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ORIGINAL ARTICLE

Combination Effect of *Spirulina fusiformis* with Rutin or Chlorogenic Acid in Lipopolysaccharide-Induced Septic Cardiac Inflammation in Experimental Diabetic Rat Model

Aman Sharma, Sumeet Gupta, Sunil Sharma[1], Meenakshi Dhanawat, Kavita Munjal

Department of Pharmaceutical Sciences, M M College of Pharmacy, MM University (Deemed to be University), Ambala, [1]Department of Pharmaceutical Sciences, Guru Jambeshwar University, Hissar, Haryana, India

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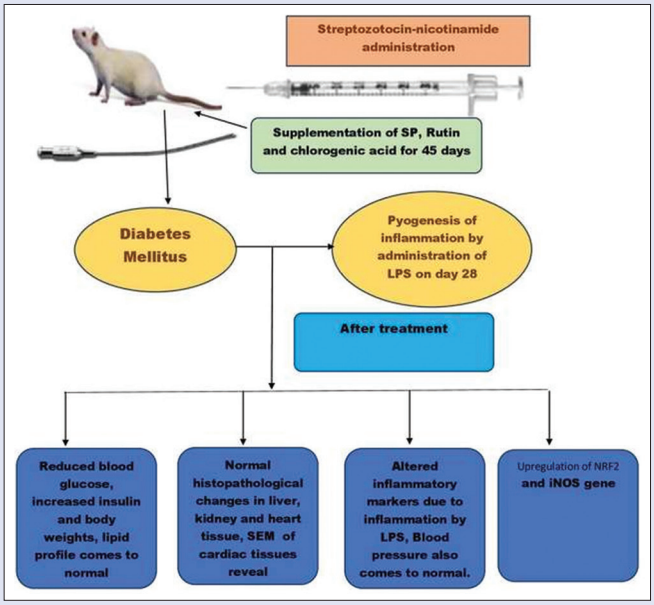
ABSTRACT

Background: The connection between inflammation and hyperglycemia leads to diabetes mellitus and its complications. Nutraceuticals are natural sources which can be used as supplement food for the prevention of many disorders. Our aim was studied to the protective role of two antioxidants (Rutin and Chlorogenic acid) with *Spirulina fusiformis* (SP) on streptozotocin-induced diabetic rats and being challenged with lipopolysaccharide to accelerate the diabetic inflammation. **Materials and Methods:** Rats were distributed into eleven groups. Each group has 6 rats**.** Lipopolysaccharide was administered to induced inflammation in diabetes mellitus rats. Orally administered *Spirulina fusiformis* (500 and 1000 mg/kg), rutin and chlorogenic acid (30 and 60 mg/kg) individually or in combination for 28 days. Metformin (200 mg/kg) was used as a standard drug. Biochemical parameters, body weight, and blood pressure levels were measured at different intervals. Histological studies, Scanning Electronic Microscopy, and gene expression analysis Real-time polymerase chain reaction of NRF2 and inducible nitric oxide synthase gene were analyzed at the end of the experiment. **Results:** Decreased in blood glucose levels and lipid profile at different intervals were noted with combination drug therapy (*Spirulina fusiformis* with rutin and *Spirulina fusiformis* with Chlorogenic acid). Improvement in antioxidant enzymes and pro-inflammatory cytokines levels were decreased on the 45th day. Maximum diastolic blood pressure levels were decreased statistically on the 45th day. Proof of evidence was also supported with these results. **Conclusion:** Combination therapy was showed excellent results than individual therapy at all parameters.

Key words: Antioxidant, lipopolysaccharide, nutraceuticals, rutin, spirulina, supplements

SUMMARY

- Actively nutraceutical drugs
- *Spirulina* with rutin or chlorogenic acid showed excellent results in controlling blood glucose and lipids levels at different intervals in LPD induced diabetic model
- Blood pressure and body weight were also maintained after treated with combination with *Spirulina*
- Proof of evidence (histology and Scanning electronic microscopy) showed protective organs against LPD induced inflammation
- Additionally molecular mechanistic study also showed defensive in combination therapy as compared to positive control group.



Abbreviations used: CAT: Catalase; BW: Body weight; GPx: Glutathione peroxidase; HDL: High-density lipoprotein; IL-6: Interleukin 6; LDL: Low-density lipoprotein; LPS: Lipopolysaccharide; MCP-1: Macrophage chemoattractant protein; RT-PCR: Real-time polymerase chain reaction; SD: Standard deviation; SP: *Spirulina plantensis*; STZ: Streptozotocin; SOD: Superoxide dismutase; TNF: Tumor necrosis factor; TBA: Thiobarbituric acid.

Correspondence:

Dr. Sumeet Gupta,
Department of Pharmacology, M. M. College of Pharmacy, Maharishi Markandeshwar University (Deemed to be University), Mullana, Ambala, Haryana, India.
E-mail: sumeetgupta25@gmail.com

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INTRODUCTION

Diabetes mellitus affects various organs leads to diabetic complications. Numerous factors are responsible for the development of this disease. Insulin resistance or destruction in pancreatic cells is the primary physiological effect noted in the body that leads to metabolic syndrome.[1,2] From the last 2 decades, diabetes mellitus is increasing very fast and nearly 90% of the cases are converted into death.[3] Increased in inflammation markers and oxidative stress levels are

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more prone risk to the development of diabetes mellitus. An imbalance in prooxidant-antioxidant status is the final development of oxidative stress that contributes to different pathological complications that lead to metabolic syndrome. These abnormalities cause multiple organ failure, especially in diabetes mellitus.[4,5]. The diabetic complication is a serious multifarious disease which effect among adults throughout the world. Diabetes patients are more susceptible to bacterial infection or sepsis. Sepsis is one of the major pathological features in ICU patients which may lead to multiple organ failure or even death also. The heart is the most sensitive organ and had a higher chance of infection. It was well established that all patients with cardiomyopathy associated with diabetes mellitus were at increased risk for bacterial infections.[6] Lipopolysaccharide originates from gram-negative bacteria and is widely used for the induction of sepsis in preclinical research.[7] The bacterial infection frequently increased due to high blood glucose levels in diabetic patients and produces poor clinical outcomes in the critically ill stage. In the preclinical study, the diabetic rats challenged with lipopolysaccharide (LPS) develop glucose-induced hyperinsulinemia[8] and also increases the production of inflammatory cytokines like interleukin (IL)-1 β, IL-18, and tumor necrosis factor-alpha (TNF-α) which directly impact on cardiac function.[9] The risk factors like hyperglycemia, metabolic endotoxemia, hypertension, hypercholesterolemia, homocystinemia, and cigarette smoking contribute to the development of cardiovascular disease.[10] Blood pressure is one of the identified factors in sepsis to maintain oxygen delivery.[11]

Due to serious complications of sepsis, heart injury is one of the first disorders known as cardiomyopathy[12] which may further cause hemorrhages, necrosis of the kidneys, and myocardial dysfunction[13,14] It has been well-recognized that prolonged activation of the inflammatory response contributes to a wide variety of chronic human diseases such as arteriosclerosis, obesity, various liver diseases, inflammatory bowel disease, autoimmune diseases, allergy, and cancer.[15] Activation of macrophages by LPS leads to the increased secretion of a large set of proinflammatory cytokines, such as TNF-alpha, IL-6, and macrophage chemoattractant protein (MCP-1).[16] LPS can trigger groups of different toll-like receptors, especially TLR4 expressed on the cell surface of immune cells. The binding of LPS to TLR4 triggers downstream signaling cascades, including mitogen-activated protein kinases and the nuclear transcription factor kappa-B pathways, which lead to the production of inflammatory mediators from macrophages. Natural compounds are always on priority basis to use against diseases. Rutin derivative (3,4-dihydroxytoluene) originated from rare natural Chinese herb were showing positive effect in the reduction of nitric oxide, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 levels in LPS stimulated murine macrophage cell line inflammatory model.[17] Other compound named as chlorogenic acid was also significantly inhibited the expression of phosphorylated NF-κB p65, IκB and TLR-4 signal induced by LPS agent in acute kidney injury rat model.[18] Commonly known as *Spirulina fusiformis* (Blue-green algae) used as organic extracts markedly decreased pro-inflammatory cytokines including granulocytes macrophage colony-stimulating factor, IL-6, MCP-1 and TNF-α in RAW 264.7 macrophage stimulated by LPS.[19] It is very difficult to manage the LPS induced sepsis inflammation associated with diabetes complications with current drug therapy in the present scenario.[20] Natural sources or Ayurveda System of Medicine maybe use as therapeutic options in treating various diseases which may improve the quality of life. Antioxidant could be useful in preventing or attenuating the adverse effects of chronic hyperglycemia like septicemia.[21]

