Litsea polyantha Extracts Ameliorate Diabetes and Oxidative Stress in Streptozocin-induced Diabetic Rats

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ABSTRACT

Objectives: Litsea polyantha (LP) is a traditional medicinal plant in India. As the bark is mildly astringent, it is traditionally used to treat diarrhoea. Powdered bark and roots are used for pain, bruises, contusions, and fractures in animals. Litsea polyantha was evaluated for its blood glucose lowering abilities as well as for its ability to curb oxidative stress. Methods: In Wistar rats, diabetes was induced by intraperitoneal administration of Streptozocin (STZ) (60 mg/kg, b.w) for four weeks. After clipping the rats’ tails, blood glucose levels were determined. Body weight and urine volume were also measured. In addition, oxidative stress markers such as thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) were measured. Biochemical estimations were further strengthened by histological evaluation. The statistical analysis of results was carried out using one-way analysis of variance (ANOVA), followed by Tukey’s post hoc with GraphPad Prism software package. Results: Compared to untreated diabetic rats, LP extracts significantly decreased blood glucose levels in diabetic rats for 4 weeks. As evidenced by the glucose levels and biochemical markers, diabetes has significantly improved. LP treated diabetic rats had significantly higher levels of antioxidant defense enzymes and lower levels of TBARS. STZ-induced elevation of glucose levels in diabetic rats was prevented by LP. Conclusion: The results of this study demonstrated that LP had antioxidative and anti-inflammatory properties that might contribute to its therapeutic effects on diabetic rats. Keywords: Litsea polyantha, Diabetes, Oxidative stress, Inflammation, Antioxidant.

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INTRODUCTION

In India, the United States, and Europe, diabetes is the most common cause of low quality of life. Diabetic patients develop neuropathy at some point in their lives, including those with type 1 diabetes (T1D). In countries with more Western-style foods, particularly in those that consume more pre-diabetes and type 2 diabetes are increasingly common.1 There will be an increase in complications among Indians and Americans with pre-diabetes and type 2 diabetes. Pre-diabetics, T1D, and T2D currently cause diabetic complications for over 20 million Americans.2 According to the World Health Organisation (WHO), 316 million people suffer from pre-diabetes, and 387 million suffer from diabetes, respectively. Though exact Figures are not available, it is speculated that at least 200 million suffer from severe complications related to diabetes.

Despite decades of research, the only modifiable treatment for diabetes is controlling blood glucose levels and improving lifestyle.3 According to a Cochrane review of all clinical studies, patients with T1D who practice rigorous glucose control are less likely to develop diabetic neuropathy. However, patients with T2D experienced little to no benefit from rigorous glucose control despite improving glucose control for more than a decade.4 Due to the pain and inability to work, along with the poor quality of life suffered by each patient, this causes a high burden on society, but the individual costs are even higher.5 It is crucial to understand and to provide early diagnosis to prevent poor patient outcomes due to the enormity of the issue, both at the individual and societal levels.6

There is an urgent need to develop new ways of preventing diabetes and treating it while reducing the side effects of conventional medications. In spite of the fact that conventional therapies are effective and satisfactory, there are an increasing number of side effects associated with them, so new drugs derived from natural sources are urgently needed with fewer side effects and as effective as conventional therapies.

In animal models, Litsea polyantha extracts showed anti-inflammatory, analgesic, and anti-diabetic properties. Litsea polyantha may be a small to medium size evergreen tree but used as stimulant, analgesic, nerves and bone tonic, and antiseptic agent and has been given to treat diabetes, diarrhoea, dysentery, and arthritis. Litsea polyantha (Lauraceae) also known as Litsea monopetala Roxb. This evergreen tree is small to medium sized. Oraon and Munda healers of Jharkhand have traditionally used the bark of this plant as a medicine. Pojo, Kakuri, Munga, and Barkukuchita are some of its popular names. Traditionally, the bark is used to treat diarrhoea as it is mildly astringent. For pain, bruises, and contusions as well as fractures in animals, powdered bark and roots are used.7 The anti-diarrheal activity of methanol extract of dried bark and aerial parts of Litsea polyantha has been evaluated in mice using different models.8 The antioxidant activity of phenolic fractions of bark extract was carried out by various chemical and enzymatic methods.9 WHO has also suggested the evaluation of traditional plant treatments for diabetes as they are effective, non-toxic, with less or no side effects, and are considered to be excellent therapeutic candidates. It has not been studied in clinical studies whether Litsea polyantha improves blood glucose control and cardiovascular health in diabetics. Litsea polyantha also lacks information regarding the molecular mechanisms responsible for its anti-hyperglycaemic and anti-inflammatory properties. Thus, Litsea...
Litsea polyantha extracts were studied for its ability to lower blood glucose and neutralise oxidative stress as well as curb diabetes.

