranges of 0.5 μg -2.5 $\mu\text{g/ml}$. Each value represents (mean \pm standard deviation) from triplicate measurements ($n = 3$)

There was no significant difference between the effect of glimepiride and spirulina [Figure 2]. However the effect of spirulina peaked at concentration between 10–20 $\mu\text{g/ml}$.

Nitric oxide scavenging activity

Spirulina was able to inhibit nitric oxide production, although the effect of BHT was higher. There was no significant difference between the effect of glimepiride and spirulina [Figure 3]. The effect was dose dependent.

Hydroxyl radical scavenging activity

Spirulina inhibited hydroxyl radical scavenging activity; the effect was more significant compared to the positive compound BHT. The effect was dose dependent, and there was no significant difference between the effect of glimepiride and spirulina [Figure 4].

Lipid peroxidation

Spirulina inhibited LPO; the effect was more significant compared to the positive compound BHT. The effect was dose dependent, and there was no significant difference between the effect of glimepiride and spirulina [Figure 5].

In vitro inhibition of diabetes-linked enzymes

Alpha-glucosidase enzyme inhibition

Spirulina was able to inhibit the activity of alpha-glycosidase enzyme; the effect was dose-dependent. The effect of the positive drug acarbose was more potent at lower doses; there was no significant difference between the effect of glimepiride and spirulina [Figure 6].

Alpha-amylase enzyme inhibition effect

Spirulina was able to inhibit the activity of alpha-amylase enzyme, the effect was dose dependent. The effect of the positive drug acarbose was more potent at lower doses; there was no significant difference between the effect of glimepiride and spirulina [Figure 7].

Dipeptidyl peptidase-4 enzyme

Spirulina was able to inhibit the activity of DPP-IV enzyme; the effect was dose dependent and was more potent than acarbose. The effect of the positive drug acarbose was more potent at lower doses; there was no significant difference between the effect of glimepiride and spirulina [Figure 8].

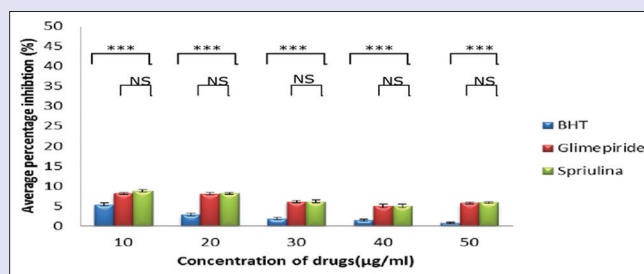


Figure 2: The effect of spirulina, BHT and glimepiride on DPPH free radical Scavenging effect. The data were statistically analyzed by two-way ANOVA followed by Dunnet's test. Each value represents (mean \pm standard deviation) from triplicate measurements ($n=3$). Values were considered statistically significant when * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

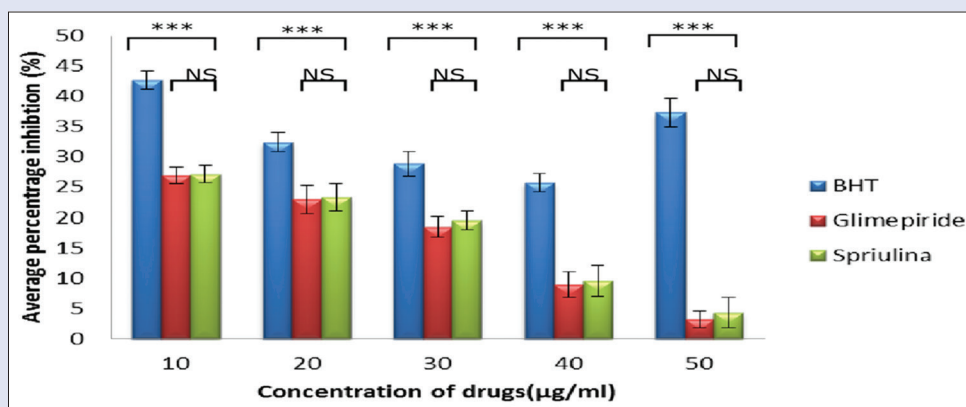


Figure 3: The effect of spirulina, BHT, and glimepiride on nitric oxide scavenging effect. The data were statistically analyzed by two-way ANOVA followed by Dunnet's test. Each value represents (mean ± standard deviation) from triplicate measurements ($n = 3$). Values were considered statistically significant when $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$

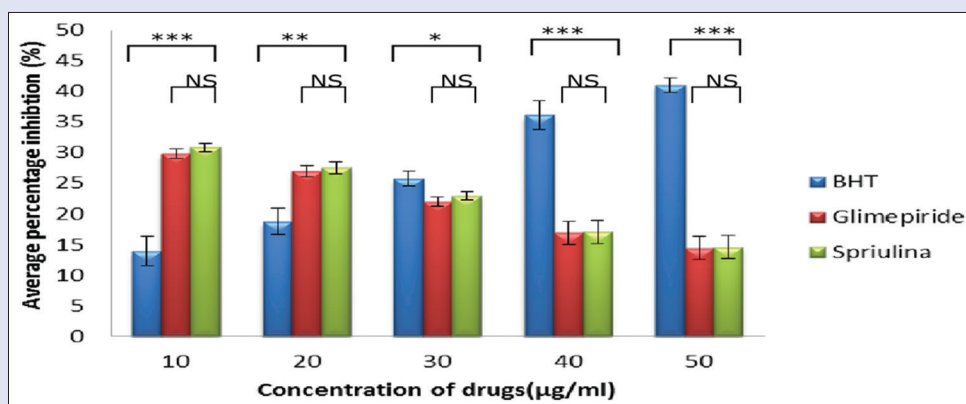


Figure 4: The effect of spirulina, BHT, and glimepiride on hydroxyl radical scavenging activity. The data were statistically analyzed by two-way ANOVA followed by Dunnet's test. Each value represents (mean ± standard deviation) from triplicates measurements ($n = 3$). Values were considered statistically significant when $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$

