



BJP

Bangladesh Journal of Pharmacology

Research Article

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Article Info

Received: 11 May 2016
Accepted: 16 July 2016
Available Online: 18 November 2016
DOI: 10.3329/bjp.v11i4.27707

Cite this article:

Punitha SC and Rajasekaran M. Cardioprotective effect of *Volvariella volvacea* in streptozotocin administered rats. Bangladesh J Pharmacol. 2016; 11: 903-10.

Abstract

The present study examined the cardioprotective role of methanol extract of the edible mushroom *Volvariella volvacea* against oxidative stress in hyperglycemic rats. Rats divided into 6 groups were administered with nicotinamide and streptozotocin intraperitoneally, except Group I (control). Group II served as diabetic control. Group III was given glibenclamide. Two groups (IV and V) of rats received (200 and 400 mg/kg) mushroom extracts orally for 30 days and Group VI received vitamin E (40 mg/kg). After the treatment period, lipid peroxides, carbonyl end products, advanced glycation end products, reduced glutathione, glutathione peroxidase, glutathione-S transferase, catalase, superoxide dismutase and non-enzymatic antioxidants (vitamin C and E) were assessed in the heart tissues of experimental animals. Glycosylated hemoglobin was estimated in blood. Electrocardiography recordings of the treated groups were also done. The results showed that mushroom extract treatment reduced the lipid peroxides, advanced glycation end products and protein carbonyls significantly and reversed the altered anti-oxidant enzymes, and the vitamins.

Introduction

Diabetes mellitus is a potent independent risk factors for cardiovascular disease mediated by increased production of free radicals (Baynes and Thorpe, 1999) and impaired anti-oxidants (Halliwell and Gutteridge, 1990). Free radicals caused oxidative stress damages cellular proteins, membrane lipids and nucleic acids (Maritim et al., 2003) which eventually accelerate micro and macrovascular impediments. Inhibition of intracellular free radical generation to prevent hyperglycemic oxidative stress and related complications in diabetic heart will be an effective therapy.

Since antidiabetic agents in use are ill reputed with side effects, nutraceuticals and functional foods are becoming popular to prevent the diabetes and its hyper-

glycemic complications (Yang et al., 2008). Natural antidiabetic drugs with antioxidant properties would be a promising alternative therapy. Mushrooms besides their rich nutritional values have been considered as remarkable therapeutic agent for diabetes and cardiovascular diseases (Wasser and Weis, 1999). Mushrooms are hypoglycemic (Hwang et al., 2005) and are also rich source of antioxidants (Ferreira et al., 2009).

The present study evaluates the antioxidant mediated cardioprotective effect of an edible mushroom, *Volvariella volvacea* in streptozotocin-induced diabetic rats. *V. volvacea* (paddy straw mushroom or Chinese mushroom) is an edible tropical mushroom and has nutritional, therapeutic (Roy et al., 2014) and antioxidant potential (Punitha and Rajasekaran, 2014).



Materials and Methods

Reagents

Streptozotocin was purchased from the Sigma Chemical Co., USA. All other reagents used were of analytical grade procured from the Himedia Lab, India.

Mushroom extract

V. volvacea was procured from the Directorate of Mushroom Research, Solan, India as dried sample. It was further dried at 50°C for 12 hours and pulverized. The powdered mushroom was extracted with methanol in soxhlet apparatus for 12 hours. The extract was concentrated using rotary vacuum evaporator and stored.

Animals

Male Wistar albino rats (150-175 g) were obtained from the Central Animal Facility, SASTRA University, Thanjavur. They were maintained in the animal room under controlled conditions.

Induction of diabetes

Diabetes was induced in overnight fasted rats by intraperitoneal injection of streptozotocin dissolved in 0.1M cold sodium citrate buffer of pH 4.5 at a dose of 60

mg/kg body weight. Nicotinamide was given (110 mg/kg ip.) 15 min prior to streptozotocin injection. To overcome the drug-induced hypoglycemic state, rats were allowed to drink 5% glucose solution overnight. Hyperglycemia was confirmed 72 hours after streptozotocin administration by measuring tail vein blood glucose with glucometer (One Touch, Switzerland). The rats with blood glucose levels beyond 250 mg/dL were considered diabetic and were used for experimentation.

