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CELL SUSPENSION OF *SPIRULINA PLATENSIS* PARTIALLY ATTENUATES ALLOXAN INDUCED ALTERATIONS IN CARBOHYDRATE AND LIPID METABOLISM IN DIABETIC MICE

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ABSTRACT: The present study evaluates the hypoglycemic and hypolipidemic effects of supplementation of cell suspension of *Spirulina platensis* (SP) in alloxan (AXN) induced hyperglycemic mice. Diabetes was induced by intra-peritoneal administration of AXN. Mice with blood glucose level above 200 mg/dl were divided into Group I (control), Group II (diabetic control), Group III (diabetic control mice fed with SP), and Group IV (control mice fed with SP). General physiology, fasting blood glucose level, oral glucose tolerance test, liver glycogen content and lipid profile were recorded in test subjects. In hyperglycemic mice, a significant ($P < 0.05$) fractional increase in food intake (41.13%), water consumption (190.56%), fasting blood glucose level (273.2%), total cholesterol (144.12%), triglyceride (124.58%), very low density lipoprotein (124.59%) and low density lipoprotein (265.4%) with concomitant decrease in body weight (25.78%), liver glycogen (10.57%) and high density lipoprotein (37.74%) when compared with control at the end of the experiment but recuperation in these parameters were observed ($P < 0.05$) when subjected to SP treatment. SP resulted in fractional increase in body weight, liver glycogen and high density lipoprotein by 33.08%, 46.36% and 47.78 respectively, and dropped off food intake and water consumption to 19.76% and 61.76% respectively while fasting blood glucose level, total cholesterol, triglyceride, very low density lipoprotein and low density lipoprotein went down to 58.55%, 49.74%, 47.68%, 47.68% and 60.02% respectively. These findings validate that SP maintains general physiological status, glucose metabolism and lipid profile of diabetic mice and hence warrants further detailed dose-dependent studies to understand its mechanism of action.

INTRODUCTION: Diabetes mellitus (DM) is a devastating and life threatening disease. Hyperglycemia causes a series of metabolic disorders that result from defects in insulin secretion or insulin action¹. It is a chronic metabolic disorder creating turbulences in carbohydrate, fat and protein metabolism. DM is usually characterized by hyperglycemia, polyuria and polydipsia.

It causes nephropathy, retinopathy, neuropathy and embryopathy². It is also associated with an increased incidence of cardiovascular disease, reduced life expectancy, significant morbidity due to microvascular complications and diminished quality of life. Dietary restrictions, planned exercise and administration of oral glucose lowering agents are applied widely to control elevated blood glucose level. Moreover, herbal supplements and other alternative medicines are widely used to treat diabetic disorders².

Spirulina platensis (SP), a filamentous cyanobacteria (Blue Green Algae) exists as a coil-like shape. It is rich in lipids, proteins, carbohydrates and some vital elements like

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manganese, zinc, selenium, magnesium, riboflavin, β -carotene, α -linoleic acid and tocopherol. The antioxidant properties of SP like scavenging of hydroxyl radicals and to inhibit lipid peroxidation have attracted attention to many researchers. These activities were largely related to phycocyanin, an active protein of SP³.

SP is investigated for anti-allergen, anti-rhinitis, anticancer, antiviral, antimicrobial, immunomodulatory, anti-inflammatory, probiotic, radiation protection effects restrict obesity, chronic fatigue prevention, chronic arsenic poisoning prevention, maintaining haematological profile and improvement of the reproductive performance as well as easing teratogenicity have already been established⁴⁻⁵.

To the best of our knowledge, there are no reports that describe holistic effects of SP in DM associated carbohydrate and lipid metabolism. Considering the therapeutic potential of SP, the aim of the present investigation is to evaluate and validate the role of SP on general physiology, fasting blood glucose level, oral glucose tolerance, liver glycogen and lipid profile in diabetic mice.

METHOD AND MATERIAL;

Plant Material:

SP powder for all experiments was procured from Sunova Spirulina Ltd., Delhi, India. It was a spray dried product, standard in quality, and a part of bulk production by the industry.