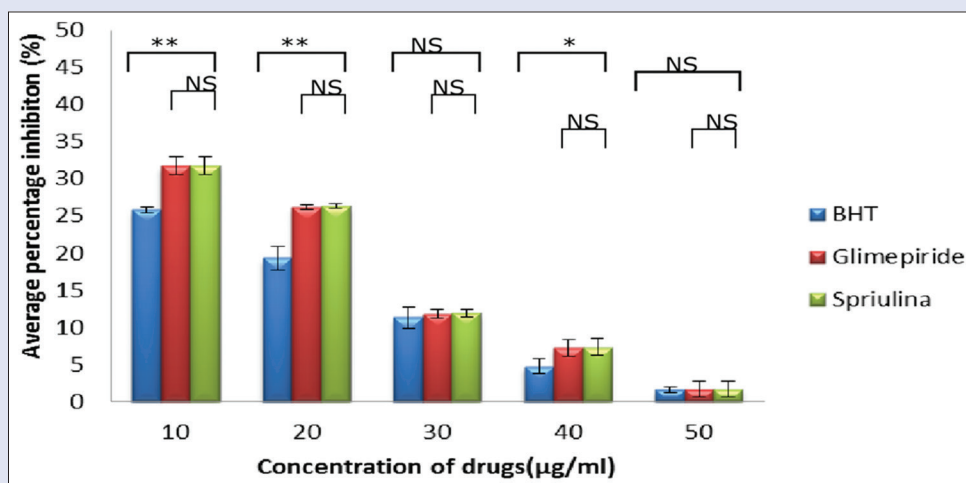


Figure 5: The effect of spirulina, BHT, and glimepiride on lipid peroxidation. The data were statistically analyzed by two-way ANOVA followed by Dunnet's test. Each value represents (mean ± standard deviation) from triplicate measurements ($n = 3$). Values were considered statistically significant when $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$

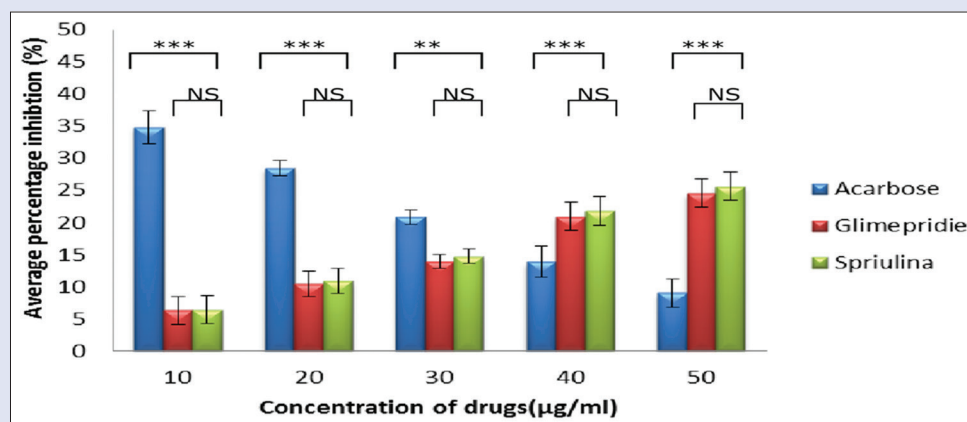


Figure 6: The effect of spirulina, acarbose, and glimepiride on inhibition of alpha-glucosidase. The data were statistically analyzed by two-way ANOVA followed by Dunnet's test. Each value represents (mean \pm standard deviation) from triplicates measurement ($n = 3$). Values were considered statistically significant when $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$