Experimental protocol

For the treatment, rats were divided into six groups of 6 rats each. Group I was considered as normal control. Group II was diabetic control. Group III received glibenclamide, a standard drug (10 mg/kg p.o.) and was considered as positive control. Group IV and V animals were administered with methanol extract of *V. volvacea* at two different concentrations, 200 and 400 mg/kg body weight, and Group VI received vitamin E (40 mg/kg body weight) by intragastric intubation. All the doses were administered for 30 days.

Biochemical estimations

At the end of the experimental period, the rats were anesthetized by CO₂ asphyxiation. Blood samples were

Box 1: Quantitative determination of glycosylated hemoglobin by ion exchange resin method

Principle

Glycosylated hemoglobin is formed continuously by the adduction of glucose by covalent bonding to the amino terminal valine of the hemoglobin beta chain progressively and irreversibly over a period of time and is stable.

A hemolysed preparation of whole blood is mixed continuously for five minutes with a weakly binding cation-exchange resin. The labile fraction is eliminated during the hemolysate preparation and binding. During mixing non-glycosylated hemoglobin binds to the ion exchange resin leaving the glycosylated hemoglobin free in the supernatant which is separated using a resin separator. Absorbance of the supernatant at 415 nm is read. The percent glycosylated hemoglobin is determined by measuring the ratio of absorbance of glycosylated hemoglobin and total hemoglobin fraction of control and test.

Requirements

Ion exchange resin (predispensed tubes 3 mL), lysing reagent, resin separator and control (10% glycosylated hemoglobin), heparinized vial, and GlycoHb kit (Kamineni Life Sciences Pvt. Ltd., India).

Procedure

Hemolysate preparation

Step 1: Dispense 0.5 mL of lysing reagent into tubes labeled as control and test

Step 2: Add 0.1 mL of reconstituted and well mixed blood sample into the respective tubes

Step 3: Mix well for complete lysis

Step 4: Allow to stand for five min

Glycosylated hemoglobin separation

Step 1: Remove the cap of ion-exchange resin solution and label control and test

Step 2: Add 0.1 mL of hemolysate to the resin tubes

Step 3: Insert a resin separator into each tube in such a way that the rubber sleeve is 3 cm above the solution

Step 4: Mix the solution using a vortex and keep for five min

Step 5: Push the resin separator into the tubes until the resin is firmly packed

Step 6: Pour the supernatant directly into a cuvette and measure the absorbance

Total hemoglobin fraction

Step 1: Dispense 5.0 mL of distilled water in two test tubes (control and test)

Step 2: Add 0.02 mL of hemolysate and read the absorbance after mixing well

Calculation

%Glycosylated Hb = Ratio of test /Ratio of control x 10 (value of control)

Video clips

Hemolysate preparation: 2 min 50 sec

Mixing, glycosylated hemoglobin separation and reading absorbance: 3 min 10 sec

Table I

Effects of *V. volvacea* on oxidative stress markers and HbA1C levels

Experimental groups	Control		Streptozotocin			
			Glibenclamide	<i>V. volvacea</i> (200 mg)	<i>V. volvacea</i> (400 mg)	Vitamin E
Lipid peroxide (nmol melondialdehyde/mg protein)	15.3 ± 0.5	31.3 ± 0.8 ^a	18.5 ± 0.7	24.1 ± 0.7 ^a	19.5 ± 0.5	20.8 ± 0.8 ^a
Protein carbonyl (nmol of carbonyl/mg protein)	1.9 ± 0.0	3.1 ± 0.2 ^a	2.0 ± 0.1	1.9 ± 0.1	2.1 ± 0.2	1.9 ± 0.1
Advanced glycation end products (AU/mg protein)	12.3 ± 0.1	24.5 ± 0.3 ^a	15.7 ± 0.2	16.5 ± 0.3	14.5 ± 0.2	19.6 ± 0.2 ^a
HbA1c (%)	7.6 ± 0.2	12.2 ± 0.2 ^a	12.2 ± 0.2 ^a	8.1 ± 0.2	8.1 ± 0.1	9.7 ± 0.1 ^a

