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ORIGINAL ARTICLE

Ameliorative Effect of *Tinospora tuberculata* in Insulin Resistance: Potential Roles of Oxidative Stress Resistance and Heat Shock Protein 70 (Hsp 70) Modulation

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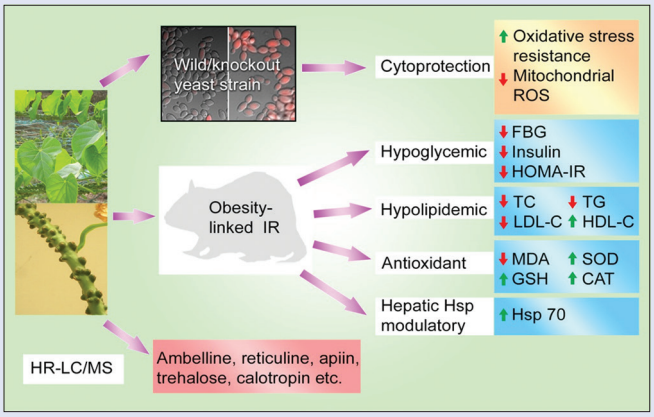
ABSTRACT

Background: *Tinospora tuberculata* Bumee is a climbing vine distributed throughout the South Asian countries and it is known to be a hypoglycaemic agent. However, its role in cytoprotection and cellular oxidation is unclear. In the current investigation, cytoprotection against cellular stress and heat shock protein 70 (Hsp 70) modulation was investigated to understand the beneficial effect of butanolic fraction of *T. tuberculata* (TTB) in hyperglycemia and hyperlipidemia. **Materials and Methods:** The cytoprotective effect of TTB was studied on wild and knock-out yeast strain (Δtrx2). The effect of TTB on cell viability and the level of mitochondrial superoxide were determined to assess the antioxidant protection against reactive oxygen species assault. IR was induced in Wistar rats by high-fat diet and a low dose of streptozotocin. Hyperglycemic rats were orally treated with TTB (50 and 100 mg/kg body weight) or metformin (200 mg/kg b.w.) daily for 2 weeks. The effect of TTB on different parameters such as body weight, glucose levels, insulin resistance, glucose tolerance, lipid profile, antioxidant status, and hepatic Hsp 70 was analyzed in IR rats. TTB was characterized using high-resolution liquid chromatography-mass spectrometer (HR-LC/MS). **Results:** TTB demonstrated significant protection against the oxidative damage caused by H_2O_2 in both wild and Δtrx2 strains. In obese rats, TTB treatment reduced insulin resistance, improved glycaemic status, glucose tolerance, lipid profile, antioxidant level, and upregulated hepatic Hsp 70. HR-LC/MS analysis of TTB showed the presence of isoquinoline alkaloids (ambelline and reticuline), flavonoid glycoside (apiin), disaccharide carbohydrate (trehalose), cardenolide (calotropin), etc., **Conclusion:** The positive effect of the TTB on IR is attributed to its cytoprotective action against cellular stress and the activation of hepatic Hsp 70.

Key words: Antioxidant, diabetes, Hsp 70, *Tinospora crispa*, yeast

SUMMARY

- In the present study, cytoprotection against cellular stress and Hsp 70 modulation was investigated to understand the beneficial effect of butanolic fraction of *T. tuberculata* (TTB) in hyperglycemia and hyperlipidemia.
- TTB has a positive impact on all the parameters of IR in rats, such as body weight, insulin resistance, glucose levels, glucose tolerance, lipid profile, oxidative stress marker, and hepatic Hsp 70.
- The activity of TTB against IR and metabolic syndrome may be related to its cytoprotective effect by countering the oxidative stress and inducing hepatic Hsp 70.



Abbreviations used: IR: Insulin resistance; T2D: Type 2 diabetes mellitus; Hsp: Heat shock protein; STZ: Streptozotocin; TTB: Butanolic fraction of *Tinospora tuberculata*; DPPH: 1,1-diphenyl-2-picrylhydrazyl radical; YPD: yeast extract peptone dextrose; TRX2: Thioredoxin genes; ROS: Reactive oxygen species; WT: Wild type; Δtrx2: TRX2 gene knockout yeast strain; H_2O_2: Hydrogen peroxide; OD_{600}: Optical density at 600 nm; CFU: Colony-forming units; HFD: high-fat diet; b.w.: Body weight; NC: normal control group; HFD-C: High-fat diet control group, MET: Metformin treated group; TTB1: TTB treated at 50 mg/kg b.w.; TTB2: TTB treated at 100 mg/kg b.w.; OGTT: Oral glucose tolerance test; AUC: area under the curve; ELISA: Enzyme-linked immunosorbent assay; HOMA-IR: Homeostasis model assessment of insulin resistance; TC: Total cholesterol; TG: Triglyceride; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol; MDA: malondialdehyde; TBA: Thiobarbituric acid; SOD: superoxide dismutase; CAT: Catalase; GSH: reduced glutathione; DTNB: 5, 5′-dithiobis (2-nitrobenzoic acid); UHPLC: Ultrahigh-performance liquid chromatography; MS: Mass spectrometer; HR-LC/MS: High-resolution liquid chromatography-mass spectrometer; Q-TOF: Quadrupole time-of-flight.

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INTRODUCTION

Insulin resistance (IR) is characterized by a systemic disruption of insulin and glucose homeostasis. This results in abnormalities in carbohydrate, fat, and protein metabolism, which eventually develop into type II diabetes (T2D). In a recent study, it was estimated that 415 million adult people (aged 20–79 years) worldwide suffer from diabetes and it is predicted that the total number will exceed 642 million by 2040.[1] Lifestyle factors, such as high-fat calorie intake and physical inactivity, lead to obesity, which is commonly associated with a state of IR. There is a strong causative link between obesity, chronic low-grade inflammation, and IR. Prolonged hyperinsulinemia-associated metabolic stress promotes the generation of Reactive oxygen species (ROS), which is a key triggering factor in the progression of metabolic syndrome. The focal point in the development and progression of IR is the mitochondrial superoxide radical, which drives the process of insulin insensitivity.[2]

One of the consequences of persistent low-grade inflammation is cellular stress which is associated with protein aggregation and misfolding. The build-up of these misfolded proteins drives the pathogenesis of T2D, which is thought to be a disorder of protein denaturation.[3] Heat shock proteins (Hsp) are a family of stress-responsive proteins, which repair damaged biomolecules during cellular stress and maintain proteostasis. Hsp 70 is one among 12 genes that are found to have significantly lower expression in diabetic subjects and low expression of Hsp 70 is inversely correlated to blood glucose level.[4] The low expression of Hsp makes tissue vulnerable to stress during T2D. Pharmacological and nonpharmacological methods to induce Hsp has shown a beneficial effect on T2D due to their cytoprotective functions.[5] Resveratrol and curcumin are examples of antioxidant phytochemicals that induce the expression of Hsp70 and have a positive effect on IR and diabetes.[6,7]

