

***Lonicera japonica* protects spargue-dawley rat against 11 β -HSD1 induced endothelial dysfunction in metabolic syndrome**

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Abstract

Objective: To study the pharmacological evaluation of *Lonicera japonica* with reference to metabolic syndrome in order to restore 11 β -HSD1 induced endothelial dysfunction in SD rats.

Introduction:

Metabolic syndrome is multidisciplinary metabolic disorder in which Diabetes Mellitus, Hypertension and Obesity are presented jointly. Chlorogenic acid is a major bioactive component in the flower buds of *Lonicera japonica* having 11 β -HSD1 property. 11 β -HSD1 induced endothelial dysfunction has been postulated to be a major contributor to the pathogenesis of Metabolic Syndrome.

Methods: Present studies were aimed towards investigating the effectiveness of this LJALE extract against STZ-HFD induced diabetes and associated complications, Fructose induced hypertension and HFD induced obesity respectively using SD rats. Parameters like ex vivo 11 β -HSD 1 inhibition assay, Vascular reactivity study, Nitric Oxide and Peroxynitrite have been measured.

Results: Chlorogenic acid isolated from LJALE could dose dependently inhibit the 11 β -HSD1 activity. LJALE attenuate the 11 β -HSD1 induced endothelium dysfunction in rats. LJALE treated obese animal reflect significant increased Nitric oxide and decreased Peroxynitrite.

Conclusion: From all above statements It has been concluded that *Lonicera japonica* protects SD rats against 11 β -HSD1 induced endothelium dysfunction in Metabolic Syndrome.

Keywords: *Lonicera Japonica*, 11 β -HSD1, Metabolic Syndrome

1. Introduction

The metabolic syndrome, one of the most common clinical conditions in recent times, represents combination of cardio metabolic risk determinants, including central obesity, glucose intolerance, insulin resistance, dyslipidemia, non-alcoholic fatty liver disease and hypertension [1].

The prevalence of metabolic syndrome is high and increasing among adults parallel with lifestyle risk factors and changes in population demographics. Based on estimates from the Centers for Disease Control and Prevention, 57 million adults were classified as having metabolic syndrome in 2008, and this number climbed to 79 million in 2011. In fact, approximately 1 in 4 adults older than 20 years have this condition [2].

It has been found that flower buds of *Lonicera japonica* has also been traditionally indicated for treatment of diabetes, hepatic disorders, antiinflammatory disorder anti cancer activity [3,4] *Lonicera japonica* Thunb. (Caprifoliaceae), a widely used traditional Chinese medicine, was known as Jin Yin Hua (Chinese), Ren Dong and

Japanese honeysuckle [5]. Since 1995, *Lonicera japonica* has been listed in the “Pharmacopoeia of the People’s Republic of China” and more than 500 prescriptions containing *Lonicera japonica* have been used to treat various diseases in China [6].

Chlorogenic acid is a major bioactive component in the flower buds of *Lonicera japonica* [7]. Chlorogenic acid received more and more attention because of its 11 β -HSD1 inhibitory activity [8], Macrovascular and microvascular diseases are currently the principal causes of morbidity and mortality in patients with Metabolic Syndrome. 11 β -HSD Enzyme plays a very important role in biosynthesis of glucocorticosteroids [9]. 11 β -HSD1 is a glycosylated membrane protein, oriented into the lumen of endoplasmic reticulum (ER). It is generally located on Adipose tissue, Liver, Blood vessels. 11 β -HSD1 catalyse the interconversion from inactive cortisone to active cortisol.

As the main site of lipid and carbohydrate metabolism, the liver has a crucial role in the pathogenesis of the metabolic syndrome. 11 β -HSD1 induced endothelial dysfunction has been postulated to be a major contributor to the pathogenesis of Metabolic Syndrome. Growing data suggest the role of hepatic 11 β -HSD1 in the development of abnormalities in carbohydrate and lipid metabolism that occur in the metabolic syndrome. Therefore, the expression and activity of this enzyme are promising therapeutic targets for the future. Chlorogenic acid is a principle chemical constituent of *Lonicera Japonica*. The hepatic and adipose G6PT-H6PDH-11 β -HSD1 triad plays a crucial role in the pathogenesis of metabolic syndrome [10]. Action of 11 β -HSD1 is maintain and regulated by Glucose 6 Phosphate Transporter (G6PT). 11 β -HSD1 activity is significantly reduced in G6PT-deficiency (GSD1b) while remarkably increased in glucose- 6-phosphatase deficiency (GSD1a) which strongly supports the contribution of G6PT to metabolic sensing [11, 12]. Inhibition of G6PT with chlorogenic acid leads to decreased cortisol production in the liver, *via* the change of cofactor supply of 11 β -HSD1 [13]. These data suggest that pharmacological inhibition of G6PT can be a hopeful therapeutic option in the metabolic syndrome and in type 2 diabetes. So it may conclude that chlorogenic is a physiological inhibitor of 11 β -HSD1.