Values are expressed as Mean ± SE (n = 6); ^aindicates significantly different from control p<0.05

collected by retro-orbital sinus puncture in heparinized vials. Hearts were excised immediately and washed with physiological saline. The heart tissue homogenate prepared using 0.1M Tris HCl buffer (pH 4.7) was used for biochemical estimations. Determination of lipid peroxides (Ohkawa et al., 1979), protein carbonyls (Levine et al., 1994), reduced glutathione (Ellman, 1959), glutathione peroxidase (Rotruck et al., 1973) glutathione-S-transferase (Habig et al., 1974), catalase (Aebi, 1984), superoxide dismutase (Kakkar et al., 1984), vitamin C (Omaye et al., 1979) and vitamin E (Baker et al., 1980) were done in the heart tissue homogenate of all groups of rats. Advanced glycation end products in heart tissues were determined (Nakayama et al., 1993) using multi-mode microplate reader (Biotek, USA). Blood samples collected in heparinized vials were used for glycosylated hemoglobin (HbA1c) estimation using GlycoHb kit, Lifechem™ purchased from Kamineni Life Sciences Pvt. Ltd., India.

Electrocardiogram

ECG was recorded using 12 channel polygraph device

(Biopack, MP100, USA). All the rats were anesthetized using ketamine (100 mg/kg i.p) before taking ECG readings. QT, QRS and ST waves were measured for all the groups.

Statistical analysis

The results are expressed as Mean ± Standard error of mean. Statistical analysis of the results was done by one way analysis of variance (ANOVA) using GraphPad Prism 5 software, followed by Dunnett's test. The difference was considered statically significant when p<0.05.

Results

Induction of diabetes with streptozotocin led to marked hyperglycemia within 3 days of injection. This hyperglycemic state was accompanied by significantly altered oxidative stress markers and antioxidant status. Streptozotocin-treated rats showed elevated levels of lipid peroxides (31.3 ± 0.8 nmol melondialdehyde/mg pro-

Table II

Effects of *V. volvacea* on anti-oxidant status (enzymatic and non-enzymatic)

Experimental groups	Control		Streptozotocin			
			Glibenclamide	<i>V. volvacea</i> (200 mg)	<i>V. volvacea</i> (400 mg)	Vitamin E
GPx (µg of gsh oxidised/min)	11.4 ± 0.5	5.7 ± 0.2 ^a	9.6 ± 0.3	9.0 ± 0.2	9.1 ± 0.4	9.2 ± 0.5
GST (µg of CDMB conjugation formed/min)	7.5 ± 0.2	4.6 ± 0.3 ^a	7.2 ± 0.2	6.3 ± 0.3	6.4 ± 0.2	7.0 ± 0.2
CAT (nmoles of H ₂ O ₂ oxidized / min/mg proteins)	59.0 ± 1.1	25.1 ± 0.6 ^a	49.4 ± 0.6	47.8 ± 1.2	47.7 ± 1.3	49.9 ± 1.1
SOD (IU/mg protein)	12.8 ± 0.4	5.3 ± 0.2 ^a	9.8 ± 0.3	7.7 ± 0.4 ^a	8.4 ± 0.3	8.0 ± 0.3
Vitamin C (µg/mg protein)	194 ± 1.6	111.9 ± 0.8 ^a	187.6 ± 2.4	169.8 ± 1.7	181 ± 0.6	189.2 ± 1.9
Vitamin E (µg/mg protein)	5.8 ± 0.1	3.0 ± 0.1 ^a	4.8 ± 0.1	4.4 ± 0.3	4.2 ± 0.1	5.3 ± 0.1

Values are expressed as Mean ± SE (N = 6); ^aindicates significantly different from control p<0.05

tein) when compared to control group rats (15.3 ± 0.5 nmol melondialdehyde/mg protein) (Table I). Extract (200 mg/kg) showed an increased level to 19.5 ± 0.5 nmol melondialdehyde/mg protein. Glibenclamide and vitamin E-treated groups also exhibited only moderate increase from the control.

Protein carbonyl was also significantly elevated (3.1 ± 0.2 nmol of carbonyl/mg protein) in streptozotocin-treated group. High dose of extract (400 mg/kg) maintained the carbonyl content (2.0 ± 0.02 nmol of carbonyl/mg protein) equal to control group. Treatment with glibenclamide and vitamin E showed only moderate elevation from the control rats.