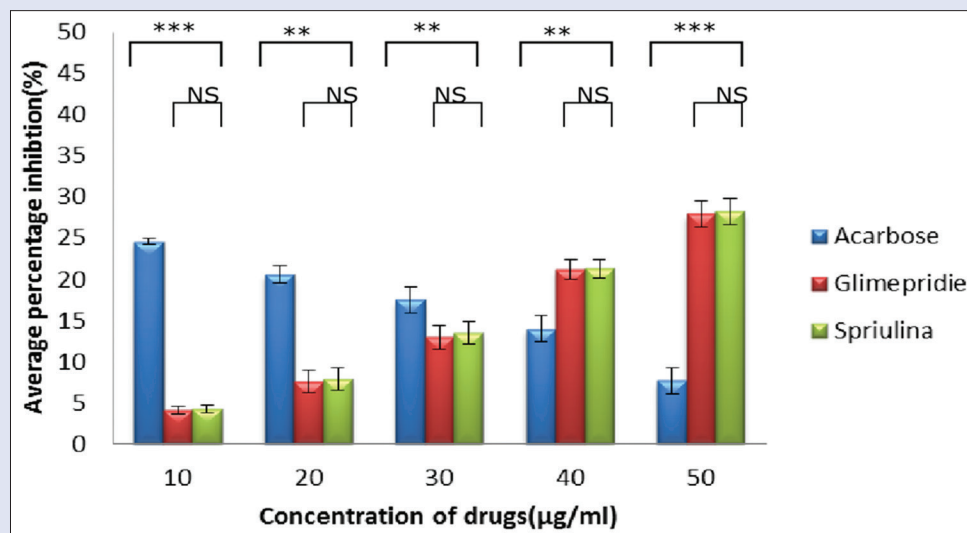


Figure 7: The effect of spirulina, acarbose, and glimepiride on inhibition alpha-amylase. The data were statistically analyzed by two-way ANOVA followed by Dunnet's test. Each value represents (mean \pm standard deviation) from triplicate measurements ($n = 3$). Values were considered statistically significant when $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$

Diabetic study

All tested parameters were found to be within the physiological reference for the normal groups. The normal group was put to serve as reference group because there was no treatment done on the group. There was a constant increase in plasma glucose level in the negative control group till the end of the 12 weeks treatment. The plasma glucose level started to decrease from week 4 of the study in metformin- and spirulina-treated group. There was no effect in the group treated with combination of metformin and spirulina [Figure 9]. There was a slight drop in the body weight in the 1st week in test groups, after which there were steady increases in the body weight. The negative control group showed a lower body weight compared to test groups [Figure 10].

Oral glucose tolerance test for normal rats

Spirulina was able to reduce the postprandial glucose level in normal rat model after 60 min, and the result is similar with metformin. The result was almost similar in with combination of spirulina and metformin [Figure 11].

Biochemical analysis

Hematology (glycated hemoglobin, red blood cell, and white cell count)

The glycated hemoglobin (HbA1c) and red blood cell (RBC) level of the negative control rats were elevated, while the white cell count (WBC) was decreased. Treatment with metformin showed a reduction in HbA1c, increase in RBC, and decrease in WBC. Treatment with spirulina alone and combination of spirulina and metformin showed a reduction in HbA1c, RBC, and increase in WBC levels. This is shown in Figures 12 and 13.

Renal profile test

Urea level was higher, while creatinine level was lower in the negative control compared to the tests groups. Treatment with metformin, spirulina, and a combination of metformin and spirulina showed a significant reduction in urea and an increase in creatinine, but rats treated with spirulina alone showed more pronounced reduction/increase as reflected in Figure 14.

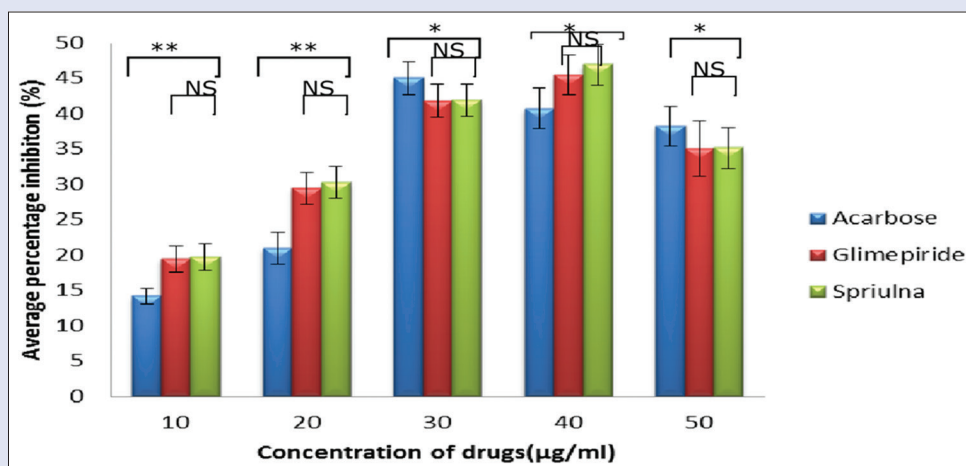


Figure 8: The effect of spirulina, acarbose, and glimepiride on inhibition dipeptidyl peptidase-4 inhibitor enzyme. The data were statistically analyzed by two-way ANOVA followed by Dunnett's test. Each value represents (mean ± standard deviation) from triplicate measurements ($n = 3$). Values were considered statistically significant when $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$

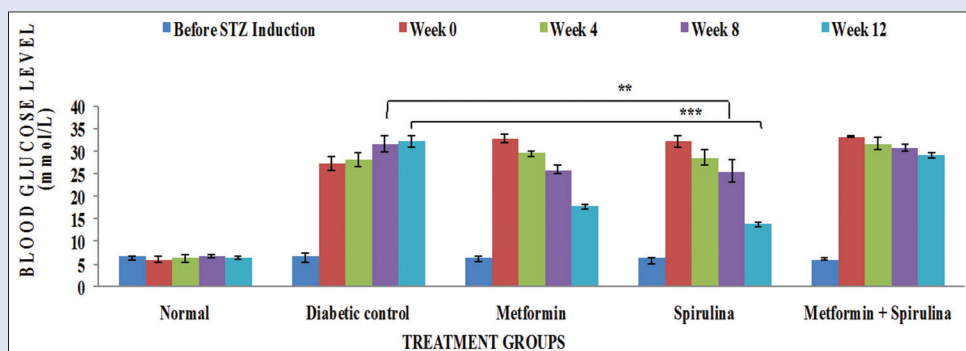


Figure 9: Mean plasma glucose level of rats per group. Rats were treated over a period of 90 days. All data were presented as mean (±) standard error mean (SEM) ($n = 6$) using SPSS. The data were statistically analyzed by two-way ANOVA followed by Dunnett's test. Values were considered statistically significant when $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$