So the present investigation was aimed to study the pharmacological evaluation of *Lonicera japonica* with reference to metabolic syndrome in order to restored endothelial dysfunction in SD rats.

2. Materials & Methodology

Metabolic syndrome is multidisciplinary metabolic disorder in which Diabetes Mellitus, Hypertension and Obesity are presented jointly. Present studies were aimed towards investigating the effectiveness of this LJALE extract against STZ-HFD induced diabetes and associated complications, Fructose induced hypertension and HFD induced obesity respectively using Spargue-Dawley (SD) rats.

2.1 *Ex vivo* 11 β - HSD 1 inhibition assay [14]

Overnight fasted male SD rats were divided in to groups (n = 6) and respective groups were treated with vehicle (Normal saline), Diabetic control , Alcoholic extract of *lonicera japonica* (LJALE) (Lowest dose & Highest Dose) and Standard drug Metformin treated diabetic animals. After 4 hour of drug administration, all the animals were sacrificed and about 100 mg of liver and adipose tissue were excised and transferred to 900 μ l phosphate buffer saline (PBS) and minced coarsely with scissor. The tissues were thoroughly washed for three times and tissue pellet was collected. About 30 mg of the various tissue pellets were transferred to 85 μ l of DMEM (Dulbecco’s modified eagles media with 5.5 mM glucose) and 25 μ l NADPH (600 μ M) and 6 μ M of cortisone was added to all the samples to get final concentration of 100 μ M NADPH and 1 μ M cortisone, and all the sample tubes were incubated for 2 h at 37 $^{\circ}$ C. At the end of incubation period, all the tubes were centrifuged at 12,000 rpm for 10 min at 4 $^{\circ}$ C and 100 μ l of the supernatant was taken and 1200 μ l of ethyl acetate was added and vortexes for 10 min at 2000 rpm, then the samples were centrifuge at 12,000 rpm for 10 min at 4 $^{\circ}$ C. The 1000 μ l of supernatant was collected, the organic layer was evaporated to dryness, and the pellet obtained was subjected to LC–MS/MS analysis. The percentage inhibition of 11 β -HSD1 activity was calculated with respect to vehicle control.

2.2 Vascular Reactivity Study [15]

2.2.1 Preparation of rat aortic spiral preparation:

After 8 weeks from STZ induction, rats were sacrificed by cervical dislocation under mild anesthesia and thoracic aorta was isolated from the diaphragm by dissection and placed in oxygenated KHS at 4 $^{\circ}$ C. The aorta was then carefully dissected out, cleaned of connective tissues with the help of sharp iris scissor and care was taken not to damage the vessel. Then the aorta is spirally cut into 2.5 cm segments. Each spiral aortic preparation was

mounted in a 10 ml organ bath containing a modified Krebs Henseleit solution (The composition of Krebs solution was in mM; KHS; NaCl-118, KCl-4.7, KH₂PO₄-1.2, MgSO₄-1.2, CaCl₂-2.5, NaHCO₃-25 and glucose-5.5, sucrose-10) of pH 7.4 (at every hour pH was checked and adjusted if required) and osmolarity (280-308mmol/Kg). The solution was continuously aerated with carbogen (95% O₂+5% CO₂) at 37°C. A resting tension of 2 g, which we had determined to be the optimal resting tensions. Changes in the isometric contraction were recorded on student Physiograph. During the equilibration period and throughout the experiment the KHS in the organ bath was changed at every 15-20 minutes to minimize the variability of the responses.

2.2.2 Experimental protocol for vascular reactivity study:

After 2 hours of equilibration, the aortic strips were challenged with 80 mM KCl until a plateau was achieved by two-equipotent response. This was done to check the viability of the tissue. The 15 min of gaps should be kept in between each response so that it can come to its maximum resting state. In strip preparation acetylcholine was then added in the range of concentrations of 10⁻²⁵ to 10⁻¹⁵ M into the organ bath containing Krebs Henseleit solution until the response reached to maximum relaxation in 10⁻⁴ M Phenylephrine precontracted rat thoracic aorta.