Advanced glycation end products showed significant elevation in streptozotocin group (24.5 ± 0.3 AU/mg protein) when compared to control group (12.3 ± 0.1 AU/mg protein). However, mushroom extract treatment groups and glibenclamide treatment group exhibited non-significant changes from that of control. Glycosylated hemoglobin (HbA1c) percent was also elevated in streptozotocin-treated hyperglycemic rats ($12.2 \pm 0.2\%$). On the other hand, groups administered with extract

($8.1 \pm 0.1\%$), glibenclamide ($6.9 \pm 0.1\%$) and vitamin E ($9.7 \pm 0.1\%$) showed HbA1c content nearer to control level ($7.6 \pm 0.1\%$).

Significant reduction of reduced glutathione (GSH) in the streptozotocin-treated group was noted. *V. voluacea* extract (400 mg/kg) administered rats showed non-significant change from the control level. Rats treated with glibenclamide and vitamin E also exhibited only marginal change from the control values. Antioxidant enzyme glutathione peroxidase (GPx) was significantly reduced (5.7 ± 0.2 μ g of gsh oxidised/min) in streptozotocin-treated group rats (Table II). Treatment with extract (400 mg/kg) offered protection and the GPx level (9.1 ± 0.4 μ g of gsh oxidised/min) was nearer to control (11.4 ± 0.5). Similar trend of results was noted in glibenclamide and vitamin E-treated groups. In streptozotocin-treated groups, hyperglycemia caused significant reduction of glutathione-S-transferase (GST) enzyme (4.6 ± 0.3 μ g of CDMB conjugation formed/min). However, mushroom extract received rats showed only moderate reduction (6.4 ± 0.2 μ g of CDMB conjugation formed/min) at 400 mg/kg. Rats of groups glibenclamide and vitamin E also exhibited recovery