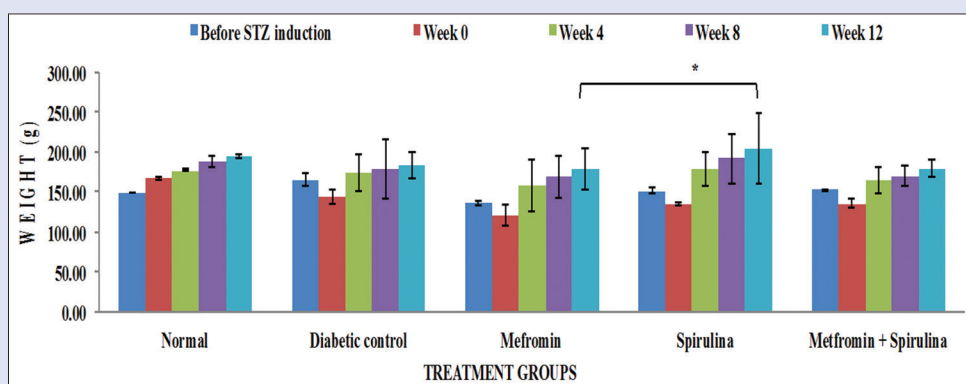


Figure 10: Mean body weight of rats per group. Rats were treated over a period of 90 days. All data were presented as mean (±) standard error mean (SEM) ($n = 6$) using SPSS. The data were statistically analyzed by two-way ANOVA followed by Dunnett's test. Values were considered statistically significant when $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$

Liver function test

The alkaline phosphatase (AP), aspartate transaminase (AST), and alanine aminotransferase (ALT) levels were high in the negative control. AP was high;

AST and ALT were reduced in metformin-treated group. Treatment spirulina alone and combination of metformin and spirulina showed a reduction in AP, AST, and ALT levels, but spirulina showed more reduction Figure 15.

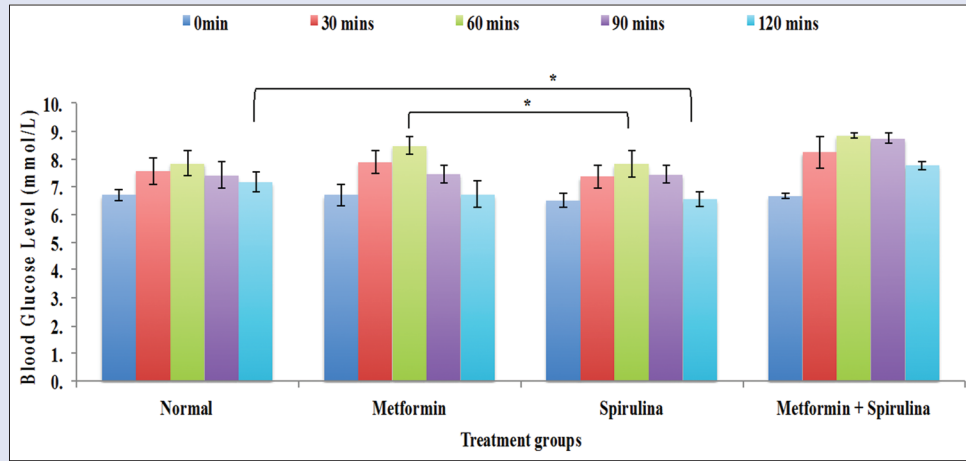


Figure 11: Mean oral glucose tolerance test of rats per group. Rats were treated over a period of 90 days. All data were presented as mean (\pm) standard error mean (SEM) ($n = 6$) using SPSS. The data were statistically analyzed by two-way ANOVA followed by Dunnett's test. Values were considered statistically significant when $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$

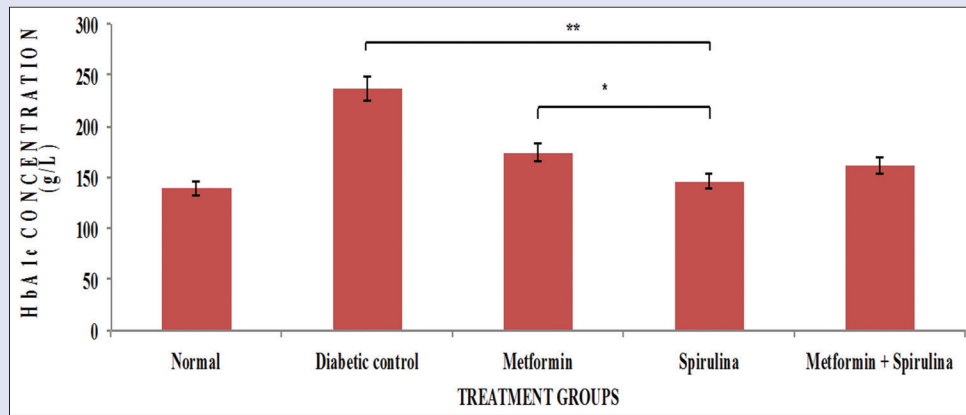


Figure 12: Mean glycated hemoglobin concentration of rats per group. Rats were treated over a period of 90 days. All data were presented as mean (\pm) standard error mean (SEM) ($n = 6$) using SPSS. The data were statistically analyzed by two-way ANOVA followed by Dunnett's test. Values were considered statistically significant when $*P < 0.05$ and $**P < 0.01$