2.3 Assay of NO by Griess Reagent [16]

2.3.1 Preparation of a Nitrite Standard Reference Curve

- Prepared 1ml of a 100µM nitrite solution by diluting the provided 0.1M Nitrite Standard 1:1,000 in the matrix or buffer used for the experimental samples.
- Designated 3 columns (24 wells) in the 96-well plate for the Nitrite Standard reference curve Dispense 50µl of the appropriate matrix or buffer into the wells in rows B–H.
- Added 100µl of the 100µM nitrite solution to the remaining 3 wells in row A.
- Immediately performed 6 serial twofold dilutions (50µl/well) in triplicate down the plate to generate the Nitrite Standard reference curve (100, 50, 25, 12.5, 6.25, 3.13 and 1.56µM), discarding 50µl from the 1.56µM set of wells. Do not add any nitrite solution to the last set of wells (0µM).

2.3.2 Nitrite Measurement (Griess Reaction)

- Allow the Sulfanilamide Solution and NED Solution to equilibrate to room temperature (15–30 minutes).
- Add 50µl of tissue homogenate to wells in duplicate or triplicate.
- Using a multichannel pipettor, dispense 50µl of the Sulfanilamide Solution to all experimental samples and wells containing the dilution series for the Nitrite Standard reference curve.
- Incubate 5–10 minutes at room temperature, protected from light.
- Using a multichannel pipettor, dispense 50µl of the NED Solution to all wells.
- Incubate at room temperature for 5–10 minutes, protected from light. A purple/magenta color will begin to form immediately.
- Measure absorbance within 30 minutes in a plate reader with a filter between 540nm.

2.4 Estimation of Peroxynitrite [17]

- Sodium nitrite (0.6 M, 10 ml) was rapidly mixed with acidified hydrogen peroxide (H₂O₂ 0.7 M; HCl, 0.7 M, 10 ml). The reaction was immediately stopped and the products stabilised by the addition of sodium hydroxide (1.5 M, 10 ml).
- All the reactions were performed on ice.
- Excess hydrogen peroxide was removed by passing the solution down a column of manganese dioxide (1.5 g)
- Removal of hydrogen peroxide was confirmed to be more than 98% efficient by measuring absorbance at 220 nm, thus eliminating any direct vasoactive actions of hydrogen peroxide. The resulting concentration of peroxynitrite was determined by measuring the absorbance at 302 nm in 1 M sodium hydroxide ($\epsilon_{302 \text{ nm}}=1670 \text{ M}^{-1} \text{ cm}^{-1}$)
- The concentration of peroxynitrite synthesised ranged from 100µM to 1nM All dilutions of peroxynitrite solutions were made in 0.5 M NaOH.
- Tissue homogenate was diluted with 0.5 M NaOH and take the absorbance at 302nm.
- Determine average absorbance value of each homogenate. Determine its concentration by comparison to the peroxynitrite Standard reference curve.

3. Results

3.1 11β-HSD 1 Assay

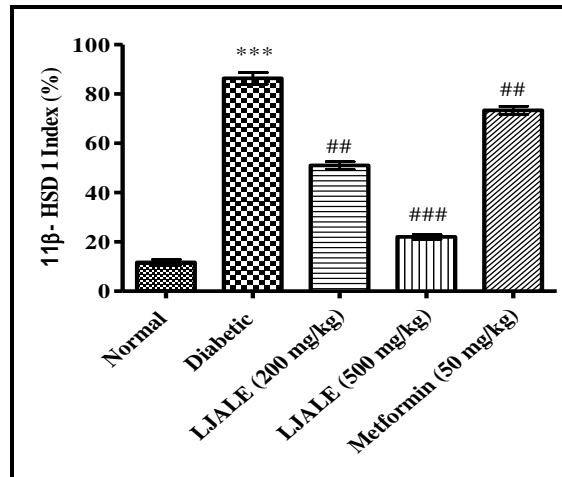


Figure 1: showing the comparison of 11β-HSD1 Index in Normal rats, Diabetic rats, LJALE (200 mg/kg), LJALE (500 mg/kg) and standard Metformin (50 mg/kg) treated diabetic rats. Values are expressed in mean ± S.E.M. n = 4-6. *** p < 0.001 Vs Normal Control and ## p < 0.01, ### p < 0.001 Vs Diabetic control.