MATERIALS AND METHODS

Plant Material and Preparation of the Extracts

The whole plant of Litsea polyantha (Lauraceae) were collected from Uttarakhand, India during the months of August and September. The plant material was identified and authenticated by Dr. K. Madhava Chetty, Botanist, Department of Botany, Sri Venkateswara University, Tirupati, A.P. A voucher specimen (0741) has been preserved for further utilization. The plant leaves were shade dried, and pulverized using a manual grinder. The powdered crude drug (1kg) was defatted by maceration with petroleum ether (at room temperature; for 48 hr). This process was repeated (three times) for complete removal of the fatty materials. It was then air dried and subsequently subjected to concurrent fractionation in Soxhlet apparatus with increasingly polarized organic solvents. After exhaustive extraction, the three extracts namely, ethanolic, aqueous and hydroalcoholic extracts were collected and concentrated under reduced pressure. Three concentrated extracts of Litsea polyantha (LP) were obtained (yield 0.62 %, 0.63 % and 0.69 % w/w with respect to the dried starting material). The final product was then stored at 4°C prior to use. The three final extracts were code-named as LP-A (aqueous extract), LP-H (hydroalcoholic extract) and LP-E (ethanolic extract).

Animals

Adult male Wister rats (weighing 150–200 g) were used for the study. The animals were obtained from the animal house, Shri Guru Ram Rai University, Dehradun, Uttarakhand. The animals were housed in polypropylene cages at 25 ± 1°C (with 12 hr light and dark cycle) at animal house, Siddhartha Institute of Pharmacy, Dehradun, Uttarakhand. All the animals were acclimatized to laboratory environment for a week prior to experiment. The animals were provided free access to standard pellet diet and water ad libitum. The study protocols were approved by the IAEC (Institutional Animal Ethics Committee) via protocol reference number SIP/IAEC/PCOL/07/2020. The care and use of laboratory animals were strictly in accordance with the guidelines prescribed by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Drugs and chemicals

Streptozotocin (STZ) and Glibenclamide were procured from from Sigma–Aldrich (St. Louis, MO, USA). All other unlabeled chemicals and reagents were of analytical grade and available commercially (SRL Mumbai, E. Merck India).

Acute toxicity study

Acute toxicity study of Litsea polyantha leaves extracts was carried out according to the Organization for Economic Co-operation and Development (OECD) guideline 425. Five adult male Wistar rats were chosen at random (one for each phase). The animals were fasted for four hours and only given water. At a dose of 2000 mg/kg, extract of Litsea polyantha leaves was given orally. The rats were monitored continuously for the first 4 hr, then at regular intervals for the next 24 hr for toxic symptoms such as sleepiness, restlessness, writhing, convulsions, piloerection, and mortality, if any. The rats were kept under observation for two weeks.

Diabetes induction

Diabetes was induced in overnight-fasted Wistar rats by single intraperitoneal (l.p.) injection of streptozotocin (STZ) at a dose of 60 mg/kg in 0.1 M citrate buffer, pH 4.5. Blood glucose level was measured prior to induction of diabetes and 48-hr post STZ/vehicle injection. Animals with blood glucose levels above 200 mg/dl were considered as diabetic and included in experiment. For diabetic rats, if the blood glucose level is greater than 450-500mg/dl, 1 unit of huminsulin was administered via subcutaneous route to decrease mortality. If the blood glucose level is greater than 600 mg/dl, 2 units of huminsulin were administered via subcutaneous route.