Animals:

Swiss Albino mice *Mus musculus* weighing 22-27 g were obtained from Central Drug Research Institute, Lucknow, India. Mice were maintained at the animal house of host University under standard conditions. Animals were fed with standard diet (Aashirwad Ltd., Chandigarh, India) and water *ad libitum*. Animals were maintained under controlled conditions (temperature [$23 \pm 1^\circ\text{C}$], humidity [$50 \pm 15\%$]), and normal photoperiod (12 h light-dark cycle). Eleven weeks old mice were acclimatized for 1-week prior to experimental studies. Rice husk was used as bedding material and changed daily. The research proposal (Reg. No. 5873/10) was approved by the Research Committee of the Department and all experimental procedures were

performed in accordance with the guidelines of Institutional Animal Care Committee and the principles outlined in the Declaration of Helsinki.

Drugs and Chemicals:

AXN-monohydrate was purchased from Spectrochem, India. All other chemicals and biochemical reagents were of analytical grade.

Induction of Diabetes:

Animals were fasted for 16-18 h with free access of water prior to induction of diabetes. DM was induced by intra-peritoneal (i.p.) administration of AXN ($450 \text{ mg kg}^{-1} \text{ bw}$) in three injections at intervals of 48 h ($150 \text{ mg kg}^{-1} \text{ bw}$ each time). Mice with blood glucose level above 200 mg/dl after 72 h were considered as diabetic and selected for further experimental investigations⁶.

Experimental Design:

The experimental mice were divided into four groups containing six mice in each group.

Group I: Control (normal saline, 10 ml/kg i.p.)

Group II: Diabetic control; diab (AXN $150 \text{ mg kg}^{-1} \text{ bw}$ for three times)

Group III: Diab + SP (diabetic control mice fed with 15 mg of SP)

Group IV: Ctrl + SP (control mice fed with 15 mg of SP)

Preparation and Administration of *Spirulina platensis* Suspension:

The suspension was prepared by supplementing 1.5 g of SP in 50 ml of distilled water. SP suspension was fed orally during entire tenure of experiment using infant feeding catheter (3 mm size) attached to a sterile syringe. Catheter was inserted into the gastric region of mice, and 0.5 ml of suspension containing of 15mg of SP was discharged gradually into each test animals.

Determination of FBGL and Oral Glucose Tolerance Test (OGTT):

Experimental animals were forced fed with $0.2 \text{ g kg}^{-1} \text{ bw}$ of glucose using baby oral feeding tube. Blood samples were collected from fasted mice at 0

min as well as from glucose loaded animals for every 15 min of interval for one hour and blood glucose level (BGL) was determined at the 15th, 30th, 45th and 60th min on 7th, 14th and 21st day Glucose Oxidase Method using glucometer (Accu-Chek Active, Mumbai, India) and compatible blood glucose strips at different time points during the course of the study. Similar method and procedure were followed to determine fasting blood glucose level⁷.

Body Weight, Food Intake and Water Consumption:

The experimental mice were weighed on 1st, 7th, 14th and 21st day using a top pan balance. A known amount of feed was introduced into each cage every day and on next day the unfed feed was collected, dried and weighed. The amount of dried food consumed was calculated from the difference between the two. Each water bottle containing 50 ml of water was inserted into the grid of each experimental cage as well as one water bottle of same amount in other separate cage without animal so as to ascertain error if any. Next day the bottles were removed from cages and the remaining quantity of water was measured and calculated from the rest volume.

Liver Glycogen (LG):

Liver extract was prepared from the known weight of liver tissue from each experimental mouse. Test tube containing 2 ml of liver extract was placed in a boiling water bath for 1h after addition of equal volume of 10N KOH which was further neutralized by 1 ml of glacial acid and brought the volume up to 20 ml with water. 4 ml of anthrone reagent was added in the test sample for colour development after cooling. The OD was taken for test sample at 650 nm against a blank and compared with standard for calculation⁸.

Biological Assays of Lipid Profile:

The serum was used for the estimation of lipid profile. Total cholesterol (TC), triglycerides (TG) and high density lipoprotein (HDL) were estimated by standard procedure (direct method). Very low density lipoprotein (VLDL) and low density lipoprotein (LDL) were calculated as per Friedwald equation⁹.

$$\text{VLDL} = \text{TG}/5$$

$$\text{LDL} = \text{TC} - (\text{HDL} + \text{VLDL})$$

Statistical Analysis:

The statistical analysis of the data was done using SPSS. Data expressed as mean \pm standard error of mean (SEM). Comparisons between two groups with normality and homogeneity of variances were performed by two-tailed unpaired Student's *t*-test. One-way analysis of variance (ANOVA) was used, followed by the *post-hoc* Tukey multiple comparison test to analyze data for control mice when the SP treated diabetic group was included. The levels of significance were set at $P < 0.05$ (almost significant) and $P < 0.01$ (significant).