Many nutraceuticals such as flavonoids, vitamins, linoleic acid, omega-3 fatty acids, α-lipoic acid, phytoestrogens, and dietary fibers are clinically used to focus on the pathogenesis of metabolic disorders and their complexities.[22] These food supplements provide protection against oxidative stress and prevent myocardium oxidative damage and rectify the number of changes in biochemical and clinical endpoints.[23]

To date nutraceuticals are one of the promising natural products that originate from plant sources having variety of pharmacological properties. Considering that both rutin and chlorogenic acid shows significant hypoglycaemic activity at the dose of 25 mg/kg and 10 mg/kg respectively in streptozotocin (STZ) induced diabetic rat model.[24] Rutin, chlorogenic acid and *Spirulina fusiformis* are very promising compounds which may show positive effect against deleterious effects induced by lipopolysaccharide induced in type 2 diabetes mellitus rat model. So, keeping in view the pharmacological properties of selected compounds, the present study was undertaken to evaluate the protective effect of individuals or in the combination of *Spirulina fusiformis* with rutin or chlorogenic acid at low doses in the treatment of septicemia in the diabetes mellitus rat model.

MATERIALS AND METHODS

Chemicals

STZ and Lipopolysaccharide were purchased from Sigma-Aldrich Co. Ltd (India) and metformin was obtained as a gift sample from Torrent Pharmaceuticals (Ahmedabad, Gujarat, India). All other solvents and chemicals were used of the highest grade from the chemical Industry.

Drugs and preparation

Spirulina fusiformis in the form of powder was gift from Recon Ltd (Bangalore). Rutin and chlorogenic acid both were purchased from Sigma Ltd, (India). *Spirulina fusiformis* was suspended in vehicle (olive oil) and was administered orally using oral gavage. Rutin and chlorogenic acid were suspended in Tween 80 in distilled water.

Animals

Adult male Wistar rats (200–250 g) were procured from the approved animal house at the National Institute of Pharmaceutical Education and Research (Punjab, India). The experimental protocol was accepted by the Institute from Animal Ethics Committee (MMCP/IAEC/15/01). The animals were housed in an animal house under a specific pathogen-free condition in a controlled laboratory environment (temperature of 24°C–28°C and relative humidity of 60%–70% with a 12 h day and night cycle. Rats were fed with standard pellet diet and were accessed to water during the whole experiment.

Experimental design

Rats were again divided into 11 groups, each contains 6 rats. Group 1 (Negative control): Tween 80 in distilled water and olive oil, Group 2 (Positive control): STZ (35 mg/kg, i.p), Group 3 (Standard control): STZ (35 mg/kg) + Metformin (200 mg/kg once daily, oral), Groups 4 (Drug treated): STZ (35 mg/kg) + Rutin (30 mg/kg once daily, oral), Group 5 (Drug treated): STZ (35 mg/kg) + Rutin (60 mg/kg once daily, oral), Group 6 (Drug treated): STZ (35 mg/kg) + Chlorogenic (15 mg/kg once daily, oral), Group 7 (Drug treated): STZ (35 mg/kg) + Chlorogenic (30 mg/kg once daily, oral), Groups 8 (Drug treated): STZ (35 mg/kg) + *Spirulina plantensis* (SP) (500 mg/kg, oral) Group 9: (Drug treated): STZ (35 mg/kg) + *Spirulina plantensis* (SP) (1000 mg/kg, oral), Group 10: (Drug treated): STZ (35 mg/kg) + *Spirulina plantensis* (SP) (500 mg/kg, oral) + Rutin (30 mg/kg once daily, oral), Group 11: (Drug treated): STZ (35 mg/kg) + *Spirulina plantensis* (SP) (500 mg/kg, oral) + chlorogenic acid (15 mg/kg once daily, oral).

Induction of diabetes and infection

Diabetes mellitus was induced by single dose[25] of STZ (40 mg/kg b. w) *via* intraperitoneal route to all rats except normal group and it was confirmed after 5 days with increase in serum blood glucose levels (>300 mg/dl). After 28 days of the experiment, LPS was dissolved in sterile, endotoxin-free 0.9% (m/v) sodium chloride solution and injected intraperitoneal at a dosage of (1 mg/kg, b. w) into all diabetic rats to stimulate sepsis.[26]

Biochemical estimations

Blood samples were drawn at weekly intervals on days 0th, 7th, 14th, 21st, and 45th. The rats were anesthetized with ether and blood sample was collected by retro-orbital puncture. About 30 µl serum was then separated for the estimation of glucose. Triglyceride, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) were also measured on the 45th day. Serum insulin was also estimated by radio immunoassay kit purchased from Stat Diagnostics (Linco Research Inc.), Mumbai, India. The cytokine levels (TNF-α and IL-6) were determined on the 40th day by the sandwich ELISA method with a commercially available kit from Fisher Thermo Scientific Co.(Rockford, Ill).[27] Glycosylated hemoglobin levels were also estimated using a standard technique on the 45th day.[28]

Body weight

Rats from each group were weighed at different intervals (0, 7th, 14th, 21st, and 42nd day) on a digital electronic balance for small animals.

Blood pressure measurement

Blood pressure was measured at different intervals on days 0th, 15th, 30th, and 45th using the tail-cuff method (Coda 6 System, Kent Scientific, USA). The rats were placed in a warmed individual restrainer, and both an occlusion cuff and a volume pressure-recording cuff were placed close to the base of the tail. The pressure recording allowed a noninvasive measurement of systolic blood pressure and diastolic blood pressure.[29]

Morphology study of organs

At the end of the experiment (45th day), three rats from each group were sacrificed under anesthesia using pentobarbitone sodium (60 mg/kg) and further by cervical dislocation if necessary. Various organs were used for histopathology and scanning electron microscopy.[30] The remaining animals were used for antioxidant and gene expression studies.

Lipid peroxidation assay of heart

Lipid peroxidation was assessed on the 45th day by measuring the thiobarbituric acid (TBA) reactivity of malondialdehyde, as an end product of fatty acid peroxidation. For this purpose, about 0.2 ml of cardiac cells were suspended in the combination of 0.8 ml of phosphate-buffered saline and 0.025 ml of butylated hydroxyl toluene (88 mg/10 ml absolute alcohol). Then 30% of trichloroacetic acid (0.5 ml) was added. After that, the tubes were vortex and then allowed to stand on ice for at least 2 hr. The tubes were centrifuged at 2000 *g* for 15 min. For each tube, about 1 ml of supernatant was transferred to a new tube and then added 0.25 ml of 1% TBA in 0.05 N sodium hydroxide. The tubes were then mixed and kept in a boiling water bath for 15 min and the concentration of the malondialdehyde-TBA complex was assessed as described earlier.[31] The TBA reactive substance values were expressed as nmol/mg protein.

Assay of superoxide dismutase of heart

Superoxide dismutase (SOD) was assayed on the 45th day with utilizing the technique based on inhibition of the formation of nicotinamide adenine dinucleotide, phenazine methosulfate, and amino blue tetrazolium formazan. A single unit of the enzyme was expressed as 50% inhibition of nitroblue tetrazolium reduction/min/mg/protein.[32]

Assay of glutathione peroxidase of heart

Glutathione peroxidase (GPx) was determined on the 45th day with the standard technique. Supernatant (1 ml) was treated with Ellman's reagent (0.5 ml) and phosphate buffer (3.0 ml; 0.2M, pH 8.0). The absorbance was read at 412 nm. GPx activity was expressed as µg of GPx consumed/min/mg protein and reduced glutathione as mm/mg of tissue.[33]

Assessment of gene expression on 45th day

Total RNA contents were extracted from heart tissue samples in 1 ml QIAzol with chloroform. The RNA pellets were rinsed with 70% ethanol, dried, and suspended in diethylpyrocarbonate (DEPC, 129112, QIAGEN Inc[34,35] RNA amount and purity were assessed using a spectrophotometer at 260 nm. The ratio of the 260/280 optical density of all RNA tested was 1.7–1.9. RNA in samples was transcripted to the corresponding cDNA with RevertAid Premium reverse transcriptase. NRF2 [forward primer 5'-TCTCCTCGCTGGAAAAAAGAA-3' and reverse 3'-AATGTGCTGGCTGTGCTTTA-5', and iNOS gene expressions [forward primer 5'-ACGCTCAGCTCATCCGGTAT and reverse primer 3'-CACTTCAGCTCCAGCTCCTG] concentration were examined with Real time polymerase chain reaction (RTPCR) using a Bio-Rad MJ Mini Opticon RTPCR System. The primer sequences for iNOS, and GAPDH (housekeeping). Data are presented relative to control values using three separate experiments.