Experimental design

A total of thirty-six rats were randomly allocated among 6 groups. The first group (Normal control) comprised normal rats receiving 0.1 % w/v CMC orally, the second group (STZ) comprised diabetic rats that represented the diabetic group, the third group (Standard treated) comprised diabetic rats that were daily administered Glibenclamide (10 mg/kg, p.o.) for 30 days. The Glibenclamide dose was selected according to a previously published study. The fourth group (treated) comprised diabetic rats receiving daily LP aqueous extract (100 mg/kg, p.o.) for 30 days, the fifth group comprised diabetic rats receiving daily LP hydroalcoholic extract (100 mg/kg, p.o.) for 30 days and the sixth group comprised diabetic rats receiving daily LP ethanolic extract (100 mg/kg, p.o.) for 30 days. One day past final drugs administration, animals were weighed, and blood glucose level was assessed by ACCU-Check. All rats were then decapitated and the pancreas was carefully excised, rinsed with ice-cold saline and fixed in 10% neutral-buffered formalin for histopathological examination. Below is a summary of the experimental design and grouping of animals:

Group I: Normal control.
Group II: Diabetic control.
Group III: Glibenclamide (10 mg/kg body weight) treated group.
Group IV: LP aqueous extract (LP-A)100 mg/kg treated group.
Group V: LP hydro alcoholic extract (LP-H) 100 mg/kg treated group.
Group VI: LP ethanolic extract (LP-E) 100 mg/kg treated group.

General Parameters

Blood was collected from overnight fasted rats every day during the study, initially at day 0, then on the 48 hrs, 8th day, 16th day, 24th day, and 30th day. A syringe was used to prick the rats’ tails, and blood glucose was measured using an electronic glucometer and glucose test strips. A blood sample of between 0.5 ml and 1 ml was collected from the retro-orbital method for the serum biochemical estimation on the 30th day of observation by the retro-orbital method. After STZ administration and before starting the study, basal blood glucose level was measured. Animals with blood glucose levels above 200 mg/dl were considered as diabetic and included in experiment. The body weight of control, diabetic, and treated rats was determined. Individual urine collection metabolic cages were used to collect urine samples after animals had acclimated for 24 hr. The baseline data was established by taking urine samples from the control, diabetic control, and treatment groups every week from the first day.

Biochemical estimation

During the 30th day of observation, a blood sample varying from 0.5 ml to 1 ml was collected from the retro-orbital method for the serum biochemical estimation. Cholesterol, total cholesterol (TC), Serum triglycerides (TG), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) etc were estimated using enzymatic calorimetric kits.

Histopathological examinations

For light microscopy evaluation, 5 μm specimens were cut from formalin-fixed pancreas paraffin blocks and stained with hematoxylin and eosin (H&E). Based on the severity of pathological damage to islets of Langerhans cells, the cell injury was assessed as follows: (-) no noticeable damage; (+) mild damage; (++) moderate damage; (++) severe damage; (+++) very severe damage.
damage, (+) minor damage affecting up to 25%, (+++) moderate damage affecting 25%–50%, and (++++) severe damage affecting > 50% 17.

Statistical analysis
The data were expressed as mean ± SD. Statistical differences at p < 0.05 between the groups were analysed by one-way ANOVA followed by Turkey as post hoc using GraphPad Prism software package. All the analyses were done using GraphPad Prism (Version 8.01, GraphPad Software, San Diego, USA).

RESULTS
In preliminary phytochemical studies, alkaloids, saponins, steroids, flavonoids, tannins, and glycosides were identified. There was no sign of mortality in the acute toxicity study, so 1/20th of 2000mg/kg dose was deemed safe and 100 mg/kg dose was chosen for the study. Significant body weight changes were observed. In comparison to normal rats, streptozotocin causes a significant loss in body weight. Rats in the diabetic control group were found to lose body weight until the end of the study while rats in groups treated with standard drugs and different extracts at a dose of 100 mg/kg, body weight showed a significant improvement. A significant reduction in blood glucose levels was observed on 16th day, 24th day and 30th day (p<0.05) in all groups receiving 100 mg/kg. Diabetic rats administered with LP-A 100 mg/kg, LP-H 100 mg/kg and LP-E 100 mg/kg and standard treatment Glibenclamide showed significant (p<0.05) decrease in the blood glucose levels and the urine volume as well as increase in the body weight in treatment groups as compared to disease control group. Diabetic rats administered with LP 100 mg/kg showed significant and better (p<0.05) effect as compared to disease control group (Table 1).