Findings:

Effects on Body Weight, Food Intake and Water Consumption:

Control mice exhibited a gradual increase in body weight (6.96%), food intake (9.01%) and water consumption (16.8%) from day 1 to 21 with the advancing age. DC was found to drop their body weight (22.78%) significantly ($p < 0.01$) from day 7 to 21 but inverse condition were recorded regarding their food intake and water consumption. The gradual reduction in body weight in diabetic mice was improved by feeding fixed dose of SP (**Fig. 1B**) and it was found to be significant ($p < 0.01$) from day 7 to 21. The C+SP also showed weight loss pattern which were comparable with control group but insignificant ($p < 0.05$). DC showed polyphagia (**Fig. 1C**) and polydipsia (**Fig. 1D**) conditions which were significant ($p < 0.01$) at day 14 and day 21. DC+SP dropped food and water consumption as compared to DC ($p < 0.05$), showing that SP could reduce the polyphagic and polydipsic condition in diabetic subjects.

Effects on FBGL, OGTT and LG:

The FBGL of control animal ranged from 78.33 \pm 3.88 to 78.00 \pm 3.58 mgdl⁻¹ during experiments which were found to be increased to 292.33 \pm 3.50 mgdl⁻¹ at 21 days in DC (**Fig 1A**). Decreasing trend (292.33 \pm 3.50 to 121.17 \pm 2.14 mgdl⁻¹) in FBGL were observed in DC+SP subjects ($p < 0.01$) at 21 days whereas C+SP showed insignificant ($p < 0.05$) change in FBGL. OGTT was performed to measure the animal's ability to utilize glucose. The OGTT was carried out on weekly basis for 21

days in all groups of test mice. Blood glucose was tested after glucose intake at every 15 min of interval for 1 h. DC subjects could not able to metabolise glucose within 1 h on the other hand the control and C+SP subjects tolerated glucose intake and showed glucose homeostasis within 1 h (Fig 2B-D). LG content ranged from 0.123 (mg%) to

0.213 (mg%) with the advancing age in control mice. Similar trends were also seen in C+SP but statistically insignificant ($p < 0.05$) when compared with the control. The gradual reduction of LG in DC was regained by feeding of SP (Fig. 2A) and it was utmost normalized to 0.23 in DC+SP at 21 day ($p < 0.01$).