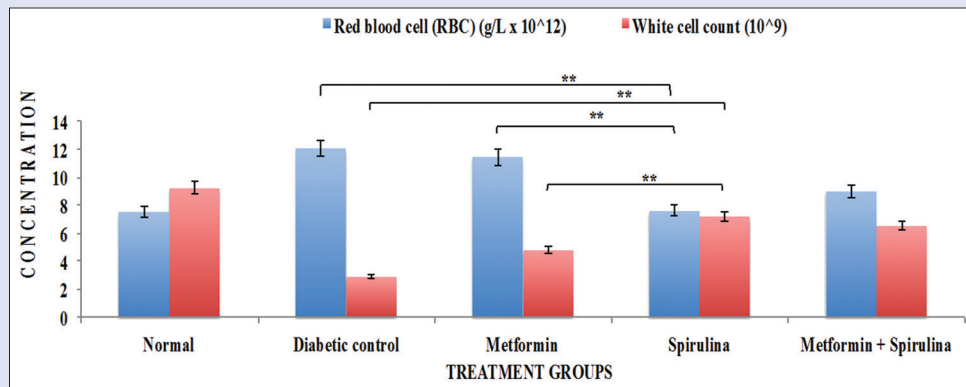


Figure 13: Mean Red blood cells concentration and white cell count of rats per group. Rats were treated over a period of 90 days. All data were presented as mean (\pm) standard error mean (SEM) ($n = 6$) using SPSS. The data were statistically analyzed by two-way ANOVA followed by Dunnett's test. Values were considered statistically significant when $***P < 0.01$

Lipid profile test

The result showed decreased levels of high-density lipoproteins (HDLs) and elevated levels of triglycerides and low-density lipoproteins (LDLs) in the negative control and metformin, although the levels were slightly higher in negative group. The group treated with spirulina and combination of spirulina and metformin caused a reduction in triglycerides and LDL and an increase in HDL group treatment with spirulina alone was more pronounced effect [Figure 16].

Insulin Concentration

The result showed an increase in concentration of insulin release groups treated with spirulina and combination of spirulina and metformin compare to group treated with metformin alone and negative control [Figure 17].

In vivo antioxidant test

Glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) activities were low and LPO was high in negative control and metformin-treated group. The activities of the antioxidant enzymes were

slightly high in the spirulina alone and spirulina + metformin; the LPO was low almost the same level as the normal [Figure 18].

DISCUSSION

Spirulina is not cytotoxic to L6 muscle cells even at high concentrations. The spirulina used in this study was manufactured and supplied by a glucagon-like peptide (GLP)-certified company, Alpha Active Industries Sdn. Bhd., and all the contents were duly quantified. Enhanced oxidative stress caused by increased generation of free radicals such as superoxide radical, hydroxyl radical and other reactive oxygen species (ROS) lead to damages or destructions in a variety of tissues, and consequent development of diabetes and its complications.^[22] Antioxidants have been attributed to various mechanisms such as prevention of chain initiation, decomposition of peroxides, reducing capacity, and radical scavenging.^[23] Spirulina was able to inhibit the generation of free radicals such as hydroxyl radical, nitric oxide, DPPH, and LPO. The significant increase in MDA level and decrease in GSH-Px, SOD, and CAT activities found in the liver and kidney of STZ-induced diabetic rat illustrate the enhanced oxidative stress in the liver and kidney. Spirulina has shown to normalize the pathologic change caused by the STZ

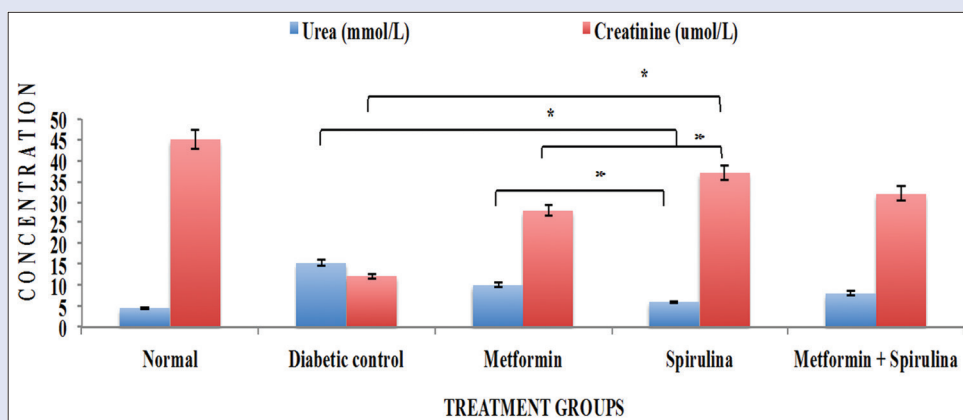


Figure 14: Mean urea and creatinine concentration of rats per group. Rats were treated over a period of 90 days. All data were presented as mean (\pm) standard error mean (SEM) ($n = 6$) using SPSS. The data were statistically analyzed by two-way ANOVA followed by Dunnett's test. Values were considered statistically significant when $*P < 0.05$