Statistical analysis

The data presented in the Tables/Figures are the mean ± standard deviation. The statistical difference between mean was analyzed using ANOVA and by Tukey's multiple comparison test. The $P < 0.05$ was considered significant.

RESULTS

Effect on blood glucose levels

Blood glucose levels [Table 1] were significantly increased from 7th day to 45th day in the positive control group, whereas in the normal control group, it was found normal level at all intervals. On the 45th day (Individual therapy), the percentage of blood glucose levels in rutin treated group was reduced up to 49.8% with 30 mg/kg and 59.7% with 60 mg/kg but in the case of the chlorogenic acid treated group, the glucose levels were noted higher reduction (66.7%) with low dose (15 mg/kg) and low reduction (53.6%) with high dose (30 mg/kg). Maximum percentage of blood glucose level was found with double dose of *Spirulina fusiformis* (1000 mg/kg) as compared to single dose of *Spirulina fusiformis* (500 mg/kg) at all intervals. In combination therapy at low dose, greater percentage reduction were noted in *Spirulina fusiformis* (500 mg/kg) with Chlorogenic acid-treated group (71.2%) > *Spirulina fusiformis* (500 mg/kg) with rutin treated group (63.4%).

Lipids levels [Table 2] were significantly changed in different treated groups as compared to the positive control group. On the 45th day at individual therapy, HDL level was found maximum with rutin treated group at dose of 60 mg/kg (47.85%) and spirulina treated group at a dose of 500 mg/kg (43.55%) respectively. In combination therapy, *Spirulina fusiformis* (500 mg/kg) with rutin (30 mg/kg) treated group showed 54.08% increases as compared to *Spirulina fusiformis* (500 mg/kg) with chlorogenic acid (15 mg/kg) treated group (51.82%). In the case of LDL and triglycerides, rutin and chlorogenic acid-treated groups

Table 1: Anti diabetic activity of *Spirulina fusiformis*, rutin and chlorogenic acid treated group at different intervals in type 2 diabetes rat model

Groups (*n*=6)	0 day (mg/dl)	7th day (mg/dl)	14th day (mg/dl)	21st day (mg/dl)	45th day (mg/dl)
Normal control	84.63±0.33	82.7±0.51#, a	83.06±3.38#, a	86.05±3.55#, a	86.6±1.95#, a
Positive control (DM)	83.53±4.98	312.35±11.47	307.2±58.9	311.2±69.05	327.1±30.45
DM+metformin (200 mg/kg)	85.616±3.90	179.9±26.80#, a (42.4)	139.71±4.06#, a (54.52)	119.21±28.67#, a (61.6)	103.3±9.05#, a (68.4)
DM+rutin (30 mg/kg)	79.76±7.34	284.75±22.31$, a (8.82)	241.31±22.9#, c,$, a (21.5)	220.6±35.05#, a,$, a (29.1)	164.15±45.22#, a,$, b (49.8)
DM+rutin (60 mg/kg)	84.01±6.63	281.9±30.74$, a (9.74)	229.7±27.8#, a,$, a (25.2)	183.19±34.7#, a,$, c (41.1)	131.64±11.57#, a (59.7)
DM+chlorogenic acid (15 mg/kg)	83.25±8.48	284.26±14.15$, a (8.99)	216.3±45.2#, a,$, b (29.5)	150.83±22.8#, a,$, NS (51.5)	108.6±12.4#,a (66.7)
DM+chlorogenic acid (30 mg/kg)	85.63±6.58	279.6±40.01 (10.45)	239.7±49.4#, c,$, a (21.9)	202.8±41.9#, a,$, b (34.8)	151.5±4.3#,a (53.6)
DM+SP (500 mg/kg)	83.2±10.8	250.53±24.93#, c,$, b (19.76)	188.33±18.17#, a,$, NS (28.9)	164.04±16.21#, a,$, NS (47.2)	126.9±24.11#, a,$, NS (61.2)
DM+SP (1000 mg/kg)	85.4±3.1	234.3±48.61#, b,$, NS (24.95)	203.6±26.12#, a,$, NS (33.72)	153.26±20.65#, a,$, NS (50.7)	104.7±27.2#a (67.9)
DM+SP (500 mg/kg) + rutin (30 mg/kg)	74.26±8.15	263.2±35.4$, a (15.84)	194.1±8.79#, a,$, NS (36.8)	135.08±19.33#, a,$, NS (56.6)	119.4±43.9#, a (63.4)
DM+SP (500 mg/kg) + chlorogenic acid (15 mg/kg)	83.16±10.2	193.07±32.44#, a,$, NS (38.2)	167±13.4#, a,$, NS (45.6)	119.03±17.64#, a,$, NS (61.6)	91.3±22.96#, a,$, NS (71.2)
Overall *P*		<0.0001	<0.0001	<0.0001	<0.0001
F		30.78	40.65	21.33	41.71

#Positive control versus all groups, $Metformin versus all treated groups, a*P*<0.001, b*P*<0.01, c*P*<0.05. One-way ANOVA was used for statistical analysis of data, further Tukey's multiple range test was used. For each group (*n*=6) values were expressed in mean±SD and *P* value which were<0.05 were significant df=70. SP: *Spirulina fusiformis*, NS: Not significant, SD: Standard deviation

Table 2: Lipid profile and insulin levels of *Spirulina fusiformis*, rutin and chlorogenic acid treated group on 45th day in type 2 diabetes rat model

Groups (*n*=6)	HDL (mg/dl)	LDL (mg/dl)	Triglycerides (mg/dl)	Insulin (µg/dl)	HBA_{1c} (%)
Normal control	46.23±3.82#, a	98.40±10.91#, a	94.05±9.19#, a	18.28±4.06#, a	6.77±2.278#, a
Positive control (DM)	17.78±2.13	193.30±4.81	221.88±22.60	38.27±7.49	12.92±2.20
DM+metformin (200 mg/kg)	40.94±3.37#, a (56.57)	90.57±9.39#, a (53.14)	106.39±9.82#, a (52.05)	20.84±8.94 (45.54)	7.48±1.21#, b (42.10)
DM+rutin (30 mg/kg)	28.15±3.47#, b (36.83)	163.63±16.37#, b (15.34)	181.27±23.67#, c (18.30)	33.89±9.10 (11.44)	10.06±2.59 (22.13)
DM+rutin (60 mg/kg)	34.10±3.39#, a (47.85)	140.98±12.71#, a (27.06)	138.26±34.36#, a (37.68)	28.07±8.42 (26.65)	9.67±2.60 (25.15)
DM+chlorogenic acid (15 mg/kg)	23.04±4.90 (22.82)	153.14±12.17#, a (20.77)	158.37±20.70#, a (28.62)	30.44±15.97 (20.45)	10.06±2.69 (22.13)
DM+chlorogenic acid (30 mg/kg)	30.71±3.69#, a (42.10)	132.00±15.20#, a (31.71)	121.89±13.15#, a (45.06)	21.14±10.59 (44.76)	9.21±2.47 (28.71)
DM+SP (500 mg/kg)	28.33±4.18#, b (37.23)	138.94±13.65#, a (28.12)	144.93±25.40#, a (34.68)	30.79±14.86 (19.54)	9.54±1.94 (26.16)
DM+SP (1000 mg/kg)	31.50±5.83#, a (43.55)	141.61±12.66#, a (26.74)	130.36±13.02#, a (19.54)	24.88±15.97 (34.98)	8.62±2.21 (33.28)
DM+SP (500 mg/kg) + rutin (30 mg/kg)	38.72±4.36#, a (54.08)	108.39±14.04#, a (43.97)	110.39±14.71#, a (50.24)	22.04±3.34 (42.04)	8.018±1.87#, c (37.94)
DM+SP (500 mg/kg) + chlorogenic acid (15 mg/kg)	36.91±7.41#, a (51.82)	102.57±12.69#, a (46.93)	104.73±8.18#, a (52.79)	21.03±3.87 (45.04)	8.13±3.28#, c (37.07)
Overall *P*	<0.0001	<0.0001	<0.0001	00243	0.0049
F	20.24	36.61	23.12	2.302	2.953