Tinospora tuberculata Bumee, syn. *Tinospora crispa*, *Tinospora rumphii* (family *Menispermaceae*) a climbing vine distributed throughout the South Asian countries such as Malaysia, Thailand, and the Philippines, while in India, it is restricted to Assam and Kerala. Stems of the plant are conventionally used for various diseases such as diabetes, ulcers, fever, malaria, hypertension, etc., The major active constituents isolated from *T. tuberculata* are furanoditerpenes, lignans, flavonoids, alkaloids, and steroids.[8,9] The beneficial effect of *T. tuberculata* on cytoprotection, cellular stress, and heat shock protein 70 (Hsp 70) modulation in obesity-induced IR is not known. In the present study, the butanolic fraction of *T. tuberculata* (TTB) was used to investigate its effect on hyperglycemia and hyperlipidemia in obese IR rat model. The hepatic Hsp 70 was evaluated as an attempt to understand the mechanism of action of the drug. The antioxidant property of the extract was investigated by a cell-based assay using wild type (WT) and null mutant yeast. Yeast cells (*Saccharomyces cerevisiae*) were used as a biological screening system to study the antioxidant capacity of compounds in which the oxidant-induced growth arrest response of yeast was measured in high throughput, 96-well microtiter plate.[10] The mitochondrial oxidative stress is thought to be the focal point in the development of IR.[2] Therefore, a reduction in mitochondrial oxidative stress in the yeast model was also monitored due to the role of superoxide free radicals in the pathogenesis of IR.

MATERIALS AND METHODS

Plant materials and preparation of the extract

The stem of *T. tuberculata* was collected from Kottayam, Kerala, which was authenticated by Dr. Peter K. Mani, Department of Botany, B.C.M. College, Kottayam, Kerala and the specimen was deposited in the herbarium (Voucher number 437/2015). The shade-dried plant materials were ground to a coarse powder (500 g) and extracted with methanol (2.0 × 3 L), at room temperature, the combined extract was evaporated under reduced pressure to yield a viscous residue (41.6 g). It was dissolved in distilled water and successively partitioned with diethyl ether and n-BuOH each three times. The ether fraction (7.1 g), butanolic fraction (17.8 g), and the aqueous residual layer were evaporated under reduced pressure (40°C). A pilot study was carried out with the three fractions to screen the antioxidant potential, by *in vitro* methods (1,1-diphenyl-2-picrylhydrazyl radical assay and reducing power method) and butanolic fraction (TTB) showed a better activity and selected for further study (data not shown here).

Chemicals and reagents

YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L dextrose) was procured from Difco Laboratories (Detroit, Michigan) and hydrogen peroxide (H_2O_2) from Merck (Mumbai, India). Milli-Q water was used in all yeast-based assays. The yeast strains (WT, Δtrx2) were purchased from Open Biosystems (Thermo Fisher Scientific Inc.). MitoSOX Red (Molecular Probes, Invitrogen, USA), streptozotocin (STZ) 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), thiobarbituric acid (TBA), were purchased from Sigma Aldrich Chemical Co., Ltd. (St. Louis, USA). All the other reagents used were of analytical reagent grade obtained commercially.

Yeast strains and optimization of assay conditions

The WT strains of *S. cerevisiae*, BY4743 (MATa/MATα his3/his3 leu2/leu2 met15/MET15 LYS2/lys2 ura3/ura3) and its null mutant Δtrx2 (*TRX2* gene knockout), were selected for the antioxidant screening assay. The stressing agent, H_2O_2, at different concentrations (1 mM, 2 mM, 4 mM, and 6 mM) was tested and it was found that 4 mM gave the optimum stressing effect, on selected strains. *S. cerevisiae* (WT) was treated with different concentrations of the TTB in triplicate (0.4, 0.8, 1.6 mg/ml) and it was found to be non-toxic to yeast.

Growth curve assay

A colony of WT and Δtrx2 yeast strains were inoculated in a liquid YPD media. The inoculum was allowed an overnight incubation at 28°C, 180 rpm and finally diluted appropriately to get an OD_{600} ~ 0.1 in a flat-bottom 96-well plate (CytoOne). Different concentrations of TTB (0.4, 0.8, 1.6 mg/ml) and ascorbic acid (10 mM) were added (10 µL) into 100µL of WT/Δtrx2 yeast culture, in 96 well plate, in duplicate. Finally, 10 µL of H_2O_2 (4 mM) was added to all the wells, except the control and incubated (28°C) in Eon microplate reader (BioTek, USA) with moderate shaking for 24 h. OD_{600} measurements were automatically recorded every 30 min. The YPD media was used as blank.[10]

Viability assay

The cultures of wild and deletion strain (WT and Δtrx2) of yeast at mid-log phase (OD_{600} nm ~ 0.6) were divided into four groups. All the groups, except the control, were added with stressing agent H_2O_2 (4 mM). Further, ascorbic acid (10 mM) and TTB (1.6 mg/ml) were added to corresponding groups. All the groups were incubated (28°C) in dark for 3 h. Finally, the harvested cells were suspended in water, which was diluted (×100) and spread (20 µl) on the YPD agar plate in triplicate. The plates were incubated at 28°C for 48 h and the colonies formed were counted and expressed as the colony-forming units (CFU). The CFU in each plate was determined and the percentage viability was calculated with respect to the control plate (untreated cells), which was considered as 100%.[11]

Estimation of mitochondrial reactive oxygen species

The level of mitochondrial ROS was measured using the fluorogenic dye, MitoSOX™ Red reagent. The WT and Δtrx2 cultures were stressed with 4 mM H_2O_2 (except the control). Further, the test groups were treated with TTB (1.6mg/ml) or ascorbic acid (10 mM). All the tubes were incubated for 3 h at 28°C, 180 rpm. The cells were harvested (12000 rpm, 30 s) washed, suspended and mixed with 0.2 µl of MitoSOX Red from the stock (5 mM), to get a final concentration of 5 µM. After 20 min incubation in dark, the cells were harvested, washed, stained, and visualized under a fluorescence microscope (Zeiss Apotome) at 100 X.

Animal study

Healthy Wistar rats of either sex (150–200 g) were used in this study. The animals were housed under controlled conditions of temperature (25°C ± 2°C) and humidity (45%–60%) on a 12-h light/12-h dark cycle. All the animals were in free access to food and water. This study was conducted with the approval of the Institutional Animal Ethical Committee (CPCSEA protocol no. PH/IAEC/VNS/2K13/011). After acclimatization for 1 week, six randomly separated rats were fed regular laboratory chow and kept as the normal control (NC) group (Group I). The remaining rats were allowed free accesses of prepared high-fat diet (HFD) containing 58% fat, 25% protein, and 17% carbohydrate, as a percentage of total kcal. The composition of HFD is described elsewhere.[12] After 6 weeks of modified food regimen of HFD, the rats were fasted overnight and administered a single injection of STZ (35 mg/kg b.w., i.p.) dissolved in freshly prepared citrate buffer (0.1 M, pH 4.5).[13] One week after STZ injection, the fasting blood glucose was measured and rats with glucose levels >160 mg/dl were selected for subsequent studies. The rats were subdivided into four groups (Group II–Group V). Group II–Group V was taken as high-fat diet control (HFD-C), metformin treated at 200 mg/kg b.w. (MET), TTB treated at 50 mg/kg b.w. (TTB1) and TTB treated at 100 mg/kg b.w. (TTB2), respectively. The doses of TTB were chosen according to the previous study, at which no signs of toxicity were observed.[14] All the groups receive their respective diet regimen until the completion of the study.