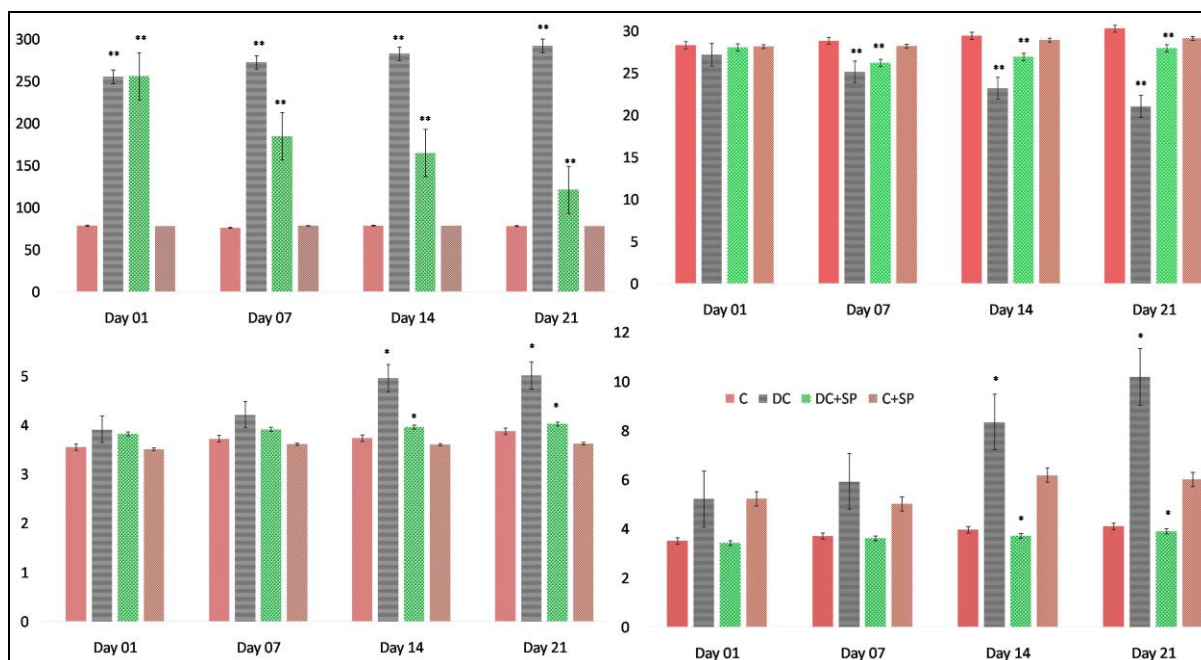


FIG.1: VALUES ARE EXPRESSED AS MEAN \pm SEM ($n=6$); SUPERSCRIPTS ** AND * ARE SIGNIFICANTLY DIFFERENT AT $P < 0.01$ AND AT $P < 0.05$ RESPECTIVELY WHEN COMPARED TO CONTROL. One-way analysis of variance was used followed by the *post-hoc* Tukey multiple comparison test. Group I: control (C), Group II: diabetic control (DC), Group III: diabetic control mice fed with SP (DC+SP), and Group IV: control mice fed with SP (C+SP). (a) Fasting blood glucose level (mg/dl), (b) Mean Body Weight (gm), (c) Mean Food Intake ($\text{g mice}^{-1}\text{day}^{-1}$), (d) Mean Water consumption ($\text{ml mice}^{-1}\text{day}^{-1}$)

Effect on Serum Lipid Profile:

There were significant ($p < 0.05$) increase recorded in TC, TG, LDL and VLDL throughout the experiment in DC when compared with control mice except HDL which were found to be decreased ($p < 0.01$) with duration of DM. It was found that TC, TG, LDL and VLDL increased to $197.33 \pm 1.21 \text{ mgdl}^{-1}$, $158.333 \pm 0.82 \text{ mgdl}^{-1}$, $150.67 \pm 1.14 \text{ mgdl}^{-1}$ and $31.67 \pm 0.16 \text{ mgdl}^{-1}$ respectively in DC at days 21 and it was maximum recorded value (Table 1; Fig 3A-D). DC+SP

showed restoring of TC, TG, LDL and VLDL values when compared to DC ($p < 0.01$). C+SP showed insignificant ($p < 0.05$) change in lipid profile when compared to control mice. The HDL ranged between $25.83 \pm 0.75 \text{ mgdl}^{-1}$ to $26.00 \pm 0.63 \text{ mgdl}^{-1}$ in control animals. Significant decrease in HDL was observed in DC when compared with control at all treatment period ($p < 0.01$). DC+SP showed significant increase ($p < 0.01$) in HDL from 15.00 ± 0.63 to $22.17 \pm 0.75 \text{ mgdl}^{-1}$ at 21 days of experiment.

TABLE 1: THERAPEUTIC POTENTIAL OF SPIRULINA PLATENSIS IN LOWERING DOWN OF TOTAL CHOLESTEROL (MG/DL) \pm SEM IN DIFFERENT GROUPS OF TEST MICE

Group of Mice	Day 01	Day 07	Day 14	Day 21
C	80.83 ± 0.98	81.17 ± 0.75	80.83 ± 0.75	81.50 ± 1.38
DC	$188.00 \pm 2.83^{**}$	$192.33 \pm 0.82^{**}$	$192.83 \pm 0.75^{**}$	$197.33 \pm 1.21^{**}$
DC+SP	188.67 ± 2.94	161.83 ± 1.17	123.50 ± 1.87	99.17 ± 2.64
C+SP	80.67 ± 0.82	80.33 ± 1.03	80.67 ± 0.82	82.33 ± 0.52

Values are expressed as mean \pm SEM ($n=6$); superscripts ** are significantly different at $P < 0.01$ when compared to control. One-way analysis of variance was used followed by the *post-hoc* Tukey multiple comparison test. Group I: control (C), Group II: diabetic control (DC), Group III: diabetic control mice fed with SP (DC+SP), and Group IV: control mice fed with SP (C+SP).