#Positive control versus all groups, $Metformin versus all treated groups, aP<0.001, bP<0.01, cP<0.05. One-way ANOVA was used for statistical analysis of data, further Tukey's multiple range test was used. For each group (n=6) values were expressed in mean±SD, and P value which were<0.05 were significant df=70. SP: *Spirulina fusiformis*, SD: Standard deviation, HDL: High density lipoprotein, LDL: Low density lipoprotein, DM: Diabetes mellitus

at higher doses were showing decrease levels and the same results were obtained with low dose of *Spirulina fusiformis* (500 mg/kg) treated group. In combination therapy, both parameters were showing decrease the levels in *Spirulina fusiformis* (500 mg/kg) with chlorogenic acid-treated group (15 mg/kg) as compared to other *Spirulina fusiformis* with rutin treated group. The results were extremely statistically higher significant ($P < 0.0001$).

Insulin level was 38.27 ± 7.49 µg/dl in the positive control group [Table 2] on the 45th day. Insulin levels were found at the range of 11% to 35% after treated with individual therapy at both doses of rutin, chlorogenic acid and *Spirulina fusiformis* group. *Spirulina fusiformis* (500 mg/kg) with chlorogenic acid (15 mg/kg) and metformin-treated group showed 45.54% decreases the level as compared to the positive control group. Glycosylated hemoglobin value was 12.92 ± 2.20 in the positive control group on the 45th day. Only combination therapy showed slightly statistical significant ($P < 0.01$) as compared with positive control group.

Blood pressure was measured at different intervals [Table 3]. The blood pressure was increasing after the 7th day in all groups except normal control group. There was no statistically significant difference was noticed in any treated group as compared to the positive control group at all the intervals. On the 45th day, 10% reduction was found in systolic blood pressure levels in both the combination therapy. In the case of diastolic blood pressure [Table 4], slight reduction was noticed at the higher dose of all individual treated groups. Combination therapy showed 13%

Table 3: Changes in systolic blood pressure levels after treatment with *Spirulina fusiformis*, rutin and chlorogenic acid at different intervals in type 2 diabetes mellitus rat model

Groups (*n*=6)	7th day (mmHg)	15th day (mmHg)	30th day (mmHg)	45th day (mmHg)
Normal control	124.92±3.83	128.75±5.78	123.59±3.69	124.42±6.49
Positive (DM)	138.22±4.73	140.05±6.19	143.39±5.34	142.05±7.06
DM+metformin (200 mg/kg)	140.08±4.03	133.75±4.19↓ (4.49)	130.42±3.55↓ (9.04)	128.75±4.79↓ (9.36)
DM+rutin (30 mg/kg)	143.12±3.09	144.12±1.99↑ (2.90)	140.95±6.57↓ (1.70)	136.79±9.55↓ (3.70)
DM+rutin (60 mg/kg)	139.72±6.56	142.72±6.42↑ (1.90)	134.05±9.24↓ (6.51)	130.89±9.22↓ (7.85)
DM+chlorogenic acid (15 mg/kg)	141.08±4.44	137.72±4.87↓ (1.66)	133.72±8.46↓ (6.73)	129.20±17.94↓ (9.04)
DM+chlorogenic acid (30 mg/kg)	142.32±2.85	140.25±3.75↑ (0.14)	135.25±7.60#,a↓ (5.67)	131.92±9.01↓ (7.13)
DM+SP (500 mg/kg)	138.40±4.64	141.65±4.11↑ (1.142)	136.82±10.56↓ (4.58)	130.15±9.64↓ (8.37)
DM+SP (1000 mg/kg)	134.92±2.84	140.03±4.11↓ (0.014)	135.03±10.56↓ (5.83)	128.36±9.64↓ (9.63)
DM+SP (500 mg/kg) + Rutin (30 mg/kg)	143.24±2.88	144.52±2.09↑ (3.19)	138.85±6.11↓ (3.166)	127.19±12.30↓ (10.46)
DM+SP (500 mg/kg) + chlorogenic (15 mg/kg)	148.25±6.31	143.22±6.10↑ (2.26)	131.55±12.13↓ (8.25)	126.89±19.37↓ (10.67)
Overall *P* value		<0.0001	0.0103	0.3435
F		2.98	2.62	1.51

#Positive control versus all groups, ↑Increase , ↓Decrease. One-way ANOVA was used for statistical analysis of data, further Tukey's multiple range test was used. For each group (*n*=6) values were expressed in mean±SD, and *P* value which were <0.05 were significant df=70. SP: *Spirulina fusiformis*, SD: Standard deviation, DM: Diabetes mellitus

Table 4: Changes in diastolic blood pressure levels after treatment with *Spirulina fusiformis*, rutin and chlorogenic acid at different intervals in type 2 diabetes mellitus rat model

Groups (*n*=6)	0 day (mmHg)	15th day (mmHg)	30th day (mmHg)	45th day (mmHg)
Normal control	86.33±3.85	85.50±7.58	86.33±6.18	89.50±8.91
Positive (DM)	94.47±8.9	98.97±15.12	95.64±14.97	96.31±16.47
DM+metformin (200 mg/kg)	95.07±10.58	94.57±10.17↓ (4.46)	93.24±10.04↓ (2.50)	84.79±12.50↓ (12.70)
DM+rutin (30 mg/kg)	94.56±7.91	96.23±13.12↓ (2.76)	93.23±12.80↓ (2.51)	89.89±9.98↓ (6.66)
DM+rutin (60 mg/kg)	95.43±8.93	96.09±11.97↓ (2.90)	93.76±10.12↓ (1.96)	84.76±7.76↓ (11.99)
DM+chlorogenic acid (15 mg/kg)	94.80±8.94	94.63±9.62↓ (4.38)	90.80±7.39↓ (5.06)	85.80±5.67↓ (10.91)
DM+chlorogenic acid (30 mg/kg)	93.39±9.88	90.73±11.65↓ (8.32)	88.39±14.3↓ (7.58)	86.27±15.09↓ (10.43)
DM+SP (500 mg/kg)	92.23±8.57	93.9±11.14↓ (5.12)	92.04±6.02↓ (3.76)	89.71±5.71↓ (6.85)
DM+SP (1000 mg/kg)	94.56±11.30	94.47±11.66↓ (4.54)	90.47±8.71↓ (5.40)	86.63±6.52↓ (10.05)
DM+SP (500 mg/kg) + rutin (30 mg/kg)	92.73±9.72	90.23±8.32↓ (18.83)	86.9±4.91↓ (9.13)	83.56±5.31↓ (13.23)
DM+SP (500 mg/kg) + chlorogenic acid (15 mg/kg)	90.83±21.73	90.67±21.79↓ (8.38)	86.33±11.36↓ (9.73)	82.83±7.49↓ (13.99)
Overall *P*		0.867	0.799	0.638
F		0.521	0.606	0.790

↑Increase, ↓Decrease. One-way ANOVA was used for statistical analysis of data, further Tukey's multiple range test was used. For each group (*n*=6) values were expressed in mean±SD, and *P* value which were <0.05 were significant df=70. SP: *Spirulina fusiformis*, SD: Standard deviation, DM: Diabetes mellitus

reduction in diastolic blood pressure levels as compared to systolic blood pressure. Maximum blood pressure decreases at all intervals (15th day, 30th day, and 45th day) in both the drug treatment combination.

Body weight (BW) [Table 5] was also measured at different intervals. Positive control group were showing constantly decreases in the bodyweight. Individual *Spirulina fusiformis* treated group and combination of *Spirulina fusiformis* with other drugs treated group showed statistically significant ($P < 0.001$) increase in the bodyweight as compared to positive control group on 45th day. The percentage rate was found in the range of 21.43% to 25.55%. In antioxidant assays [Table 6], the CAT, TBARS, and SOD were showing statistical significant difference ($P < 0.001$) in both the combination group i. e *Spirulina fusiformis* with rutin and *Spirulina fusiformis* with chlorogenic acid as compared to positive control group. TNF-α and IL-6 were also showing marked improvement in both drug combination-treated groups and found statistically significant ($P < 0.001$). Chlorogenic acid at a dose of 30 mg/kg showed a slightly statistical significant ($P < 0.05$) decrease in IL-6.