Oral glucose tolerance test

Oral glucose tolerance test (OGTT) was performed at the end of the study period. The overnight fasted animals in all groups were challenged with an oral glucose load (2 g/kg b.w.) and blood samples were collected from the tail vein at 0, 30, 60, and 120 min after glucose administration. The fasting blood glucose levels were measured using the blood glucometer. The area under the curve (AUC) was calculated by the trapezoidal rule.

Preparation of serum and tissue homogenates

After the treatment period, blood samples were collected from the eye pit of the overnight fasted rats after mild anesthesia. Samples were centrifuged at 3000 rpm for 10 min to separate the serum, which was then stored at −80°C to estimate biochemical parameters. After collecting the blood, rats were euthanized, and the liver was dissected out and washed in ice-cold saline solution. A weighed portion of tissues was homogenized in cold phosphate buffer (0.05 M, pH 7.4) to prepare 10% w/v homogenate. The homogenates were centrifuged at 12000 rpm for 10 min at 4°C and the supernatant was used for the estimation of hepatic oxidative stress and Hsp 70. The protein content of liver homogenate was measured.[15]

Estimation of blood glucose and insulin

The fasting blood glucose level was measured using a one-touch glucometer (Accu-Chek Active, India). Serum insulin was determined according to the manufacturer's protocol using a commercial rat ELISA kit (Ray Biotech, Inc., Norcross, GA, USA). Furthermore, IR was assessed by the homeostasis model assessment for insulin resistance (HOMA-IR) and the following formula was used.[16]

HOMA-IR = Serum Insulin (µU/ml) × Serum glucose (mg/dl)/405.

Estimation of lipid profile

Total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were estimated with colorimetric kits procured from Span Diagnostic (Surat, India) following the manufacturer's instructions, using biochemical analyzer (Star - 21 Plus autoanalyzer, Rapid Diagnostics Pvt. Ltd., New Delhi).

Antioxidant status and oxidative stress biomarkers

Determination of malondialdehyde (MDA), the end product of lipid peroxidation, was done based on its colorimetric reaction with TBA reagent and expressed as nmol/mg protein.[17] The superoxide dismutase (SOD) activity was determined by the rate of reduction of nitro blue tetrazolium at 560 nm.[18] The catalase (CAT) activity was measured as the rate of decrease in H_2O_2 absorption at 240 nm, as previously described by Aebi 1984.[19] The determination of GSH content was done following Ellman *et al.* 1959.[20] It is based on spectrophotometric measurement (412 nm) of chromogen produced by the reaction of 5,5-dithiobis-2 nitro benzoic acid (DTNB) with compounds containing sulfhydryl groups.

Assay of hepatic Hsp 70

The Hsp70 level in liver homogenates was detected by an ELISA assay. The absorbance was measured at 450 nm in a microplate reader (Multiskan™ FC, Microplate Photometer Thermo Scientific™). The concentration of Hsp70 in the samples was calculated using a standard curve and expressed in ng/mg protein.

Phytochemical characterization by high-resolution liquid chromatography-mass spectrometer

The bioactive fraction was subjected to phytochemical characterization with a high-resolution liquid chromatography-mass spectrometer (HR-LC/MS) system (Q-Exactive Plus Biopharma-High-Resolution Orbitrap, Thermo Fisher, Waltham, MA). The system consists of ultrahigh-performance liquid chromatography (UHPLC) column, Hypersil GOLD C_{18} (100X2.1 mm, 3 µm) (Thermo Fischer Scientific Pvt. Ltd.), maintained at a column temperature of 40°C. The analysis was done by gradient elution of mobile phase, which comprises of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), at a flow rate of 0.3 ml/min, over a period of 25 min. Following sample injection (5µl), an Agilent quadrupole time-of-flight (Q-TOF) mass spectrometer with an electrospray ionization source operating in both positive/negative mode was used as the detector. MS analyses were performed using a full scan mode of mass range 150-1000 *m/z*. The data was acquired in Agilent Mass hunter Data Acquisition software (Version B.05.00) and METLIN database.

Statistical analysis

The statistical significance between groups was performed by one-way analysis of variance followed by Dunnett's test (for yeast-based assays) or Tukey's multiple comparison test (for animal study data)

using GraphPad. $P < 0.05$ were considered significant. The statistical analysis of the results was performed using Graph Pad InStat, 3.06 programs (Graphpad Software Inc., San Diego, CA).

RESULTS

Growth curve assay

The growth arrest induced by H_2O_2 was halted by TTB (1.6 mg/ml), in both the WT and Δtrx2 strain. Even though the TTB treated cells demonstrated a lag phase initially, after 6 h, it was able to overcome the H_2O_2 induced stress and the recovered cells showed a normal exponential phase, similar to control. There was a marked reduction in the yeast cell population in H_2O_2 treated culture of both the strains, compared to the control group. Similar to the standard (ascorbic acid), TTB (1.6 mg/ml) treated cultures of both strains, significantly ($p < 0.01$) overcome the growth inhibitory effect of the H_2O_2 after 12 h incubation, meanwhile lower concentration of TTB (0.4 and 0.8 mg/ml) failed to revert the H_2O_2 induced growth arrest [Figure 1].

Viability assay

On exposure to H_2O_2, the viability of untreated WT cells was reduced significantly ($p < 0.01$, 29.91% ± 3.81%). Meanwhile, the cells pretreated with TTB and ascorbic acid exhibit viability of 58.57% ± 4.15% and 68.62% ± 3.52%, respectively, demonstrating the protective effect against H_2O_2 challenge. Similarly, cells of Δtrx2 strain showed 23.35% ± 3.48% viability in the H_2O_2 group, while ascorbic acid (64.35% ± 2.25%) and TTB (53.91% ± 4.03%) treatment significantly ($P < 0.01$) improved the cell viability [Figure 2].