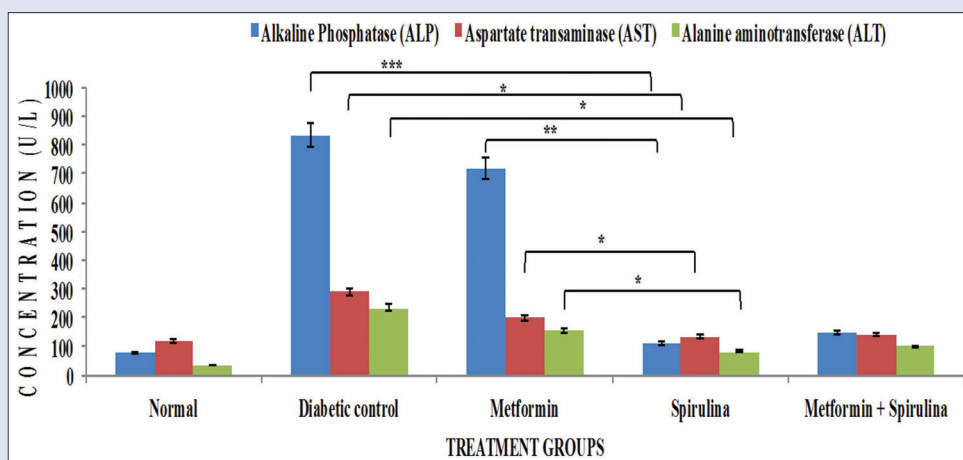


Figure 15: Mean ALP, aspartate transaminase, and alanine aminotransferase concentrations of rats per group. Rats were treated over a period of 90 days. All data were presented as mean (\pm) standard error mean (SEM) ($n = 6$) using SPSS. The data were statistically analyzed by two-way ANOVA followed by Dunnett's test. Values were considered statistically significant when $*P < 0.05$

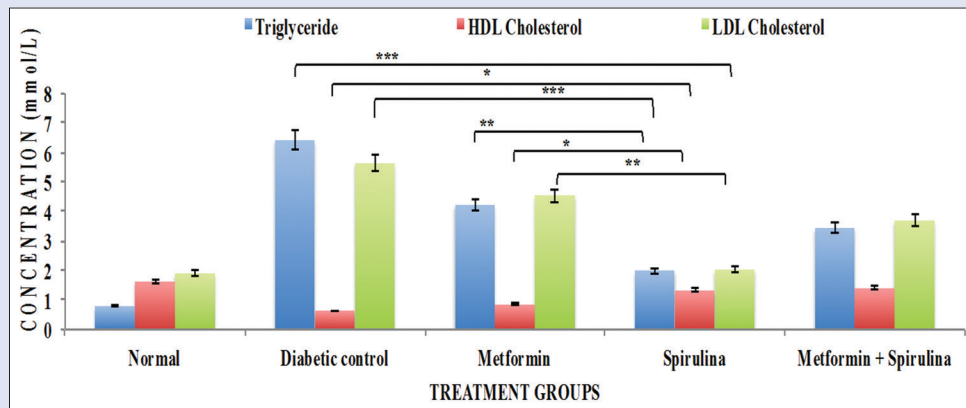


Figure 16: Mean triglyceride, high-density lipoproteins, and low-density lipoproteins cholesterol concentrations of rats per group. Rats were treated over a period of 90 days. All data were presented as mean (\pm) standard error mean (SEM) ($n = 6$) using SPSS. The data were statistically analyzed by two-way ANOVA followed by Dunnett's test. Values were considered statistically significant when $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$

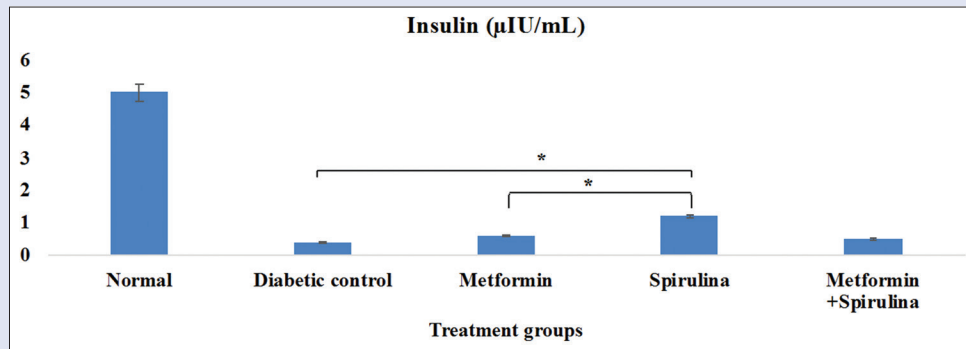


Figure 17: Mean insulin concentration of rats per group. Rats were treated over a period of 90 days. All data were presented as mean (\pm) standard error mean (SEM) ($n = 6$) using SPSS. The data were statistically analyzed by two-way ANOVA followed by Dunnett's test. Values were considered statistically significant when $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$