Assessment of oxidative stress markers
STZ treatment and induced diabetes in rats significantly reduced the activity of CAT, SOD, GSH (p<0.01) whereas amplified TBARS levels (**p<0.001) in pancreatic tissues as compared to normal control rats. Generation of free radicals and oxidative stress increased the lipid peroxidation as evident by higher levels of TBARS, leading to diminution in the antioxidant defence system in terms of reduction in the levels of CAT, SOD and GSH. The LP extracts administration significantly increased and restored the antioxidant defence enzyme system by restoring the CAT, SOD and GSH levels to normal. The LP treatment (100 mg/kg bw) showed significant upsurge of CAT, SOD and GSH levels as compared to diabetic control group animals (*p<0.05, **p<0.01, ***p<0.001). The results are presented in Table 3.

Histopathological examination
The effects of LP extracts (100 mg/kg) were recorded by dosing the animals for 4 weeks and studying their histopathological sections. The histopathological analysis of the pancreatic tissue in the 4th week showed normal tissue without any sign of inflammation or degenerative changes in the control group (non-diabetic, A). HE staining showed normal structure in normal control group and histopathological photomicrograph of the pancreas showed normal acini, and normal cellularity in the islets of Langerhans of control pancreas group (A). Severe degeneration in the pancreas appeared in the STZ-induced diabetic rat, including lymphocyte infiltration, hypochromatosis and the disappearance of cell borders in the pancreatic islets (Figure 1). Diabetic control group showed extensive damage to islets of Langerhans and decreased number of islet cells. After treatment with all the LP extracts (100 mg/kg) improvement in the pancreatic tissues could be observed.

Table 1: Effect of LP-A, LP-H and LP-E on blood glucose levels.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Blood glucose levels (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 day</td>
</tr>
<tr>
<td>Normal control</td>
<td>88.32±1.31</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>87.11±1.24</td>
</tr>
<tr>
<td>Standard treatment</td>
<td>89.42±1.21</td>
</tr>
<tr>
<td>LP-A 100 mg/kg</td>
<td>87.5±1.61</td>
</tr>
<tr>
<td>LP-H 100 mg/kg</td>
<td>89.23±1.41</td>
</tr>
<tr>
<td>LP-E 100 mg/kg</td>
<td>90.6±1.49</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ±SD (n=6 number).

*a indicate significance from the normal control group at **p<0.01, ***p<0.001 probability level.

*b indicate significance from the Diabetic control group at **p<0.01, ***p<0.001 probability level.

Table 2: Effect of LP-A, LP-H and LP-E on lipid profile.

<table>
<thead>
<tr>
<th>Groups</th>
<th>TC (U/l)</th>
<th>TG (mg/dl)</th>
<th>HDL (mg/dl)</th>
<th>LDL (mg/dl)</th>
<th>VLDL (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>60.6±1.71</td>
<td>31.2±1.86</td>
<td>12.5±0.30</td>
<td>54.2±1.94</td>
<td>6.23±0.37</td>
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<tr>
<td>Diabetic control</td>
<td>70.5±1.41**</td>
<td>40.1±1.84**</td>
<td>11.1±0.11**</td>
<td>67.5±3.88**</td>
<td>8.63±0.36**</td>
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<tr>
<td>Standard treatment</td>
<td>61.2±2.94</td>
<td>32.4±1.95</td>
<td>13.0±0.20</td>
<td>57.6±2.08</td>
<td>6.82±0.25</td>
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<tr>
<td>LP-A 100 mg/kg</td>
<td>63.7±0.95*</td>
<td>33.7±1.26*</td>
<td>12.8±0.13*</td>
<td>58.5±1.29*</td>
<td>6.87±0.20</td>
</tr>
<tr>
<td>LP-H 100 mg/kg</td>
<td>64.3±1.34*</td>
<td>33.0±0.86*</td>
<td>13.0±0.46*</td>
<td>57.8±2.15**</td>
<td>6.38±0.27*</td>
</tr>
<tr>
<td>LP-E 100 mg/kg</td>
<td>63.8±1.62*</td>
<td>32.3±0.31*</td>
<td>12.8±0.11*</td>
<td>57.5±1.55**</td>
<td>6.45±0.06*</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ±SD (n=6 number).