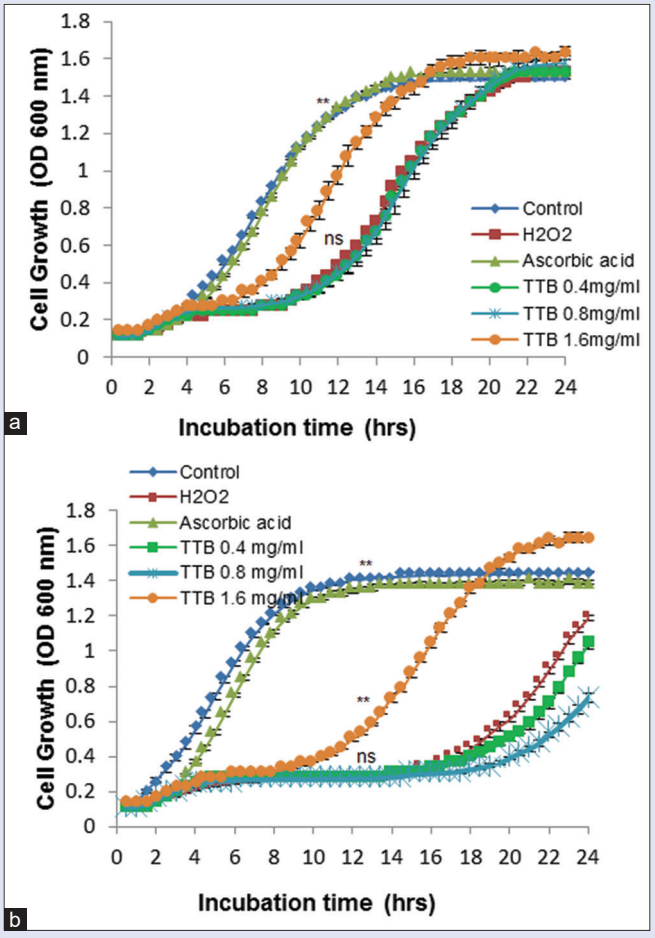

Figure 1: (a) Exponential growth curve for WT yeast strains; (b) Exponential growth curve for Δtrx2 yeast strains (**$P < 0.01$, ns = not significant ($P > 0.05$) compared to H_2O_2 group)

Mitochondrial oxidative stress

The cellular fluorescence indicates the presence of mitochondrial superoxide due to oxidative stress. The number of fluorescent cells was significantly lower in TTB (5.2% ± 1.2%) and ascorbic acid-treated WT yeast cells (6.9 ± 0.98%) in comparison to H_2O_2 treated group (22.7% ± 1.27%). As expected, Δtrx2 strain was more severely affected by H_2O_2 induced oxidative stress and resulted in 33.3% ± 3.9% of cells being florescent. Ascorbic acid was able to reduce the number of fluorescent cells by three times (11.75% ± 1.8%) and TTB by four times (8.35% ± 1.73%) compared to the negative control [Figure 3].

Bodyweight

Bodyweight of rats fed with HFD showed a gradual increase, as compared to the rats of NC group. However, after the treatment period, the mean body weight of MET, TTB1 and TTB2 groups showed a significant ($P < 0.05$) reduction as compared to HFD-C rats [Figure 4].

Oral glucose tolerance test

In HFD-C group rats, blood glucose levels reach its peak at 30 min after the glucose load and the glucose level was persistent till the end of the 2 h period. At the same time, the NC group, exhibit a hike in the blood glucose level initially but further returned to its basal level after 2 h. In both the treatment group (TTB1 and TTB2), glucose level peaked after 30 min and dropped thereafter, to a near basal level by the end of the 2 h period. OGTT-AUC was increased in the HFD-C group compared to NC group. The AUC in the TTB1 and TTB2 groups were 24165.0 and 22489.5, respectively, which were significantly ($P < 0.05$) lower than that of the HFD-C group [Figure 5].

Blood glucose and insulin

As reported earlier, HFD/STZ treatment resulted in altered glucose metabolism in the HFD-C group, causing a significantly higher ($P < 0.001$) fasting blood glucose levels compared with the NC group. The rats treated with two doses of TTB (50 and 100 mg/kg) for 14 days significantly ($P < 0.05$) reduce the fasting blood glucose level (31.37 and 31.70% respectively) compared to the HFD-C group. A similar effect was observed in the MET group, with a significant ($P < 0.05$) improvement

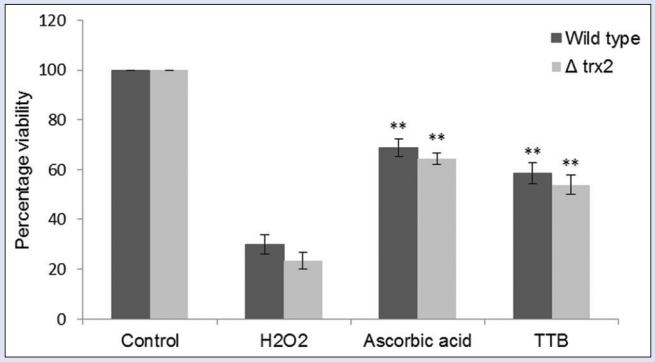

Figure 2: Cell viability assay: The effect of TTB (1.6 mg/ml)/ascorbic acid (10 mM) treatment on WT/Δtrx2 strains under H_2O_2 stress (4 mM). The number of CFUs was used to express the cell viability. The total number of colonies observed on the control plate (untreated cells) was considered as 100% survival (**$P < 0.01$, compared to H_2O_2 group)