Real-time polymerase chain reaction of NRF2 gene

RT-PCR results [Figure 1a] showed up-regulation in NRF2 gene expression in response to the treatment with *Spirulina fusiformis* with rutin (500 mg/kg + 30 mg/kg) had 1.43-fold as compared with normal control group 1.11-fold [Figure 1b]. 1.09-fold found as compared with standard treated group 1.04 fold [Figure 1c] and 1.066 fold was achieved as compared with positive control group 0.99 fold [Figure 1d]. Second drug combination *Spirulina fusiformis* with chlorogenic acid (500 mg/kg + 15 mg/kg) showed marked downregulation of NRF2 gene expression when compared with positive control group 0.79 fold while other treatments did not show showed any marked upregulation but activity was almost down regulated to positive control group 0.99. Results showed significant activity with *Spirulina fusiformis* with rutin treated group.

Real time polymerase chain reaction of inducible nitric oxide synthase gene

RT-PCR results [Figure 2a] revealed up and downregulation of INOS gene expression in various groups when compared to the normal, standard, and positive control group. Upregulation was noted (1.24 fold) in the treatment of *Spirulina fusiformis* with rutin (500 mg/kg + 30 mg/kg) when compared with normal group 1.6-fold [Figure 2b]. Downregulation was found with both the drug combination-treated group (1.34 and 1.18) fold as compared with metformin 2.2 fold [Figure 2c]. Expression showed 1.54 times higher with *Spirulina fusiformis* with rutin as

Table 5: Changes in body weight after treatment with *Spirulina fusiformis*, rutin and chlorogenic acid at different intervals in type 2 diabetes mellitus rat model

Groups (n=6)	0th day (g)	7th day (g)	15th day (g)	30th day (g)	45th day (g)
Normal control	279.66±6.31	278.50±14.80	276.66±35.89	276.33±35.44	278.16±35.33
Positive control (DM)	257.50±19.50	251.66±13.98	243.83±10.45	230.66±15.83	218.50±12.75
DM+metformin (200 mg/kg)	262.50±9.56	258.50±14.72↑ (2.71)	261.166±12.81↑ (7.10)	256.333±15.62↑ (11.12)	260.33±16.42#,b↑ (19.12)
DM+rutin (30 mg/kg)	272.33±19.24	267.66±14.00↑ (6.35)	250.50±13.21↑ (2.73)	243.50±10.13↑ (15.56)	245.16±13.01↑ (12.20)
DM+rutin (60 mg/kg)	263.83±26.25	259.50±19.41↑ (3.11)	243.66±9.87↑ (0.06)	241.83±10.45↑ (4.84)	240.83±9.02↑ (10.21)
DM+chlorogenic acid (15 mg/kg)	262.33±11.65	258.66±15.46↑ (2.78)	241.83±14.56↑ (0.82)	238.83±10.62↑ (3.54)	238.66±10.68↑ (9.22)
DM+chlorogenic acid (30 mg/kg)	281.50±15.74	275.83±15.68↑ (9.60)	254.33±14.71↑ (4.30)	250.33±11.32↑ (8.52)	252.16±8.65↑ (15.40)
DM+SP (500 mg/kg)	278.66±13.09	271.66±19.57↑ (7.94)	264.50±13.83↑ (8.47)	264.16±13.64↑ (14.52)	265.33±13.47#,a↑ (21.43)
DM+SP (1000 mg/kg)	292.5±15.46	286.33±13.67#,c↑ (13.77)	275.33±36.94↑ (12.91)	271.50±19.07#,b↑ (17.70)	275.66±18.74#,a↑ (26.16)
DM+SP (500 mg/kg) + rutin 30 mg/kg	282.33±16.09	276.83±7.30↑ (10)	268.16±13.74↑ (9.97)	263.16±13.92↑ (14.09)	265.16±14.13#,a↑ (21.35)
DM+SP (500 mg/kg) + chlorogenic acid (15 mg/kg)	291.83±41.98	281.83±17.01#,c↑ (11.98)	273.33±19.15↑ (12.09)	270.83±18.78#,b↑ (17.41)	274.33±19.99#,a↑ (25.55)
Overall P	0.0025	0.0103	0.0103	< 0.0001	<0.0001
F	3.21	3.21	2.64	4.623	7.051

#Positive control versus all groups, $Metformin versus all treated groups, aP<0.001, bP<0.01, cP<0.05. One-way ANOVA was used for statistical analysis of data, further Tukey's multiple range test was used. For each group (n=6) values were expressed in mean±SD, and P value which were<0.05 were significant df=70. SP: *Spirulina fusiformis*, SD: Standard deviation, DM: Diabetes mellitus

Table 6: Antioxidant and cytokinines levels after treatment with *Spirulina fusiformis*, rutin and chlorogenic acid on 40th day in type 2 diabetes mellitus rat model

Groups	CAT (units/mg protein)	TBARS (units/mg protein)	SOD (units/mg protein)	TNF-α (pg/ml)	IL-6 (pg/ml)
Normal control	29.82±2.41	1.48±2.13	10.17±5.15	10.68±0.74	5.26±0.52
Positive control (DM)	19.19±2.79	4.10±1.31	3.76±5.08	27.52±0.59	12.63±1.03
DM+metformin (200 mg/kg)	26.66±6.15#,a	1.76±5.13	5.04±4.24	13.10±0.91#,b	6.52±0.63
DM+rutin (30 mg/kg)	17.41±2.62	2.28±1.03	5.26±3.27	20.18±2.55	10.05±1.36
DM+rutin (60 mg/kg)	19.64±5.16	1.77±2.01	6.87±3.18	16.51±2.28	9.18±1.20
DM+chlorogenic acid (15 mg/kg)	16.76±4.34	2.16±5.42#,b	4.39±3.04	15.42±0.80	9.53±1.57
DM+chlorogenic acid (30 mg/kg)	19.88±3.79#,a	1.85±3.13	5.14±3.20	18.95±0.92	8.32±1.18#,c
DM+SP (500 mg/kg)	22.52±4.22	2.10±2.19	4.86±1.45	17.28±1.38	9.82±1.20
DM+SP (1000 mg/kg)	24.14±3.29	2.13±5.13	5.27±1.18	16.78±2.25	8.19±2.21
DM+SP (500 mg/kg) + rutin (60 mg/kg)	24.31±1.21#,a	2.24±4.20#,a	6.21±5.10#,b	11.25±0.26#,a	7.05±0.29#,a
DM+SP 500 (mg/kg) + CHL (30 mg/kg)	23.62±4.13#,a	1.35±1.75#,a	5.52±5.28#,b	12.13±0.53#,a	6.08±1.12#,a
Over all P	<0.0001	<0.0001	<0.000	<0.0001	<0.0001
F	4.36	5.68	7.23	3.067	4.031

#Positive control versus all groups, $Metformin versus all treated groups, aP<0.001, bP<0.01, cP<0.05. One-way ANOVA was used for statistical analysis of data, further Tukey's multiple range test was used. For each group (n=6) values were expressed in mean±SD, and P value which were <0.05 were significant df=70. SP: *Spirulina fusiformis*, SD: Standard deviation, CHL: Chlorogenic, CAT: Catalase, SOD: Superoxide dismutase, TNF: Tumor necrosis factor, IL-6: Interlukin 6, TBARS: Thiobarbituric acid reactive substances, DM: Diabetes mellitus

compared with positive control group (1.02). *Spirulina fusiformis* with chlorogenic acid found upregulation as compared with positive control group [Figure 2d]. The other treatments did not show any significant activity when compared to the normal, standard, or positive group.