*a indicate significance from the normal control group at **p<0.01, ***p<0.001 probability level.

*b indicate significance from the Diabetic control group at **p<0.01, ***p<0.001 probability level.

TC-Total Cholesterol, TG- Triglycerides, HDL- High density lipoprotein, LDL- Low density lipoprotein and VLDL – Very Low-density lipoprotein
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Table 3: Effect of LP-A, LP-H and LP-E on markers of oxidative stress.

<table>
<thead>
<tr>
<th></th>
<th>CAT (U/min)</th>
<th>SOD (U/mg protein)</th>
<th>GSH (μM/g tissue)</th>
<th>TBARS (nM/min/mg protein)</th>
</tr>
</thead>
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<tr>
<td>Normal Control</td>
<td>5.08±0.75</td>
<td>23.51±1.03</td>
<td>4.75±0.04</td>
<td>30.10±1.14</td>
</tr>
<tr>
<td>Diabetes Control</td>
<td>a 3.53±0.13***</td>
<td>a 12.71±0.71***</td>
<td>a 0.52±0.01***</td>
<td>a 54.08±1.21***</td>
</tr>
<tr>
<td>Standard treatment</td>
<td>b 5.61±1.14**</td>
<td>b 21.68±0.74***</td>
<td>b 4.64±0.47***</td>
<td>b 28.86±1.30***</td>
</tr>
<tr>
<td>LP-A 100 mg/kg</td>
<td>b 5.25±0.83*</td>
<td>b 17.32±0.85**</td>
<td>b 3.83±0.39**</td>
<td>b 4.13±1.14***</td>
</tr>
<tr>
<td>LP-H 100 mg/kg</td>
<td>b 5.45±0.76**</td>
<td>b 20.41±0.86***</td>
<td>b 4.56±0.43***</td>
<td>b 33.08±1.05***</td>
</tr>
<tr>
<td>LP-E 100 mg/kg</td>
<td>b 5.34±0.81***</td>
<td>b 22.83±0.81***</td>
<td>b 4.70±0.30***</td>
<td>b 29.07±1.18***</td>
</tr>
</tbody>
</table>

Values are expressed as Mean±SD (n=6 number).

a indicate significance from the control group at **p<0.01, ***p<0.001 probability level.
b indicate significance from the Diabetic control group at ** p<0.01, ***p<0.001 probability level.

Diabetes-induced weight loss was also observed in diabetic rats. The accumulation of amino acids could be due to a decrease in insulin levels causing tissue protein turnover to increase as a source of metabolic energy.22 Treatment with LP extracts reduced body weight loss, blood glucose levels. Many mechanisms, including improved insulin secretion and sensitivity, increased glucose uptake, and inhibition of α-glucosidase activity may underlie LP's anti-hyperglycaemic actions. Aside from regulating key enzymes responsible for hepatic glucose homeostasis, LP extracts might also attributed to decrease glucose production.23 In comparison to the diabetic control group, all the LP extracts treated groups exhibited substantial reduction in TC, TG, LDL and VLDL (Table 2). When compared to the diabetes control group, HDL levels increased considerably. This could be due to the anti-hyperlipidaemic effects of LP extracts that are linked to AMPK activation, which increases glucose uptake.