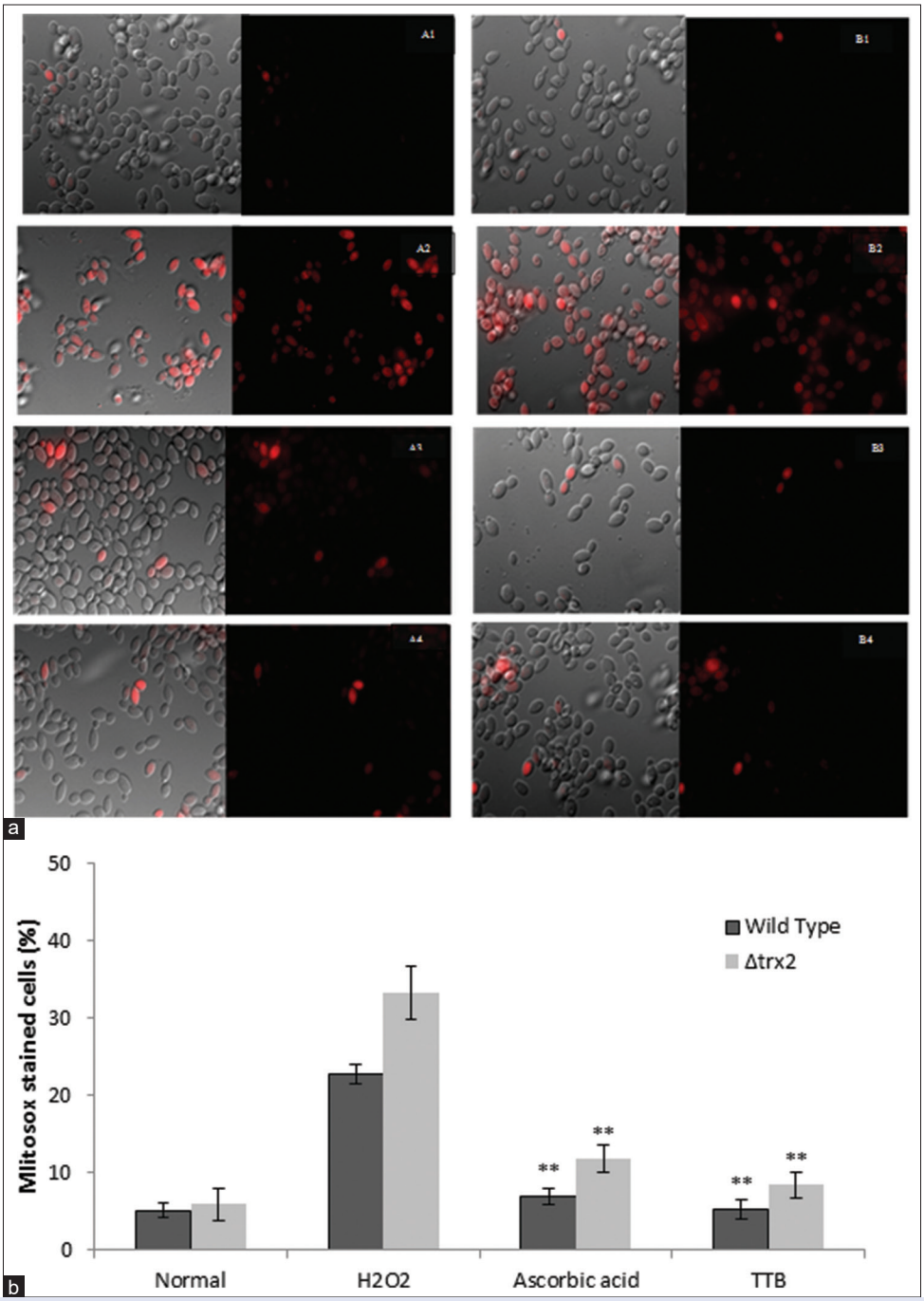

Figure 3: (a) Visualization of Reactive oxygen species produced in WT (A1-A4) and Δtrx2 (B1-B4), stained with MitoSOX. A1&B1: Cells of normal control; A2&B2: Cells after stressed with H_2O_2; A3&B3: Cells pretreated with ascorbic acid and stressed with H_2O_2; A4&B4: Cells pretreated with TTB and stressed with H_2O_2; (b): Percentage of yeast strains (WT and Δtrx2), stained with MitoSOX (**$P < 0.01$ compared to H_2O_2 group)

in the glycaemic status of the animals. At the end of the experiment, rats of the HFD-C group exhibited significant elevation in the serum insulin level compared to the NC group ($P < 0.001$). The disturbed insulin homeostasis was partially corrected in the treatment groups, with 20.9% reduction in the insulin level of TTB1, while TTB2 showed 21.7% reduction, which is comparable with the standard metformin. The IR, as depicted by HOMA-IR, was increased in the HFD-C group when compared with the NC group and it was attenuated by TTB treatment, in a dose-dependent manner [Table 1].

Serum lipid profile

The HFD feeding altered all parameters of serum lipid profile, the results showed that it significantly ($P < 0.001$) raised the levels of TC, TG and, LDL-C, while the level of HDL-C was significantly ($P < 0.01$) lowered. A fall of 17.3% in TC, 24.96% in TG and 16.98% in LDL-C were observed in TTB1 group and also there was 53.66% increase in HDL-C level compared to HFD fed rats. Similarly, TTB2 group showed a reduction of 22.54% in TC, 27.88% in TG and 21.63% in LDL-C and improvement of 58.13% in HDL-C group [Figure 6].

Hepatic oxidative stress parameters and antioxidant markers

After the 8 weeks of the experimental period, HFD causes a trigger in oxidative stress, which was indicated by a significant ($P < 0.01$) increase in hepatic MDA contents (74.66%); and also resulted in depletion of SOD, CAT, and GSH contents by 50.18%, 67.01% and 48.77%, respectively, compared to the NC group. At the same time, the animals in the treatment groups, TTB1 and TTB2, exhibit a marked enhancement in hepatic SOD (66.58% and 71.74%) CAT (92.37 and 110.19%) and GSH (57.63% and 64.84%), respectively, compared to HFD-C group. Moreover, in both the groups, lipid peroxidation was abated and MDA content is found to be comparable to that of the NC group [Table 2].

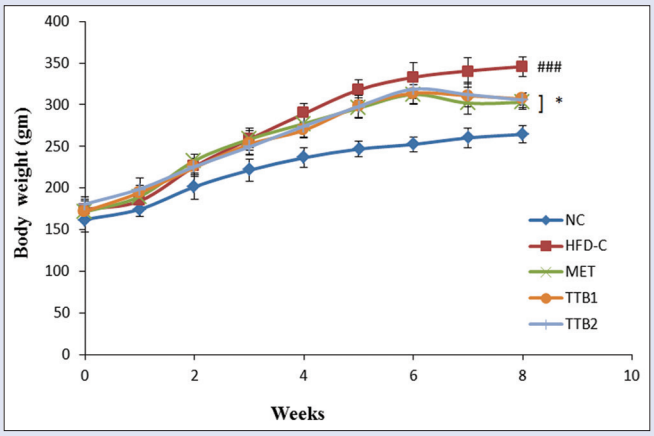

Figure 4: Bodyweight of rats during the study period. Values are presented as the mean ± standard error of mean of 6 rats. ###$P < 0.001$, compared to the NC, *$P < 0.05$, compared to the HFD-C. NC: Normal control, HFD-C: High-fat diet control, MET: Metformin (200 mg/kg), TTB1: TTB treated at 50 mg/kg b.w., TTB2: TTB treated at 100 mg/kg b.w.

Assay of hepatic Hsp 70

Previous studies suggest that agents that upregulate Hsp 70, plays a crucial role in the management of IR/T2D. In this context, we determined the possible effect of TTB in hepatic Hsp 70. The hepatic Hsp 70 in HFD-C rats was significantly ($P < 0.01$) reduced than rats of NC group. Two weeks of treatment with TTB restored the reduced Hsp 70 level in HFD fed rats in a dose-dependent manner. At the same time,

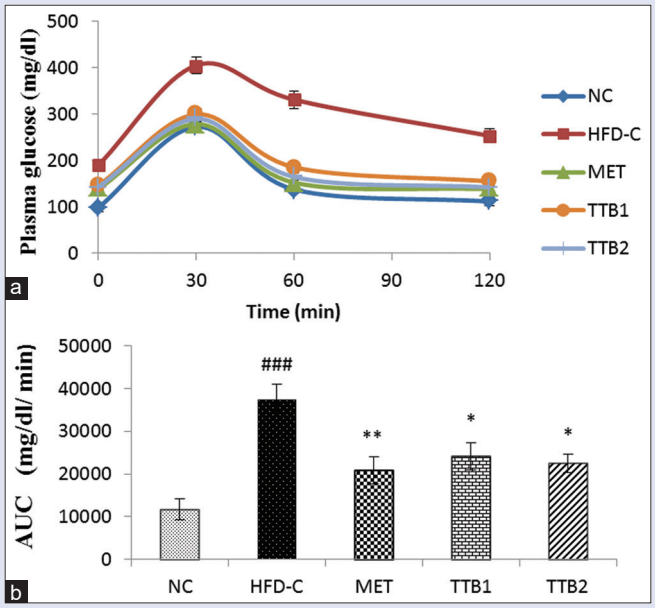

Figure 5: (a) Effects of TTB treatment on OGTT blood glucose levels; (b) The area under the curve of blood glucose levels during OGTT. Data are represent the mean ± standard error of mean. *$P < 0.05$, **$P < 0.01$ compared to the HFD-C, ###$P < 0.001$, compared to the NC. NC: Normal control, HFD-C: High-fat diet control, MET: metformin (200 mg/kg), TTB1: TTB treated at 50 mg/kg b.w., TTB2: TTB treated at 100 mg/kg b.w.