Scanning electronic microscopy

The ultrastructure of the rat heart by Scanning Electron Microscopy is represented as shown in Figure 3. In the normal control group [Figure 3a], myofibrils were observed intact with thick and definite architecture and linearly arranged mitochondria. The gap junction was very minimum and dense. Normal mitochondria were noted and the ridges were closely associated with the membrane. Positive group [Figure 3b] demonstrated a thin myofibril, disarranged mitochondria and inter-myofibrillar spaces. There was a reduction in the fibrin bundles in the cytoplasm and a number of bundles had dissolved in the sarcoplasm. Several mitochondria were small, circular, and pyknotic in appearance. Figure 3c metformin-treated group showed a drastic improvement in myofibrils which was intact as similar to the normal group. Figure 3d-h showed little improvement in the distorted architecture of myofibrils. Large inter-myofibrillar spaces and swollen mitochondria were noted. Large gaps were seen between the sarcomere. In combination-treated group, morphological structure of intermyofibrillar was improved. Abnormalities were significantly very less and maintained inter-myofibrillar architecture. Less fibrosis of mitochondria space indicated the cardioprotective effect of both drug combination *Spirulina fusiformis* with rutin [Figure 3j] and *Spirulina fusiformis* with chlorogenic acid [Figure 3k] in diabetic rats.

Histological of heart

The histological investigation was done in all groups [Figure 4] and revealed the morphological alterations and fibrosis development in cardiac tissues. The light microscope image of normal control heart shows prominent single oval-shaped cardiomyocytes. In the positive control group, an image demonstrated morphological changes that show the diabetes-induced by LPS encouraged cardiac architecture

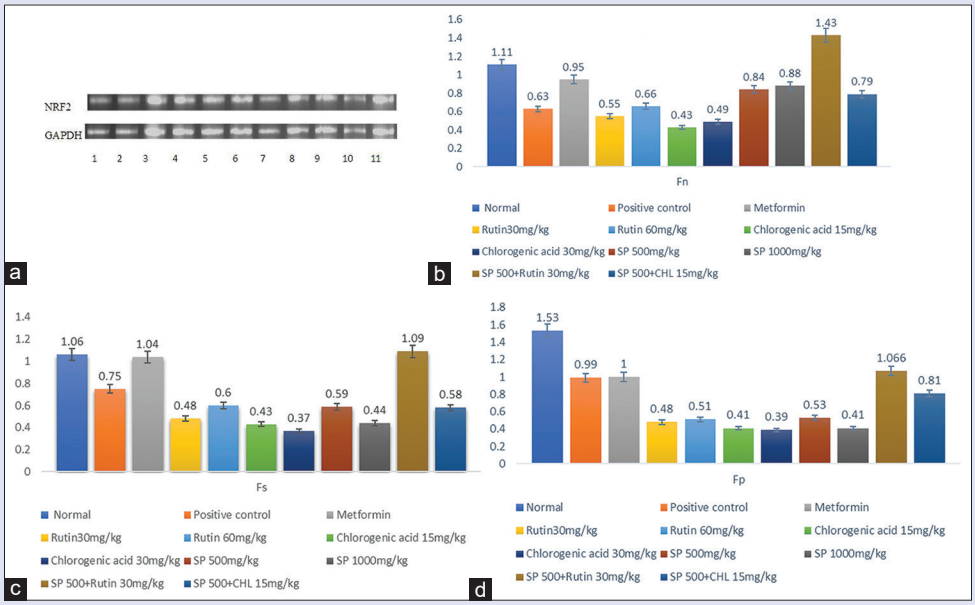

Figure 1: (a) Gel Picture of NRF2 gene of rat heart tissue (b) Graph depicting fold changes in various groups after treatment with *Spirulina*, rutin and chlorogenic acid when compared to normal (c) Graph depicting fold changes in various groups after treatment with *Spirulina*, rutin and chlorogenic acid when compared to standard (d) Graph depicting fold changes in various groups after treatment with *Spirulina*, rutin and chlorogenic acid when compared to Positive

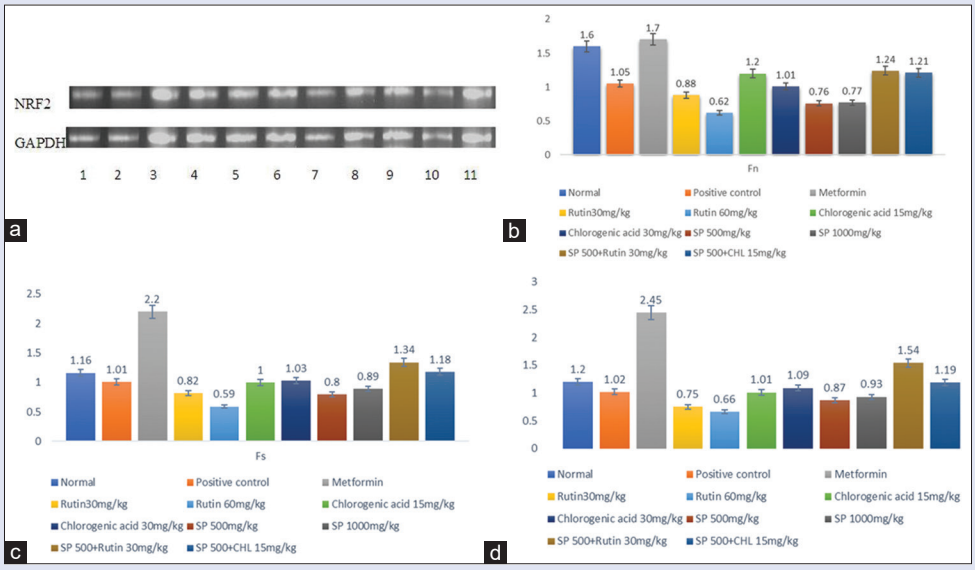

Figure 2: (a) Gel Picture of inducible nitric oxide synthase gene of rat heart tissue, (b) Graph depicting fold changes in various groups after treatment with *Spirulina*, rutin and chlorogenic acid when compared to normal (c) Graph depicting fold changes in various groups after treatment with *Spirulina*, rutin and chlorogenic acid when compared to standard (d) Graph depicting fold changes in various groups after treatment with *Spirulina*, rutin and chlorogenic acid when compared to positive

deformities due to loss of myofibrillar, cytoplasmic vacuolization, and development of interstitial fibrosis and also observed unsystematic collagen structure in the myocardium tissue, along with the perivascular and interstitial spaces. However, following combined treatment with *Spirulina fusiformis* with rutin and *Spirulina fusiformis* with chlorogenic acid evidently improved the fibrotic structure in the heart. Maximum recovery was observed in *Spirulina fusiformis* treated group at both doses and in combination with chlorogenic acid-treated group. The myocardial architecture was improved in degenerative changes as nuclei appeared in uniform shape were noted in *Spirulina fusiformis* with chlorogenic acid.

Histological of liver

In the histological [Figure 5] investigation of the present study, the hepatic tissue of the normal control rats arranged well mannered. The hepatic cell has prominent nucleoli with acidophilic cytoplasm with euchromatic vesicular nuclei. The thin-walled sinusoids arranged by flat endothelial cells with clear Von Kupfer cells nuclei. The histological

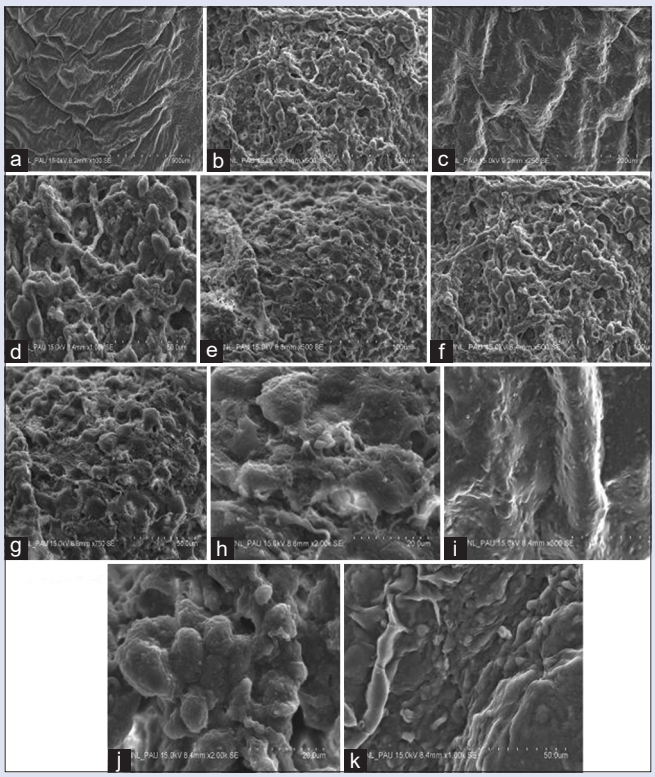

Figure 3: Scanning electronic microscopy of heart tissue in different groups. (a) Normal Control, (b) Positive control, (c) Metformin group (d) Rutin (30 mg/kg) (e) Rutin (60 mg/kg (f) Chlorogenic (15 mg/kg) (g) Chlorogenic (30 mg/kg) (h) *Spirulina plantensis* (SP) (500 mg/kg) (i) *Spirulina plantensis* (SP) (1000 mg/kg) (j) *Spirulina plantensis* (SP) (500 mg/kg) + Rutin (30 mg/kg) (k) *Spirulina plantensis* (SP) (500 mg/kg) + Chlorogenic (15 mg/kg)