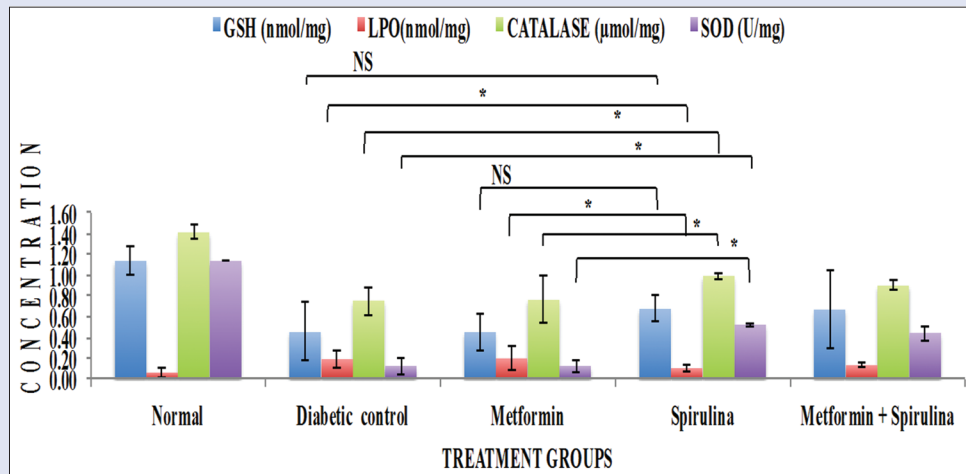


Figure 18: Mean *in vivo* antioxidant activities of rats per group. Rats were treated over a period of 90 days. All data were presented as mean (\pm) standard error mean (SEM) ($n = 6$) using SPSS. The data were statistically analyzed by two-way ANOVA followed by Dunnett's test. Values were considered statistically significant when $*P < 0.05$

probably to damages related to increased generation of free radicals.^[22,24] Research has shown that spirulina contains significant amounts of phytoconstituents such as phycocyanins, carotenoid, Vitamin E,

chlorophyll, flavonoids, saponins, and phenolic compounds. It has been reported that phycocyanins which is one of the constituents of spirulina prevents the overproduction of ROS and enhancing the activities of

SOD and GSH peroxidase.^[25] Carotenoid, Vitamin E, phycocyanin, and chlorophyll contained in spirulina are known to decrease DPPH radicals due to their hydrogen-donating ability.^[26] Flavonoids and phenolic compounds have good antioxidant potentials and mechanism of action of flavonoids is through scavenging or chelation, while phenolic compound are important because of their hydroxyl groups scavenging ability.^[27] According to a study by Romay *et al.*, 2003,^[28] phycocyanins inhibit the increase in lipid peroxides in rat liver. Neelutpal and Biman^[29] show that bioactive components from *Cascabela thevetia* L. plant possess antidiabetic, antioxidant, and lipid-reducing activity. The reduction of the plasma glucose level by spirulina may be due to the delay of the breakdown of carbohydrate into the oligosaccharides and glucose, which affects the absorption of food through the small intestine and helps in decreasing the postprandial hyperglycemia. The inhibition of the oral glucose tolerance test and sustained release of insulin further buttress the claim of reduction of postprandial hyperglycemia. There is evidence from this study that spirulina inhibits alpha-amylase and alpha-glucosidase enzymes which causes the breakdown of carbohydrate into the oligosaccharides, later to glucose, and increases the absorption of glucose through the small intestine and helps increase postprandial hyperglycemia.^[30] It may have also increased the release of insulin and GLP-1 through the activity of incretin, because spirulina was able to inhibit the DPP-IV activity. GLP-1 is an incretin hormone that results in glucose-dependent insulin secretion, suppression of glucagon secretion, a delay in gastric emptying, and a decrease in caloric intake likely secondary to centrally mediated signaling.^[31] The majority of known biological actions of GLP-1 depend on the presence of the two N-terminal amino acids; these are removed by the enzyme, DPP-4, whose substrates are polypeptides with an alanine or a proline at the second position from the N-terminal side.^[32] The activity, affinity, and wide distribution of DPP-4 results in GLP-1 having a half-life of approximately 1 min in the circulation.^[33] DPP-4 inhibitors, inhibits the principal enzyme responsible for the degradation of endogenous GLP-1, by decreasing clearance of GLP-1, concentrations of active GLP-1 are increased, resulting in a lowering of fasting and postprandial glucose concentrations.^[34] In practice, DPP-4 inhibitors increase concentrations of active incretin hormones, GLP-1 and glucose-dependent insulinotropic polypeptide (secreted by the enteroendocrine L and K cells, respectively, which are substrates for DPP-4). This results in improved cell responsiveness to prevailing glucose concentrations and suppression of glucagon secretion.^[35] Streptozotocin induction in rat model destroys the β -cells causing a reduction in plasma insulin or sensitivity to insulin mimicking a condition known as insulin resistance and an upsurge in plasma glucose concentrations. It also causes the generation of free radicals which has negative effect on the kidney, liver, and β -cells.^[36] Metformin is a biguanide, which has been reported to improve glycemic control by insulin sensitization. It prevents the liver from releasing excess glucose and stimulates the muscle and fat cells to remove glucose from the blood.^[37] Metformin reduces plasma cholesterol and triglyceride levels and does not cause weight gain.^[38] Spirulina was equipotent with metformin in the reduction of plasma glucose level in the STZ-induced rat model. The plasma glucose lowering effect observed with the treatment of spirulina could be as a result of chromium. This is because chromium lowers the plasma glucose by binding to peptide known Apo-LMWCr.^[5,39] The presence of high fiber content and generation of polypeptides from the digestion of high protein contents in spirulina, helps generate polypeptides that interfere with glucose absorption, thereby lowering plasma glucose.^[40] However, there appears to be no synergism between metformin and spirulina. HbA1c and RBC elevation as observed in the negative control maybe in response to oxidative stress generation by STZ induction.^[41,42] Liver damage has been associated with decreased WBC, this maybe the