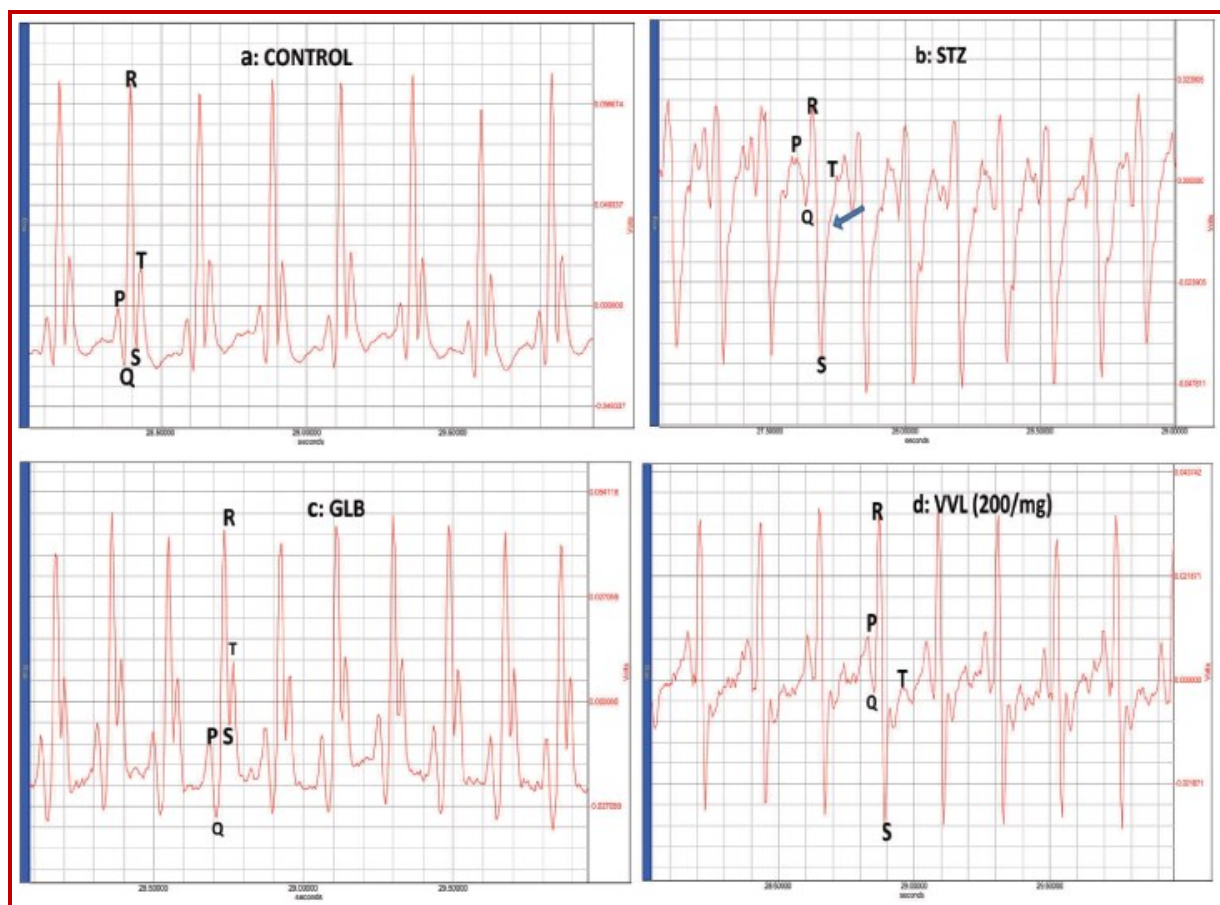


Figure 1: (a) Control group shows normal ECG pattern of QRS, QT and ST waves. (b) STZ treated group rats shows elongated QRS and QT. Prolonged ST interval exhibits with a deep notch of S (arrow). (c, d) Group rats show only moderate alterations of QRS, QT and ST intervals

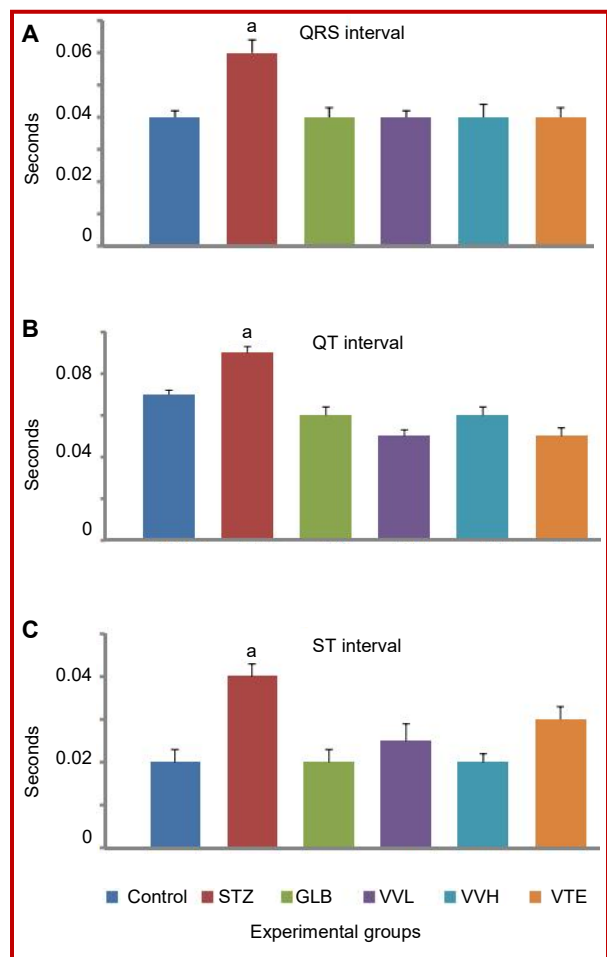


Figure 2: (A) Shows the QRS interval in treatment groups. Elongated pattern is evident in streptozotocin (STZ)-treated group. (B) Shows QT interval duration. STZ groups show elongated interval. (C) Shows prominent ST interval alteration in STZ treated group.