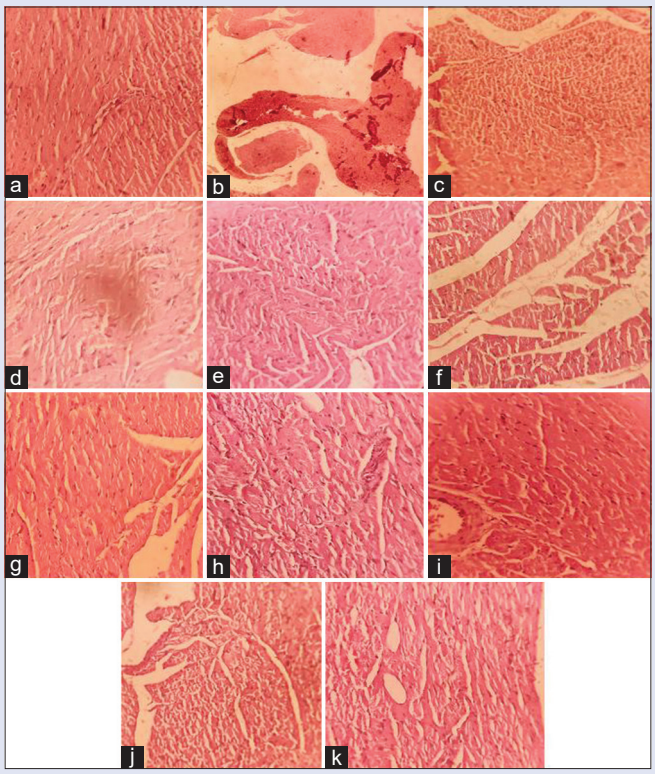

Figure 4: Histology of rat heart tissues. (a) Normal Control, (b) Positive control, (c) Metformin group (d) Rutin (30 mg/kg) (e) Rutin (60 mg/kg (f) Chlorogenic (15 mg/kg) (g) Chlorogenic (30 mg/kg) (h) *Spirulina plantensis* (SP) (500 mg/kg) (i) *Spirulina plantensis* (SP) (1000 mg/kg) (j) *Spirulina plantensis* (SP) (500 mg/kg) + Rutin (30 mg/kg) (k) *Spirulina plantensis* (SP) (500 mg/kg) + Chlorogenic (15 mg/kg)

examination of diabetic rat's hepatic tissue [Figure 5b] showed deeply stained nuclei with vacuolated hepatocytes and leucocytic aggregation. Hepatic necrosis also appeared in some regions. The liver sections of the group treated with *Spirulina fusiformis,* rutin and chlorogenic acid at individual doses [Figure 5d-i] showed disarrangement of the architecture of the hepatic tissue, cell boundaries are lost, degeneration of the hepatocytes and dark stain nuclei has been observed. Interestingly, the liver tissue of the group received treatment with *Spirulina fusiformis* and rutin at the dose of 500 mg/kg and 30 mg/kg revealed normal hepatic ultrastructure with axially arranged hepatocytes towards the central vein. Von Kupfer cells lining sinusoidal spaces were well prominent. Hepatocytes appeared active and healthy vesicular nuclei same as those observed in the normal control group. Both the combination-treated group showed almost normal hepatic structure with well-arranged von Kupfer cells. Evidently, the combined treatment of *Spirulina fusiformis* with rutin and chlorogenic acid demonstrated protective effect against LPS induced sepsis in diabetic rats.

Histological of kidney

The histopathology of renal tissues in normal control rats observed a normal structure of the renal cortex and medulla [Figure 6]. The simple squamous epithelium cells clearly lined the Bowman's capsule. The inner wall of the Bowman's capsule was covered by bunch of capillaries and lined by cuboidal epithelium cells. Prominent blood capillaries were seen in tubules. The section from the positive control diabetic rats showed glomerular capillaries atrophy with deformed renal cells, unstained degenerated cytoplasmic regions were seen in renal tubules. Sections from the renal tissue from the treatment group with *Spirulina fusiformis* with rutin showed marked protection against diabetic changes. They look quite similar with the normal control group. The groups received individual doses of *Spirulina fusiformis*, rutin and chlorogenic acid showed mild proteinuria and slightly improved in renal tubules epithelium cells. Excellent improvement was noted in combined treatment which has the potential to ameliorate diabetic nephropathy.

DISCUSSION

Septicemia is a popular disease with growing morbidity around the world. Chances of sepsis and inflammation are more in critically ill patients resulting from bacterial infection.[36] It is well known that acute infections lead to uncontrolled blood glucose levels in diabetic Mellitus patients.[6] Ketoacidosis is one of the factors to induce infection at faster rate in diabetes mellitus. There are several markers like pro-inflammatory cytokines which are freely circulated in the body targeted aggressively on the organs. Free radicals and other reactive oxygen species are also participated in many diseases include cancer, diabetes mellitus, liver injury, infections and immune deficiency diseases.[37] Prevention and management of severe infection in diabetic patients remain unsuccessful with current drug therapy which may show adverse effects. Lipopolysaccharide originates from Gram-negative bacteria. It binds to the CD14 receptor with LPS-binding protein after entering the mammalian bloodstream. As a result, a nuclear factor-κB

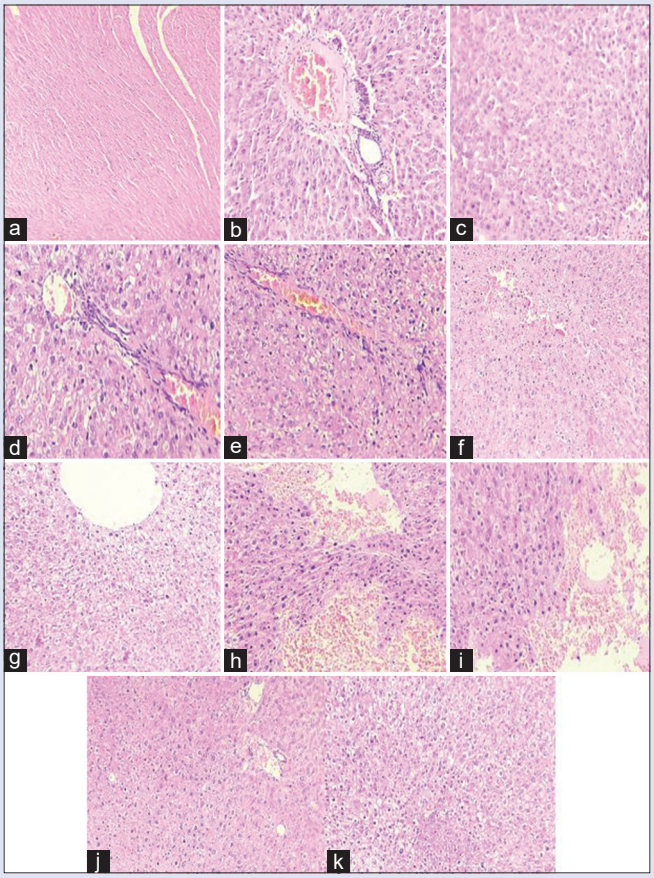

Figure 5: Histopathology of Liver. (a) Normal Control, (b) Positive control, (c) Metformin group (d) Rutin (30 mg/kg) (e) Rutin (60 mg/kg (f) Chlorogenic (15 mg/kg) (g) Chlorogenic (30 mg/kg) (h) *Spirulina plantensis* (SP) (500 mg/kg) (i) *Spirulina plantensis* (SP) (1000 mg/kg) (j) *Spirulina plantensis* (SP) (500 mg/kg) + Rutin (30 mg/kg) (k) *Spirulina plantensis* (SP) (500 mg/kg) + Chlorogenic (15 mg/kg)