reason for the observed low WBC in negative control after STZ induction.^[43] Reduction of the plasma glucose level by spirulina automatically led to the reduction of reduction in HbA1c and RBC and increase in WBC. Karnchanasorn *et al.*, 2016^[44] reported that the reduction of plasma glucose level with automatically leads to reduction in plasma HbA1c and RBC. Spirulina may have caused the reduction of HbA1c and RBC because of the antioxidant property and rich iron content in spirulina. The elevation of WBC in the spirulina-treated group maybe because of the presence of phycocyanins, polysaccharides, and allophycocyanins in spirulina which have been previously reported to help to improve the number of WBC, immunity, and provide antiviral activity (nutrex-hawaii.com). Plasma urea and creatinine are established markers of glomerular filtration.^[45] Elevation of urea in diabetic rats is indication of kidney damage to the kidney or kidney malfunction. An increase in plasma urea level is directly proportional to the increment of plasma glucose level; this condition clearly indicates damage to the kidney.^[39,46] The modification of urea in the spirulina-treated group maybe because of its content of β -carotene. β -carotene is a precursor of a potent antioxidant and phycocyanins both compounds moderates the decline in glomerular filtration rate by improving the urea level.^[36,47] Harita *et al.*, 2009^[48] reported that an increase in plasma glucose level decreases the creatinine level, a condition which is hypothesized to reflect a lower volume of skeletal muscle. Low skeletal volume will negatively affect diabetes because skeletal muscle plays a major role in the removal of glucose from the blood. The malfunctioning or inability of the skeletal muscle to clear the glucose from the blood is one of the major factors that lead to insulin resistance and eventually type 2 diabetes.^[49] The reduction in creatinine may reflect why the rats in the negative group were constantly reducing in weight. Treatment with metformin and spirulina restores the creatinine volume, this maybe because their antioxidant effect. Liver function tests are commonly used in clinical practice to screen for liver disease, supervise the progression of known diseases, and monitor the effects of potentially hepatotoxic drugs.^[50] ALP is a marker of biliary function and cholestasis and reflects liver synthetic function, while AST and ALT reflect the concentration of intracellular hepatic enzymes that have leaked into the circulation and also serve as a marker of hepatocyte injury.^[46] Elevated ALP, AST, and ALT in liver function test are associated with diabetes, and it has been linked to oxidant stress from reactive LPO, peroxisomal beta-oxidation, and recruited inflammatory cells.^[50] Elevation in ALP, AST, and ALT in the negative control may be due to oxidative stress generated by STZ induction. This observation is supported by a study by Elizabeth,^[50] which reports that elevated levels of ALP, AST, and ALT are due to oxidative stress from reactive LPO. The modification of the liver markers by spirulina treatment maybe because of the presence of β -carotene, which have been previously reported to possess hepatoprotective activity.^[40] The antioxidant properties of spirulina may have caused the improvement of the renal function by decreasing the oxidative stress damage and thereby protecting the liver and kidney cells. The lipid profile result was similar to a study by Luciane *et al.*, 2008,^[51] which reported that the distinguishing features of abnormal lipid levels in diabetes are high plasma triglyceride concentration, low HDL cholesterol concentration, and increased concentration of small dense LDL-cholesterol particles. According to a study by McKinley Health Center, increase in glucose level causes glucose to be attached to LDL and triglycerides but not HDLs. These LDLs and triglycerides coated with glucose stay longer in the bloodstream and cause sticky plaques. This could be the reason for the increase of triglycerides and LDL in the negative control. Phycocyanins, phenolic compounds, and polyunsaturated fatty acids presents in spirulina may have caused the reduction of triglycerides and LDL and increase HDL.^[52]

CONCLUSION

Spirulina is not cytotoxic to the cells, it possesses *in vitro* and *in vivo* antioxidant and antidiabetic activities and able to moderate the biochemical lipid, liver, and kidney disease markers in STZ-induced diabetic rat models. The mechanism of action of spirulina may be through the increase in concentrations of active incretin hormones, GLP-1 and glucose-dependent insulinotropic polypeptide. It also possesses a very strong *in vitro* and *in vivo*-antioxidant property which plays active role in type 2 diabetes treatment. Antioxidant effect of spirulina maybe because of the presences of significant amounts of phytoconstituents such as chromium, iron phycocyanins, carotenoid, Vitamin E, chlorophyll, flavonoids, saponins, and phenolic compounds which have previously shown antioxidant activity. Based on this finding, spirulina can be a very good potential natural alternative for the treatment and management of diabetes-associated liver and kidney complications.

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Conflicts of interest

There are no conflicts of interest.

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