Values are Mean \pm SE of three observations. ^aindicates significantly different from control ($p < 0.05$)

nearer to control. Enzyme catalase (CAT) activity was significantly reduced (25.1 ± 0.6 nmoles of H_2O_2 oxidized /min/mg proteins) in streptozotocin-treated rats when compared to control group rats (59.0 ± 1.1 nmoles of H_2O_2 oxidized/min/mg proteins). Mushroom extract (400 mg/kg) treatment, however, reversed the level nearer to control. Treatment with glibenclamide and vitamin E also showed marked increase towards control level. Superoxide dismutase (SOD) was significantly reduced by hyperglycemia in streptozotocin-treated groups when compared to control rats (12.8 ± 0.4 IU/mg protein). Moderate increase to control level (8.4 ± 0.3 IU/mg protein) was noted in *V. voluacea* (400 mg/kg), glibenclamide (9.8 ± 0.3 IU/mg protein) and vitamin E (8.0 ± 0.3 IU/mg protein) administered rats.

Non-enzymatic anti-oxidants vitamin C and E in the

hearts of streptozotocin-treated groups were also significantly reduced. Vitamin C and E contents in streptozotocin groups were 111.9 ± 0.8 and 3.0 ± 0.1 $\mu\text{g}/\text{mg}$ protein, respectively when compared to control group (194 ± 1.6 and 5.8 ± 0.1 $\mu\text{g}/\text{mg}$ protein). VVH treatment (400 mg/kg) showed recovery of vitamin C (181 ± 0.6 $\mu\text{g}/\text{mg}$ protein) nearer to control. Glibenclamide and vitamin E-treated groups also exhibited marked increase over to control group rats.

ECG recording

The ECG pattern of control group showed normal QRS, QT and ST intervals (Figure 1). However, streptozotocin-treated group exhibited elongated QRS waves. VVH treated rats showed no changes from that of control. Glibenclamide and vitamin E-treated groups also recorded normal pattern. QT interval of streptozotocin-treated rats showed prolonged interval whereas *V. voluacea* treated rats showed no abnormalities. Similar pattern was observed in glibenclamide and vitamin E groups. Prolonged ST interval with deep notch of S was noted in streptozotocin-treated group. ST segment intervals in other groups *V. voluacea*, glibenclamide and vitamin E were nearer to control group (Figure 2).

Discussion

Diabetic hyperglycemia caused oxidative stress has been undoubtedly related to cardiac problems. In the present study, oxidative stress markers such as malondialdehyde, protein carbonyls and advanced glycation end products were significantly elevated in streptozotocin-treated groups. These changes were accompanied by reduction in anti-oxidant enzymes and vitamins. Free radicals lead to protein oxidation forming carbonyl groups and are possibly the most immediate vehicle for inflicting oxidative damage on cells (Dalle-Donne et al., 2003). The oxidatively modified protein causes protein fragmentation, crosslinking and unfolding which leads to loss of protein function resulting in cellular impairment (Bulteau et al., 2001). Increased level of malondialdehyde and protein carbonyl in the heart tissues of streptozotocin-treated rats reflect oxidative stress as the consequence of increased free radical generation. Attenuation of lipid peroxidation and protein oxidation was noted in the *V. voluacea* treated groups, an evidence of prevention of oxidative stress reaction.

Chronic hyperglycemia usually results in the formation of advanced glycation end products which play a key role in diabetic cardiomyopathy through over production of reactive oxygen species (Norton et al., 1996). In the present study, advanced glycation end level was significantly elevated in the heart of streptozotocin group showing hyperglycemic oxidative

stress. Deposition of advanced glycation end has been reported in atherosclerotic plaques and within myocardium fibers (Sakata et al., 1995) leading to systolic and diastolic dysfunctions and heart failure. Treatment with mushroom extracts at both dose levels didn't cause significant changes in the advanced glycation end level suggesting that the mushroom is effective in preventing advanced glycation end formation either by exerting hypoglycemic effect or minimizing free radical formation, or by both mechanisms.

Streptozotocin-treated rats had shown a significant increase in the percentage of HbA1c due to persistent hyperglycemia. Increased non enzymatic glycosylation is one of the possible mechanisms linking hyperglycemia and vascular complications of diabetes. During diabetes, the excess glucose present in the blood reacts with hemoglobin to form HbA1C (Kondeti et al., 2010) and is considered as an index for chronic hyperglycemia. Administration of mushroom extracts displayed a remarkable decrease in glycosylated hemoglobin levels which might be due to its normoglycemic activity. The effect is comparable to the hypoglycemic drug glibenclamide.