3.2 Effect of *Lonicera japonica* alcoholic extract (LJALE) (200 mg/kg), LJALE (500 mg/kg) and standard Metformin (50 mg/kg) treatment on Acetylcholine induced relaxation in STZ-HFD diabetic rat.

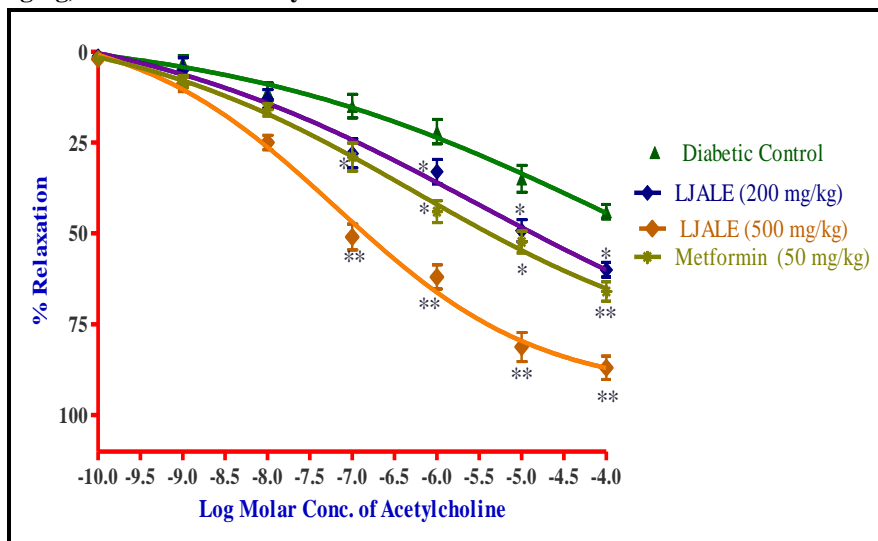


Figure 2: showing CRCs of Acetylcholine in endothelium intact aortic spiral preparations obtained from STZ-HFD diabetic rats and 4 weeks treated diabetic rats with LJALE (200 mg/kg), LJALE (500 mg/kg) and Metformin (50 mg/kg).

Values are expressed as mean ± SEM n = 4 - 6. For unpaired t test *p < 0.05, **p < 0.001 Vs diabetic Control.

Table 1: pD₂ and R max (%) of Ach. in Diabetic Control & treated diabetic rat thoracic aorta

Groups	Acetylcholine	
	pD ₂ values	R max %
Diabetic	6.13 ± 0.026	43.29 ± 1.99
LJALE (200 mg/kg)	6.49 ± 0.042*	60.05 ± 2.0*
LJALE (500 mg/kg)	7.18 ± 0.032**	87.02 ± 3.21*
Metformin (50mg/kg)	6.65 ± 0.063**	65.17 ± 2.6**

3.3 Effect of *Lonicera japonica* alcoholic extract (LJALE) (200 mg/kg), LJALE (500 mg/kg) and standard Metformin (50 mg/kg) treatment on Acetylcholine induced relaxation in Hypertensive rat.

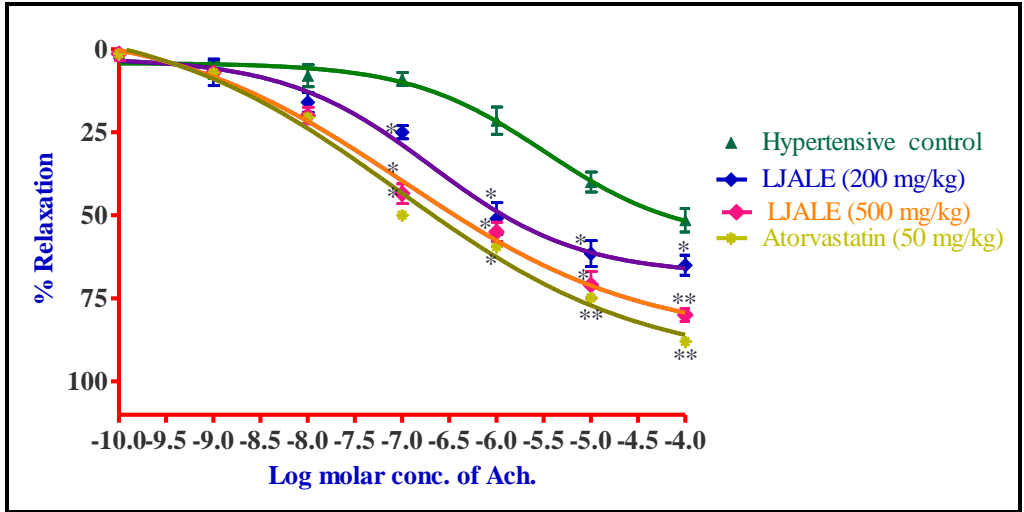


Figure 3: showing CRCs of Acetylcholine in endothelium intact aortic spiral preparations obtained from Hypertensive rats and treated hypertensive rats with LJALE (200 mg/kg), LJALE (500 mg/kg) and Atorvastatin (50 mg/kg).