Table 1: Effects *Tinospora tuberculata* on blood glucose, insulin, homeostasis model assessment of insulin resistance index and hepatic heat shock protein 70

	Glucose (mg/dl)	Insulin (µU/ml)	HOMA-IR	Hepatic Hsp 70 (ng/mg protein)
NC	98.1±8.9	38.3±5.11	9.27±2.28	3.31±0.11
HFD-C	189.9±12.63###	70.4±5.78###	32.85±3.93###	1.71±0.17##
MET	138.8±11.37*	47.5±4.87*	16.18±2.42***	1.64±0.41 (NS)
TTB1	145.7±9.58*	49.5±4.06*	17.67±2.56**	2.88±0.34*
TTB2	142.4±8.21*	48.7±5.22*	17.69±2.13**	2.92±0.18*

Values were represented as mean±SEM (n=6). ###P<0.001 compared to NC, *P<0.05, **P<0.01, ***P<0.001, NS (P>0.05) compared to the HFD-C group. NC: Normal control; HFD-C: High-fat diet control; MET: Metformin (200 mg/kg); *T. tuberculata*: *Tinospora tuberculata*; TTB: *T. tuberculata*; TTB1: TTB treated at (50 mg/kg) body weight; TTB2: TTB treated at (100 mg/kg) body weight; HOMO-IR: Homeostasis model assessment of insulin resistance; Hsp 70: Heat shock protein 70; SEM: Standard error of mean; NS: Not significant

Table 2: Effect of *Tinospora tuberculata* on hepatic lipid peroxidation, glutathione, superoxide dismutase and catalase

Groups	LPO (nmol MDA/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	GSH (mM/mg protein)
NC	2.96±0.23	8.17±0.91	38.96±3.431	21.1±2.27
HFD-C	5.17±0.43##	4.07±0.32##	12.85±1.653###	10.81±1.04###
MET	2.98±0.58**	7.13±0.82*	30.75±2.769**	18.57±1.12**
TTB1	3.24±0.22*	6.78±0.34*	24.72±2.521*	17.04±1.49*
TTB2	3.03±0.37**	6.99±0.63*	27.01±3.16*	17.82±1.23*

Values are represented as mean±SEM, (n=6). The oxidative markers of liver were significantly (###P<0.001, ##P<0.01) affected in HFD-C group, compared to NC, while the oxidative markers were significantly (*P<0.05, **P<0.01) improved in MET, TTB1 and TTB2 groups compared to HFD-C group. NC: Normal control; HFD-C: High-fat diet control; MET: Metformin (200 mg/kg); *T. tuberculata: Tinospora tuberculata*; TTB: *T. tuberculata*; TTB1: TTB treated at (50 mg/kg) body weight; TTB2: TTB treated at (100 mg/kg) body weight; LPO: Lipid peroxidation; MDA: Malondialdehyde; SOD: Superoxide dismutase; GSH: Glutathione; CAT: Catalase; SEM: Standard error of mean

improvement in the Hsp 70 level in the MET group was insignificant, compared to HFD-C rats, which indicates a different mechanism of action [Table 1].

Phytochemical characterization by high-resolution liquid chromatography-mass spectrometer

The phytochemical analysis of TTB was carried out by HR-LC/MS analysis, in both the positive and negative mode. From the molecular weight comparison, major compounds identified belong to different classes like heterocyclic isoquinoline alkaloids (ambelline and reticuline), cardenolide (calotropin), flavonoid glycoside (apiin), disaccharide carbohydrate (trehalose). The flavonoid aglycone, apigenin was previously reported in *T. tuberculata*.[21] Reticuline was isolated from an allied species of the plant, *T. sagittata*.[22] The phytoconstituents, predicted from HR-LC/MS analyses having relevant pharmacological activity, are mentioned in Table 3 and their fragmentation pattern is presented in Figure 7.

DISCUSSION

Yeast cells were used as biological systems for assaying antioxidant capacity. In recent years these methods have been widely used for evaluating the antioxidant capacity of many biomolecules like resveratrol, hesperidin.[11,23] In *S. cerevisiae* antioxidant enzymes SOD, CAT, glutathione peroxidase, and non-enzymatic protein such as glutathione and thioredoxins play a crucial role in the maintenance of redox balance. The yeast thioredoxins system comprise mainly isoforms, trx1 (encoded by *TRX1* gene) and TRX2 (encoded by *TRX2* gene), which protects cells against oxidative and reductive stress. Oxidative stress and inflammation cause upregulation of thioredoxin, which has been found to be positively correlated with IR.[24] The seemingly important role played by the *TRX2* gene in IR makes the Δtrx2 an invaluable model for screening the cellular antioxidant activity of the extracts. In the present study, TTB was subjected to different yeast-based assay to elucidate the mechanism of cytoprotection of this fraction on eukaryotic cells.

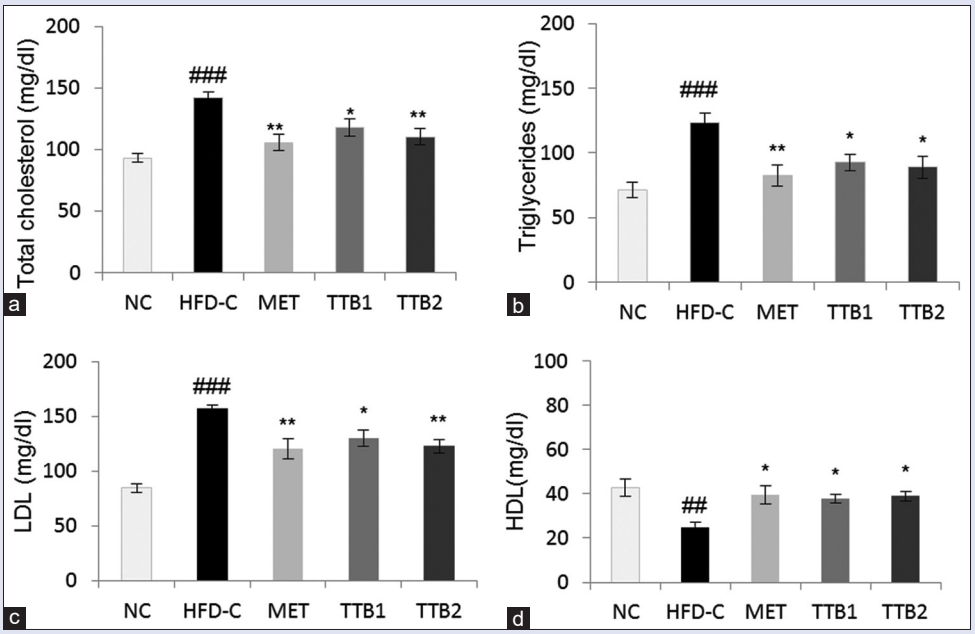

Figure 6: Effects of TTB on serum lipid profile. Each value is expressed as mean ± standard error of mean ($n = 6$). ###$P < 0.001$ when compared to NC, *$P < 0.05$, **$P < 0.01$, when compared to HFD-C. LDL-C: Low-density lipoprotein cholesterol, HDL-C: High-density lipoprotein cholesterol, NC: Normal control, HFD-C: High-fat diet control, MET: Metformin (200 mg/kg), TTB1: TTB treated at 50 mg/kg b.w, TTB2: TTB treated at 100 mg/kg b.w.