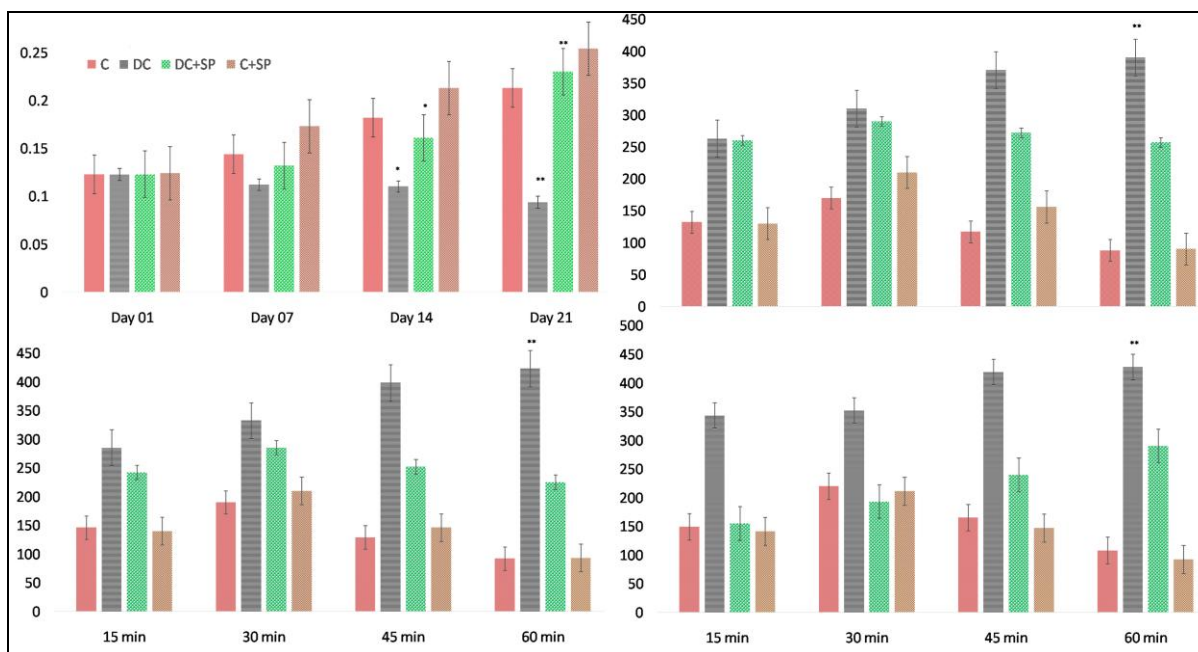


FIG. 2: VALUES ARE EXPRESSED AS MEAN±SEM (n=6); SUPERSRIPTS ** AND * ARE SIGNIFICANTLY DIFFERENT AT P < 0.01 AND AT P < 0.05 RESPECTIVELY WHEN COMPARED TO CONTROL. One-way analysis of variance was used followed by the *post-hoc* Tukey multiple comparison test. Group I: control (C), Group II: diabetic control (DC), Group III: diabetic control mice fed with SP (DC+SP), and Group IV: control mice fed with SP (C+SP). (a) Liver glycogen content (mg%), (b-d) Oral glucose tolerance level (mg/dl)