Values are expressed as mean \pm SEM n = 4 - 6. For unpaired t test *p < 0.05, **p < 0.001 Vs hypertensive Control.

Table 2: pD₂ and R max (%) of Hypertensive Control and treated rat thoracic aorta.

Groups	Acetylcholine	
	pD ₂ values	R max %
Hypertensive	5.77 \pm 0.035	51.65 \pm 3.12
LJALE (200 mg/kg)	6.77 \pm 0.054*	65.89 \pm 2.50*
LJALE (500 mg/kg)	6.93 \pm 0.056**	79.32 \pm 2.0*
Atorvastatin (50mg/kg)	7.01 \pm 0.043**	86.94 \pm 2.0**

3.4 Effect of *Lonicera japonica* alcoholic extract (LJALE) (200 mg/kg), LJALE (500 mg/kg) and standard Orlistat (200 mg/kg) treatment on Acetylcholine induced relaxation in obese rat.

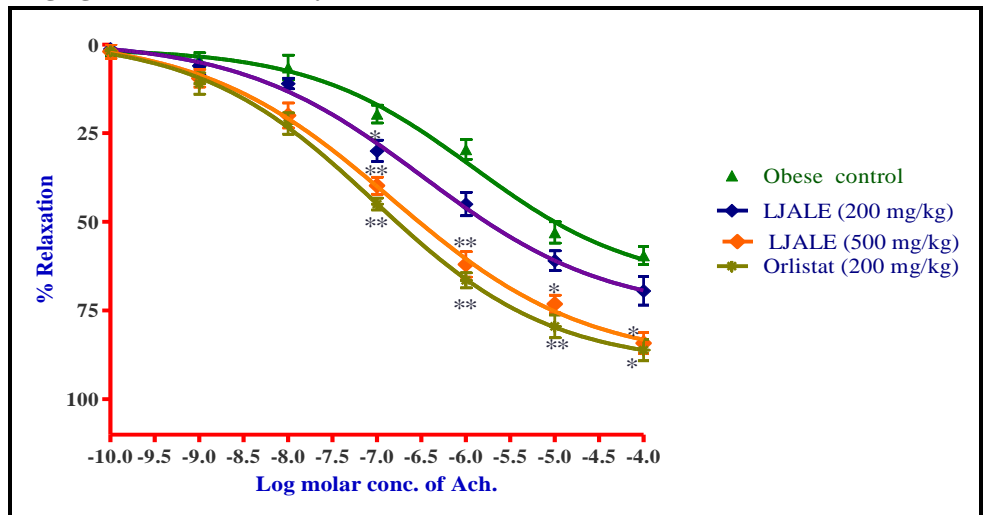


Figure 4: showing CRCs of Acetylcholine in endothelium intact aortic spiral preparations obtained from Obese rats and treated rats with obese *Lonicera japonica* alcoholic extract (LJALE) (200 mg/kg), LJALE (500 mg/kg) and Atorvastatin (50 mg/kg).

Values are expressed as mean \pm SEM n = 4 - 6. For unpaired t test *p < 0.05, **p < 0.001 Vs Obese Control.

Table 3: pD₂ and R max (%) of Obese Control and treated rat thoracic aorta.

Groups	Acetylcholine	
	pD ₂ values	R max %
Obese Control	6.1 ± 0.07	61 ± 2
LJALE (200 mg/kg)	6.9 ± 0.06	69 ± 4
LJALE (500 mg/kg)	6.9 ± 0.03**	83 ± 3
Orlistat (200mg/kg)	7.0 ± 0.04**	86 ± 3