Table 3: List of identified compounds of *Tinospora tuberculata* butanolic fraction by liquid chromatography-mass spectrometer/mass spectrometer with reported pharmacological activities

Name of metabolites	Empirical formula	*m/z*	Retention time	Class	Pharmacological activity
Apiin	$C_{26}H_{28}O_{14}$	563.1441	4.958	Flavonoid glycoside	Antioxidant, anti-inflammatory, antidiabetic and anti-obesity[31,34,35]
Ambelline	$C_{18}H_{21}NO_5$	314.1415	3.748	Crinane Amaryllidaceae alkaloid	Antidiabetic[29,30]
Desacetylcolchicine	$C_{20}H_{23}NO_5$	340.1567	4.811	Alkaloid	Hypolipidemic[33]
Hydroxy-3-O-methyl-6β naltrexol	$C_{21}H_{27}NO_5$	356.1891	6.032	Aporphine alkaloids	Antidiabetic and antiobesity[42]
Calotropin	$C_{29}H_{40}O_9$	537.237	8.954	Cardenolide	-
(S)-Reticuline	$C_{19}H_{23}NO_4$	330.173	4.599	Isoqinoline	Antioxidant anti-inflammatory[36]
Trehalose	$C_{12}H_{22}O_{11}$	377.087	1.02	Disacchride	Antidiabetic hypolipedemic[32]
Xylocarpus A	$C_{31}H_{38}O_{11}$	603.1917	7.479	Limonoids	-
Dextrorphan sulphate	$C_{17}H_{23}NO_4S$	338.1415	6.051	Morphinane alkaloid	Antidiabetic[43]

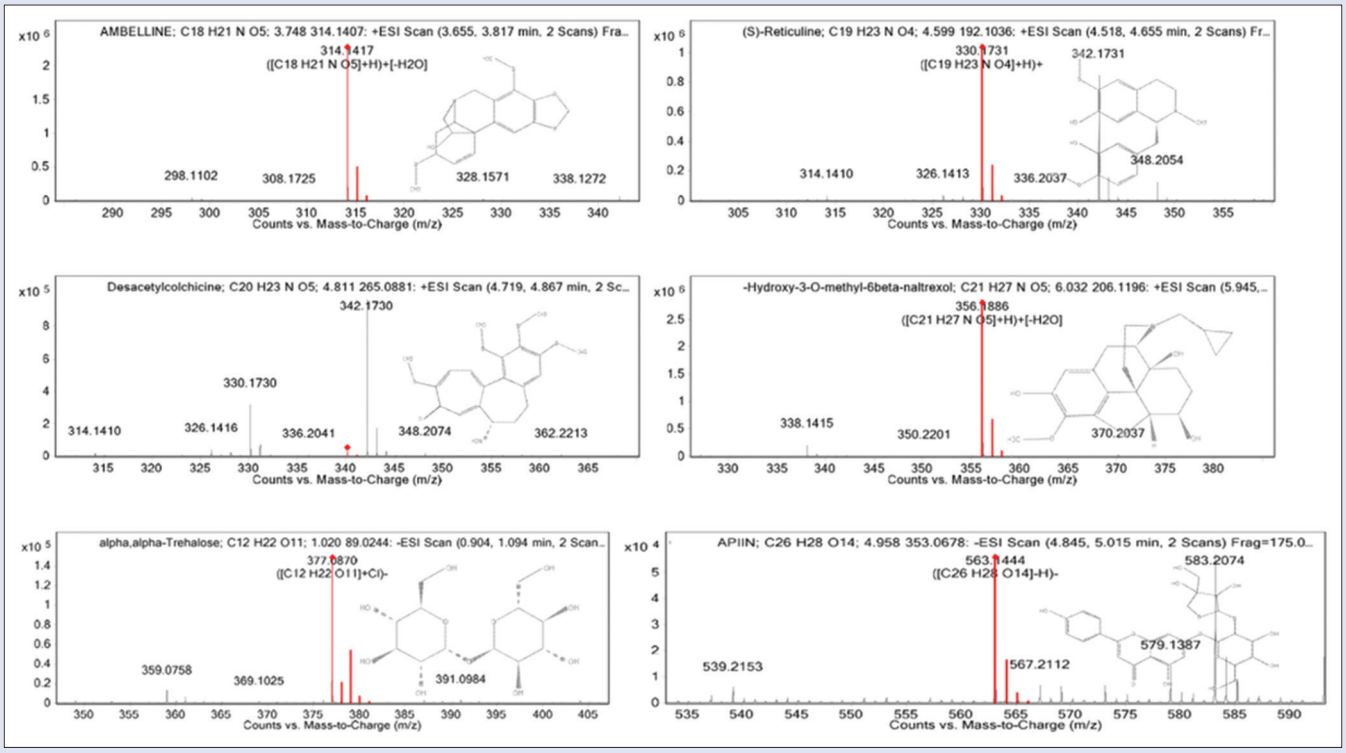

Figure 7: Mass fragmentation patterns of compounds, identified from butanolic fraction of *T. tuberculata*

TTB treatment strongly increased the cell survival of the Δtrx2 strain, despite the cells being very sensitive to H_2O_2 stress. This was exhibited by a decreased growth arrest in the growth curve assay and a significant increase in viability, in the cell viability assay. This effect is more evident in mutant strain Δtrx2, indicating its ability to protect the cell even in the absence of key antioxidant gene *TRX2*. Due to the impermeable nature of superoxide, its activity is restricted to mitochondria and is an important biomarker of mitochondrial oxidative stress. The beneficial effect of *T. tuberculata* on mitochondrial oxidative stress has not been studied, which is regarded as a pivotal element in the generation of IR. The level of mitochondrial ROS was measured using the fluorogenic dye, MitoSOX™ Red reagent. This reagent is oxidized by mitochondrial superoxide and the oxidation products on binding with nucleic acid resulted in the formation of a highly fluorescent compound, which is directly proportional to the level of mitochondrial superoxide. The yeast cells of negative control groups showing a high incidence of fluorescence as a result of the higher level of mitochondrial superoxide. The decrease in fluorescence observed in the TTB/ascorbic acid-treated strains of WT and Δtrx2 strains are indicative of the decreased mitochondrial superoxide level. It is hypothesized that the link between superoxide and IR is due to its possible role in the translocation of glucose transporters (GLUT4) and the role of mitochondria as a checkpoint in insulin signaling to GLUT4.[2,25] Therefore, superoxide scavenging property may be an additional mechanism behind the diabetes management of TTB. The radical scavenging activity of the compounds such as apiin, reticuline, and trehalose may account for the antioxidant activity of the fraction.