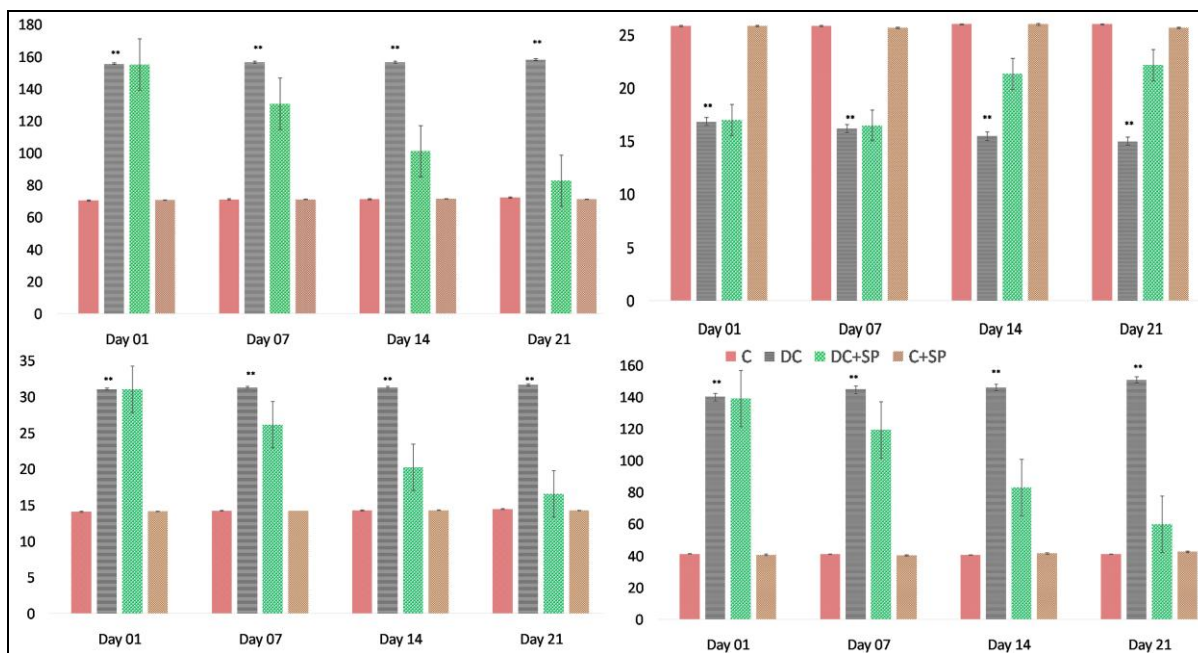


FIG. 3: VALUES ARE EXPRESSED AS MEAN±SEM (n=6); superscripts ** ARE SIGNIFICANTLY DIFFERENT AT P < 0.01 WHEN COMPARED TO CONTROL. One-way analysis of variance was used followed by the *post-hoc* Tukey multiple comparison test. Group I: control (C), Group II: diabetic control (DC), Group III: diabetic control mice fed with SP (DC+SP), and Group IV: control mice fed with SP (C+SP). (a) Triglyceride (mg/dl), (b) HDL (mg/dl), (c) VLDL (mg/dl), (d) LDL (mg/dl)

DISCUSSION: DM is primarily a metabolic disorder which results into remarkable changes in general physiology, glucose metabolism and lipid profile due to anomalies in lipid, carbohydrate and protein metabolism¹. Significant weight loss was observed in DC when compared with control mice at all experimental periods (p<0.01) except day 01 (Fig. 1B). The gradual reduction in body weight

was regained in DC by feeding of SP. In DM, body cells are unable to utilize glucose as a source of energy due to which proteins are spared as energy source, this leads to decrease in protein storage which in turn reduces body weight. No mice were found obese and gain in body weight was insignificant (p<0.01) in C+SP. Results of our present work is in accordance with the results of

other workers who have taken different therapeutic agents to treat DM¹⁰⁻¹². Insulin is the major activator of energy storage in adipose tissue. High levels of insulin consequently lead to obesity and vice versa. Differences were observed among treated groups versus control group in weight gain showing that SP maintains protein, carbohydrate and fat digestion and its utilization by mice. All mice survived till the end of 21st day so it is clear that SP were well accepted by mice. Experiments have also been carried out for the toxicological evaluation of single cell proteins (*Spirulina sp.*), mainly in rats as well as in farm animals and found non-toxic even at higher doses¹³. It was well established that SP supplementation helped diabetic patients to maintain their body weight contrary an increase in body weight was noticed when treated with the stem extract of *Opuntia fuliginosa* in rats¹⁴.

The food intake (**Fig. 1C**) was found to be increased in DC due to polyphagic condition in mice. This condition was treated in DC+SP group. As glucose is not up taken by cells, the cells remained in starved conditions thus stimulus reaches to brain and command for feeding behaviour. DC group were also observed for polydipsia (**Fig. 1D**) to maintain the solute solvent ratio in their body which were maintained in Group III. SP is reported to reduce food craving in human subjects. It was noticed that the increased water intake in the mice which fed on SP in both DC+SP and C+SP subjects induced thirst, due to its high nutritive and mineral content.