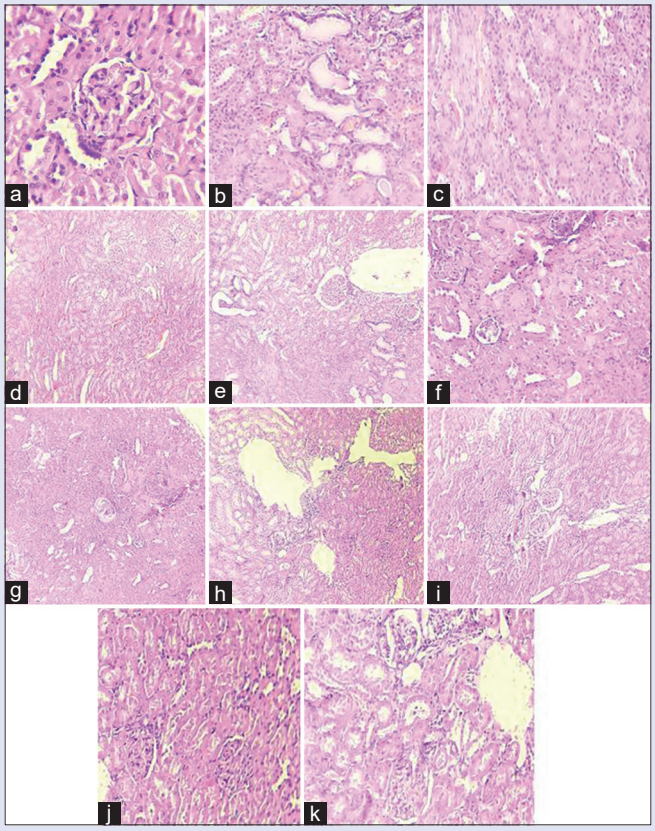

Figure 6: Histopathology of Kidney. (a) Normal Control, (b) Positive control, (c) Metformin group (d) Rutin (30 mg/kg) (e) Rutin (60 mg/kg (f) Chlorogenic (15 mg/kg) (g) Chlorogenic (30 mg/kg) (h) *Spirulina plantensis* (SP) (500 mg/kg) (i) *Spirulina plantensis* (SP) (1000 mg/kg) (j) *Spirulina plantensis* (SP) (500 mg/kg) + Rutin (30 mg/kg) (k) *Spirulina plantensis* (SP) (500 mg/kg) + Chlorogenic (15 mg/kg)

is activated to induce monocytes, macrophages, and endothelial cells to release cytokines such as TNF-α and causes tissue damage.[38] In the other side, hyperglycemia also favors though the activation of NF-κB, an increased expression of iNOS, which is accompanied by increased generation of nitric oxide. Nitric oxide can react with superoxide to produce the strong oxidant peroxynitrite, which in turn can increase lipid peroxidation, protein nitration and LDL oxidation and many signal transduction pathways.[39,40]

STZ is a chemical agent which induced diabetes in rats via the destruction of the pancreas resulting shortage of insulin. It boosts ATP dephosphorylation, which in turn generates superoxide anions, hydrogen peroxide, and hydroxyl radicals. This leads to an elevation in the intracellular peroxides in pancreatic islets which may induce damage due to the reactive oxygen species.[41]

Inflammation markers are produced by LPS in STZ induced type 2 diabetes mellitus rat model. In our study, we postulate that inflammatory markers augmented in type 2 diabetes mellitus rats after induced by LPS. The results were found to be statistically significant ($P < 0.001$) in combination therapy as compared to individual therapy. Two combinations of *Spirulina fusiformis* with rutin and S*pirulina fusiformis* with chlorogenic acid showed decreased in the highest percentage of blood glucose levels throughout different intervals. Interesting results were obtained at 21st and 45th day, similar percentage of blood glucose levels were noted in both the groups (*Spirulina fusiformis* with chlorogenic acid combination and metformin-treated group).

Our results were supported by another study conducted on diabetic mice which showed decreased in glucose levels and improvement in liver enzymes.[2,42] BWs drastically decreased in the positive control group at all intervals (7th to 45th day). In diabetes, body cells are unable to utilize glucose and proteins are instead used as energy sources. This leads to a protein deficiency and reduces BW.[43] The highest percentage improvement in BWs was found in *Spirulina fusiformis* (1000 mg/kg) and *Spirulina fusiformis* (500 mg/kg) with the chlorogenic acid-treated group.

Diabetic Mellitus is associated with various interlinked metabolic disorders, instead of this lipid metabolism is affected by high values of triglycerides and cholesterol and low levels of HDL, as found in various cases.[44] Among lipid profile data on the 45th day, both HDL and LDL was statistically significant reduction as compared to the positive control group but the percentage reduction in triglycerides levels was found exactly the same as noted in metformin-treated group. Similar to the case of insulin levels, it was statistical non-significant reduction when we compared it with the metformin-treated group. Glycosylated hemoglobin is formed through the nonenzymatic binding of circulating glucose to hemoglobin. Higher levels of blood glucose contribute to more binding and consequently increased levels of glycosylated hemoglobin.[45] A Glycylated hemoglobin level was also measured and

about 37% was found in both combination-treated groups as compared to the positive control group. Chronic systemic inflammation contributes to the pathogenesis of hypertension.[46] In case of blood pressure levels, systolic and diastolic levels were reduced in significantly at the 45th day. About 10% to 13% reduced in both systolic and diastolic blood pressure levels. In the metformin-treated group, 12% was reduced in diastolic blood pressure level and 9% measured in systolic blood pressure level.

The present study showed that the TNF-α was significantly lowered in STZ-induced diabetic rat compared to the normal rats. It has been reported that neither INF-γ nor TNF-α alone induces β-cell death under *in vitro* condition.[47] TNF-α and IL-6 level were found to be statistically significant in both combination-treated groups ($P < 0.001$) on 45th day as compared to the positive control group. Slightly significant change in individual Chlorogenic acid-treated group ($P < 0.05$) as compared to positive control group. Similar results were reported conducted by Liu *et al.* 2019.[48] at the dose of 100 μM of rutin which decreased IL-6 level and results were dose-dependent manner. Another study also supported similar results.[49] Increased concentration of antioxidant enzymes protected stress-induced infection by LPS. Two studies were conducted by Rakshit *et al.* 2021 and Xianchu *et al.* 2018[49,50] and supported our results of antioxidant and antihypertensive of rutin and one of the studies reported the effect of Chlorogenic acid on LPS-induced nitric oxide and interleukin factors.[51]

Herbal or ayurvedic formulas have multi plants constituents which can give positive and negative effects at later stage. The limitation of the ayurvedic formulation is unexplored toxicity studies and without approval, it comes in the market also through ayurvedic practitioners. We choose only two active known compounds have pharmacological properties and one full source of nutraceutical compound have multipotential pharmacological effect.

In our study, the results were found excellent at the specific treated groups in combination as compared to individual therapy. It was also noted that the effect varies at different biochemical parameters. Histological evidence also supported by other study which proved that Chlorogenic acid and rutin showed better results in combination as compared to individual therapy. The significant role of rutin was to inhibited cytokine production. It was also demonstrated that rutin suppressed the activity of pro-inflammatory cytokines by reducing TNF-α and IL1 β production in cells. In addition, rutin as the ability to reduced NF-κB activation which is a key regulator of inflammatory responses through pro-inflammatory genes. It may be possible to direct effect as antioxidant activity as free radical scavengers and singlet oxygen quenchers. The probable mechanism of flavonoids is to suppression of ROS formation by either inhibition of enzymes involved in the production, scavenging of ROS, and upregulation or protection of antioxidant defenses.[52] Chlorogenic acid may also show the effect *via* inhibition of phosphor-IκB expression and NF-κB p65 activity.[53]

CONCLUSION

Finally, it is concluded that both combinations of drug-treated group showed excellent results, it may give to diabetic patients for the management of inflammation-induced diabetes. Still, the study needs more specific molecular mechanism for final conclusive data for further clinical trials. The results of this study definitely proved as a reference material and can be treated as the preclinical report.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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