HFD induces the intracellular accumulation of lipid metabolites, thereby, increased ROS and mitochondrial stress.[26] The increased ROS activate stress kinases, which inhibit phosphorylation of insulin receptor substrate, a key mediator in insulin action leading to IR and hyperglycemic condition.[27] HFD with a single dose STZ is an established animal model which gradually impairs insulin secretion similar to the natural progression of T2D. OGTT showed that administration of TTB at the dose 50 mg/kg and 100 mg/kg was effective in controlling the increase in blood glucose level and improved glucose tolerance, indicating the ability of TTB to accelerate the glucose uptake and utilization. As reported earlier, the HFD-C rats showed an increase in fasting blood glucose level, accompanied by an increase in serum insulin level. Oral administration of TTB for 14 days produced a dose-dependent reduction in blood glucose, insulin concentration and HOMA-IR, in TTB1 and TTB2 group, which is comparable with that of standard drug metformin. The previous study by Abu *et al.* 2016, with dry powdered stem, also showed a similar effect in obese rats.[28] The HR-LC/MS analysis of TTB, reveals the presence of ambelline. The esters of ambelline show a potent selective butyrylcholinesterase (BuChE) inhibitory activity in cell line studies. BuChE inhibitors are reported to have beneficial effects on IR and T2D.[29,30] Apiin has a positive effect on IR and diabetes, by decreasing hepatic gluconeogenic enzymes activities and improving glucose tolerance.[31] It is reported that trehalose, a disaccharide present in TTB, can improve HFD induced IR in mice model, by reducing insulin secretion and the HOMA-IR index.[32] The pharmacological activity of these phytoconstituents may account for the improved glucose homeostasis showed by TTB.

Abnormalities in serum lipid profile have an important pathological change in T2D. Thus, the dyslipidemic effect of TTB was evaluated by determining the concentrations of serum TG, TC, LDL-C and HDL-C. Our results showed that there is a significant change in serum lipid profiles of HFD-C group compared to NC group [Figure 6]. The chronic administration of TTB showed a remarkable decrease in serum levels of TG, TC, LDL-C and concomitant increase in the levels of HDL-C compared to HFD-C group. These effects could be attributed to the collective actions of the phytochemicals present in TTB such as apiin, N-deacetylcolchicine, and trehalose. Apigenin reported having an anti-obesity activity, by downregulating the expression of lipogenic

genes and by improving hepatic lipid metabolism.[31] Trehalose improved obesity in HFD fed mice by suppressing adipocyte hypertrophy.[32] N-deacetylcolchicine decreases serum cholesterol and LDL-C in rats by modulating the metabolism of lipoprotein and cholesterol.[33]

The systemic antioxidant system consists of enzymatic and non-enzymatic antioxidants, which are an indicator of redox status. In our study, we measured MDA, an oxidative stress marker as well as SOD, CAT and GSH as indicators of the active antioxidant system. There was an elevation of MDA level and decrease in the activities of SOD, CAT, as well as levels of GSH in the liver tissues of HFD, fed animals, demonstrating the augmented oxidative stress. The present study showed that TTB was able to reduce oxidative stress in the liver at both concentrations (50 and 100 mg/kg b.w.), indicated by a reduced MDA level and improved activities of SOD, CAT and GSH level thus protecting the liver from oxidative damage. Apigenin shows potent radical scavenging activity in *in-vitro* models. It is also reported that apiin can significantly improve the activities of SOD, GSH-Px and CAT, with a reduction in MDA level, in animal model; and it also enhanced the total antioxidant capacity of various tissues in mice.[34,35] Reticuline showed antioxidant and anti-inflammatory activity by inhibiting the expression of pro-inflammatory cytokines, such as TNF-α and IL-6.[36] Recent studies have shown that treatment with trehalose could inhibit oxidative stress and inflammation.[37] These phytochemicals in TTB could be responsible for the inhibition of free radicals generated by diabetes-induced hyperglycemia.

Inducers of heat shock response have been used as an effective strategy to reverse IR and ensuing T2D. Methods to induce a heat shock response like hot tub therapy, use of bioactive compounds, or genetic overexpression, improve all the parameters of associated with T2D.[38] Both clinical and preclinical studies indicate that IR/hyperglycemic subjects have low expression of Hsp 70.[5] We evaluated the ability of TTB to modulate stress response *via* induction of Hsp 70 to study its effect on cellular stress and cytoprotection against metabolic stress. The liver is the main organ which utilizes glucose and hence, the hepatic Hsp 70 was evaluated by ELISA. HFD-C group showed a decreased level of Hsp 70 in liver tissue, compared to the NC group. This finding is consistent with other studies in literature in which it has been shown that there was a significant reduction in expression of Hsp 70 from nonhuman primates with T2D.[39] Hsp 70 has a cytoprotective role against cellular inflammation. There was a significant elevation of liver Hsp 70 levels in both treatment groups (TTB1 and TTB2) in a dose-dependent manner in comparison to metformin. Therefore, the mechanism of TTB may be different from the standard drug metformin. The cytoprotective effect of phytochemicals against cellular stress in yeast and mammalian cells have been attributed to an increase in antioxidant response and heat shock response.[40] The cytoprotective effect of TTB was found to be the possible mechanism for its activity against metabolic disorders. The drug offered significant protection against oxidative stress and inflammation, which is exemplified by its effect on hepatic Hsp 70. Trehalose has been shown to have anti-oxidant, anti-inflammatory and cytoprotective action and was found to modulate Hsp expression in spinal cord injury.[41]

CONCLUSION

In the current study, we investigated the mechanistic aspect of *T. tuberculata* extract by evaluating various *in vitro* and *in vivo* parameters related to IR and cytoprotection. The mitochondrial oxidative stress, especially the generation of superoxide radicals is the starting point in the pathogenesis of IR. We tested the effect of butanolic fraction of *T. tuberculata* on oxidative status and cell viability on WT and knock out yeast strains after challenging them with a physiologically relevant oxidant H_2O_2. Results of our study show that TTB was able to counter the oxidative insult induced by H_2O_2 on cell survival and viability. TTB showed the protective effect to the WT as well as the deletion strain Δtrx2, which is highly sensitive to H_2O_2. TTB has a positive impact on all the parameters of IR in rats and the effect was found to be comparable to metformin. The mechanism of TTB seems to be due to its antioxidant and anti-inflammatory activity of apiin, reticuline, ambelline and N-deacetylcolchicine. The significant effect of TTB on hepatic Hsp 70 demonstrates that its effect on inflammation and glucotoxicity may be due to the drug-induced expression of hepatic Hsp 70 as the liver is an important organ in glucose metabolism. Taken together, our results suggest that the activity of TTB against IR and metabolic syndrome may be related to its cytoprotective effect by countering oxidative stress and inducing hepatic Hsp 70.

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Conflicts of interest

There are no conflicts of interest.

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