In this concern, linear relationship between SP dosage and water intake were also reported in cattle. The results of polydipsia and polyphagia in rats were overcome by administration of antidiabetic agents such as aqueous extract of *Anacardium occidentale*, *Smallantus sonifolius*, *Ceiba pentandra*, *Ficus racemosa* to treat diabetic mice¹⁵. The three parameters studied on the glucose metabolism in this work were FBGL, OGTT and LG. The diabetic mice have shown an increased FBGL from the day 1 onwards and continued till the end of experiment. The results on the decreased FBGL were indicative of the curative effect of SP in induced diabetic mice models. Treatment with SP for 21 days resulted in the

utmost reversal of the hyperglycemic condition in diabetic mice. In this regard, aqueous extract of SP has already been established for its hypoglycemic effect in AXN and STZ (Streptozotocin) diabetic rodents¹⁰. Identical findings were also reported by many researchers¹⁰⁻¹². Pre-treated rats with SP for four weeks when subsequently induced ischemic brain damage showed controlled blood sugar³. The results from AXN and STZ induced diabetic mice confirms the scientific fact that diabetic animals show decreased glucose tolerance.

The intake of glucose resulted in acutely elevated FBGL both in the diabetic and the normal subjects. The sugar level returned to the almost original level within an hour in control and C+SP. The DC subjects were unable to regulate the FBGL on oral administration of glucose within an hour. The DC+SP had improved its glucose tolerance ability (**Fig. 2B-D**) and it was shown as a function of the duration of the experiment.

Various theories validating the hypoglycemic effect of SP have been proposed. One such theory attributed about its fiber content which leads to reduced glucose absorption while another theory suggested the possible action of peptides and polypeptides generated by the digestion of SP proteins³.

A significant reduction in LG among DC mice was observed and became more prominent with time. Feeding of SP had reversed this trend in diabetic mice. On the 21st day of the experiment, the glycogen content was nearly 23.58% less in DC when compared to the control. Yadav et al. (2008) reported 80% reduction of LG in diabetic animals and such reduction were attributed to the changed activity levels of metabolic enzymes like hexokinase glyceraldehyde-3-phosphate dehydrogenase, glycogen synthetase and glucose -6- phosphate dehydrogenase¹⁶. Report suggested that decreased LG content is due to insulin deficiency and associated glycogenolysis process¹¹.

The TC was found to be increased in DC due to hyperlipidemic condition and such pathological condition was treated in DC+SP (**Table 1**). Prolonged administration of SP leads to significant reduction in FBGL and serum lipid level which are

in agreement with other studies¹⁶. Balanced insulin level stimulates fatty acid biosynthesis which in turn incorporates fatty acid into TG in the liver and adipose tissue. The TG was found to be increased in DC and this condition was cured in DC+SP (**Fig. 3A**). The strong hypolipidemic activity of SP could be achieved through its control of DM, as this is a major determinant of TG and TC levels. HDL levels were also found to be decreased in diabetic group¹⁶. The present work also validates the work of Layam and Reddy, 2006¹⁷ that SP at a dose of 15 mg kg⁻¹ bw decreased the TG and increased HDL in diabetic mice (**Fig. 3B**).

It is well known that altered LDL value plays an important role in arteriosclerosis and hypercholesterolemia. The decrease of TC and LDL levels were achieved by oral dose of SP which demonstrates a possible protection against hypercholesterolemia. The LDL and VLDL were found to be increased in DC and this condition was treated in DC+SP (**Fig. 3C-D**). This attributed effect could be partly due to certain specific phytochemicals present in SP such as phycocyanin, phytonutrients and fiber which might be playing a role in control of hyperglycemia and rectifying the associated abnormalities¹¹⁻¹⁶.

Increased serum lipids which are responsible for secondary complications including cardiovascular and reproductive disorders were reported in diabetic mice¹⁷. Higher lipid levels in diabetic mice and increased mobilization of free fatty acids from peripheral depots are the determinants to lipolysis caused by hormones. In this context, a number of other medicinal plants or plants extracts have also been reported to have antihyperglycemic, antihyperlipidemic and insulin stimulatory effects¹⁸⁻¹⁹.

SP powder contains strong antioxidants which increased the antioxidant status in mice blood and tissues. Such components might be capable of modulating oxidation of LDL. Studies have also demonstrated that vitamin C is more potent in preventing LDL oxidation thereby reduced the risk of development of atherosclerosis in DM. SP contains good quality proteins which might stimulate insulin secretion and such effect may be responsible for the reduction in FBGL²⁰⁻²¹.

CONCLUSIONS: From this study, it can be concluded that oral therapeutic treatment with SP suspension to diabetic mice modulates physiological status, carbohydrate and lipid metabolism. Results clearly unmasked therapeutic effects of SP against DM and warrants further investigation at wider dose regimens in other test animals.

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