Increasing oxidative stress and reactive oxygen species caused nerve damage and dysfunction in diabetes due to metabolic and vascular insults.24 Possibly, the polyol pathway is responsible for the increased consumption of NADPH in diabetes, whereas mitochondrial superoxide production, activation of PKC, and glucose oxidation may also contribute to oxidative stress.25-26 Moreover, previous studies have reported impaired cellular anti-oxidant defences in patients with diabetes.27-28 Therefore, antioxidants may assist in treating diabetes and related complications by reducing oxidative stress. LP treatment might have attenuated lipid peroxidation, protein carbonylation, and increased antioxidant activities in the present study. Diabetes may be caused by oxidative damage to tissues, which predominantly consists of lipids.28 LP has been found to be a potent free radical scavenger, enhancer of scavenger enzyme activity and inhibitor of enzymes that were involved in catalysing free radical generation. This antioxidant property could be attributed to the structural characteristics of the phenolic and flavonoid phytoconstituents present in it. The phytoconstituents in LP possessed...
phenolic nuclei as well as structures with unsaturated side chains which
eases the ability to form a phenoxy radical due to resonance stabilization
of the molecules.39 Additionally, several studies reported that the Keap1-
Nrf2-ARE signalling pathway supporting cellular redox balance might
be responsible for the protective effects of phytoconstituents against
oxidative stress.39

In the pancreatic tissue of diabetic animals, TBARS, a lipid peroxidation
biomarker, showed a remarkable elevation. In order to fight free
radical damage, superoxide dismutase (SOD), catalase (CAT) and GSH
(Glutathione), three endogenous antioxidants, are considered early
remedies against oxidative stress. In this study we identified a significant
reduction in SOD, CAT, and GSH content in diabetic rats’ pancreatic
tissue. The LP extracts significantly improved the levels of SOD, CAT,
and GSH. Endogenous defence mechanisms against free radicals, which
include antioxidant enzymes (SOD, CAT and GSH) are considered to be
major players involved in the antioxidative process, and are believed to
primarily contribute to the body’s natural defence mechanisms against
free radicals. Nonetheless, SOD protects tissues from highly reactive
superoxide anions (O2−) by converting them into hydrogen peroxide
(H2O2). Additionally, elevated blood glucose levels decrease the level of
SOD in pancreatic tissue of animals due to nonenzymatic glycosylation.36
Based on these results, decreased SOD, CAT, and GSH activity was
observed in the pancreas of diabetic rats. In contrast, CAT is crucial to the
catalytic decomposition of harmful H2O2 to O2 and H2O. When diabetes
is present, CAT activation is reduced, reducing cellular protection and
making tissues more susceptible to free radicals. As evident, diabetes
and oxidative stress are linked when the enzyme activity was inhibited
in diabetic rats. The concurrent down-regulation of the pancreatic
antioxidant system renders the tissue more vulnerable to oxidative stress
due to the concurrent decline in the antioxidant system. The treatment
with the LP extracts restored the antioxidant defence system by levelling
up the antioxidant enzymes and demonstrated the protective effect.

CONCLUSION

On the basis of the findings, LP appears to be a novel and promising
complementary approach to the treatment of diabetes, offering potential
benefits for diabetes management. The best dose of LP extract for
controlling blood sugar and managing diabetes and its associated
complications could be determined by future animal studies and human
studies. By using LP extracts alone for mechanistic studies, current
findings would be validated and detailed phytochemical screening could
be performed for bioactivity-guided assays. The effects of LP on glucose
uptake in skeletal muscle cells, independent of insulin signalling, will
require further investigation. The present study demonstrated that LP
could be beneficial for managing diabetes through an improved protective
effect, and provide a mechanistic account for the anti-inflammatory and
anti-diabetic effects traditionally attributed to it.

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for providing necessary support and arrangements for successful
conduction of the experiments.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

LP: Litsea polyantha; LP-A: LP aqueous extract; LP-H: LP hydro
alcoholic extract; LP-E: LP ethanolic extract; STZ: Streptozotocin; SOD:
Superoxide Dismutase; CAT: Catalase; GSH: Glutathione Peroxidase;
OECD: Organisation for Economic Co-operation and Development;
CPCSEA: Committee for the Purpose of Control and Supervision of
Experiments on Animals; IACUC: Institutional Animal Ethic Committee;
TBARS: Thiobarbituric Acid Reactive Substances; TC: Total Cholesterol;
TG: Serum Triglycerides; LDL: Low-Density Lipoprotein; HDL: High-
Density Lipoprotein; ANOVA: Analysis of Variance.

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