Reduced glutathione (GSH), a ubiquitous anti-oxidant synthesized in all living cells and functions as an effective intracellular reductant (Rahman and MacNee, 1999), is significantly reduced in streptozotocin-treated groups. Reduced availability of GSH may also reduce the activities of GPx and GST since it acts as an important substrate for GPx, GST and several other enzymes involved in the free radical scavenging action (Rathore et al., 2000). The extract of *V. volvacea* and glibenclamide treated rats showed increased levels of GSH, suggesting that the extract has the potency to increase biosynthesis of GSH and reduce the oxidative stress.

Streptozotocin-treated groups in the present study showed drastic reduction of GPx and GST indicating severe oxidative stress. GPx maintains the structural and functional integrity of the cytosolic and mitochondrial compartments whereas GST, a group of selenium independent enzyme removes the stable peroxides from the system, resulting in the reduction of peroxide-induced damage. GPx and GST along with glutathione catalyze the reduction of hydrogen peroxide into non-toxic metabolites (Bruce et al., 1982). The reduced GST activity may be due to the inactivation caused by reactive oxygen species (Andallu and Varadacharyulu, 2003). The recovery of these anti-oxidant enzymes in the *V. volvacea* treated group can be attributed to protection mediated to organs of synthesis and by increased GSH production.

SOD and CAT enzymes are important scavengers of superoxide ion and hydrogen peroxide. These enzymes prevent the generation of hydroxyl radical and protect

the cellular constituents from oxidative damage. A decrease in SOD activity can result in the decreased removal of superoxide anions, which can be harmful to the myocardium (Sharma et al., 2001). Reduction in the activities of these enzymes in the heart tissues of streptozotocin-treated hyperglycemic rats revealed excessive formation of superoxide and hydrogen peroxide anions. Treatment with *V. volvacea* either impaired the free radical generation or enhanced the activities of SOD and CAT to scavenge the excessive free radicals.

Vitamins C and E were significantly reduced in the heart of streptozotocin-treated groups indicating hyperglycemia caused damages and reduced bioavailability. Vitamin C, a powerful water soluble reducing agent directly scavenges singlet oxygen, superoxide and hydroxyl radicals (Kitts, 1997). Vitamin E, the major lipophilic anti-oxidant present in cell membranes and lipoproteins protects myocardial membranes and inhibiting the oxidation of lipoproteins (Upston et al., 1999). Oral administration of *V. volvacea* significantly increased the levels of vitamin C and vitamin E in heart tissues.

In the present study, QRS, QT and ST intervals were prolonged in streptozotocin-treated hyperglycemic rats. Widening of QRS is a sign of abnormal interventricular conduction and prolonged QT interval results in ventricular arrhythmia, an indicator of increased CVD risk (Whitsel et al., 2000). Rats treated with *V. volvacea* showed only minor alterations of the ECG pattern when compared to control indicating protection by the mushroom.

Mushroom compounds, polysaccharides, β -glucans, lectins, lactones, terpenoids, alkaloids, sterols and phenolics (Chung et al., 2010) are found to have anti-oxidant potential. *In vitro* studies of *V. volvacea* also showed antioxidant activity (Punitha and Rajasekaran, 2014). Mushroom polysaccharides restore pancreatic cell function to increase insulin input leading to reduction of glucose and improve sensitivity of peripheral cells to insulin (Misra et al., 2009). The bioactive polysaccharide β -glucan is abundant in *V. volvacea* (Cheung, 1996). The cardioprotection offered by the mushroom *V. volvacea* could primarily be attributed to its hypoglycemic role thereby reducing the oxidative stress and secondarily by reducing the free radicals.

Conclusion

Regulating the blood glucose level and balancing the oxidant-anti-oxidant levels, *V. volvacea* extract could normalize the cardiac tissue damage in hyperglycemic rats. In addition to its extensive use as delicious food, *V. volvacea* can be used as a remedy for the prevention

and management of cardiovascular complications during diabetes.

Financial Support

Self-funded

Ethical Issue

All the procedures were performed in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines and approved by the Institution Animal Ethical Committee (51/IAEC/SASTRA/RPP).

Conflict of Interest

Authors declare no conflict of interest

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