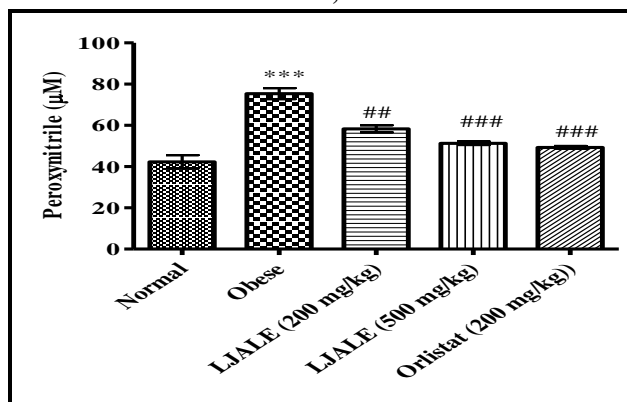
3.5 Comparison Peroxynitrite between Normal Control, Obese Control and Various Treatment groups:

Figure 5: showing the comparison of Peroxynitrite in Normal rats, Obese rats, *Lonicera japonica* alcoholic extract (LJALE) (200 mg/kg), LJALE (500 mg/kg) and standard Orlistat (200 mg/kg) treated Obese rats. Values are expressed in mean ± S.E.M. n = 4-6. *** p < 0.001 Vs Normal Control and ## p < 0.01, ### p < 0.001 Vs Obese control.

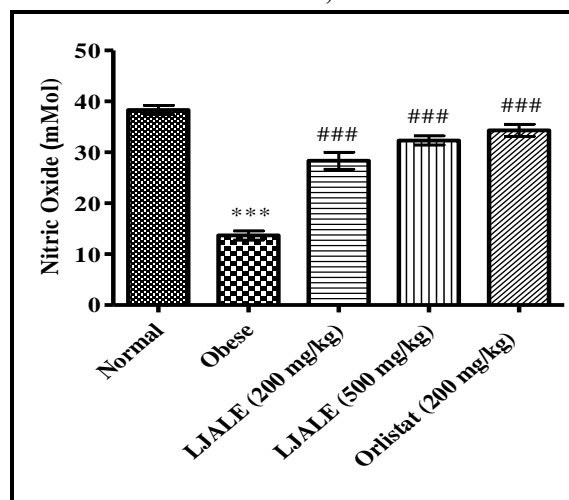
3.6 Comparison of Nitric Oxide between Normal Control, Obese Control and Various Treatment groups:

Figure 6: showing the comparison of Nitric oxide in Normal rats, Obese rats, *Lonicera japonica* alcoholic extract (LJALE) (200 mg/kg), LJALE (500 mg/kg) and standard Orlistat (200 mg/kg) treated Obese rats. Values are expressed in mean ± S.E.M. n = 4-6. *** p < 0.001 Vs Normal Control and ### p < 0.001 Vs Obese control.

4. Discussion**4.1 11β-HSD 1 Index**

- 11β-HSD 1 is mainly found in the tissues such as liver, blood vessels, adipose, skeletal muscle, brain and immune system. Over expression or increased activity of 11β-HSD 1 leads to increased in cortisol levels in the body. It has been postulated that prolonged hyperglycemia itself will activate the 11β-HSD 1 activity and ultimately leads to obesity, diabetes and other health problems [18].

- STZ-HFD treated diabetic animals showed significant ($p < 0.001$) increased in expression of 11β -HSD 1 enzyme compared to normal control animals. LJALE treated diabetic animals showed significant dose dependent inhibition of 11β -HSD1 activity in liver in ex-vivo conditions. Furthermore, at higher dose LJALE (500 mg/kg) have offered maximum inhibition ($p < 0.001$) of 11β -HSD 1 in liver compared to LJALE (200 mg/kg) ($p < 0.01$) (Fig. 1). LJALE (500 mg/kg) was found more potent than LJALE (200 mg/kg).
- Chlorogenic acid is a physiological inhibitor of 11β -HSD 1. These findings suggest that the Chlorogenic acid isolated from ethanolic extract of *Lonicera Japonica* could dose dependently inhibit the 11β -HSD 1 activity and hence thought to be useful in the treatment of diabetes.

4.2 Vascular Reactivity Study

- Enhance contractility in diabetic rat aorta could be due to deficient endothelial activity, enhancement of oxidative stress due to excessive production of oxygen free radicals and decreased antioxidant defense systems [19].
- Cortisone / cortisol ratio is an indicator of 11β -HSD 1 activity. 11β -HSD1 knockout mice possess a normal aortic contractory response [20]. These studies suggest that pre-receptor modulation of glucocorticoids in both ECs and VSMCs plays an important role in the regulation of vascular tone. Cortisol downregulate the expression of the inducible form of nitric oxide synthase within vascular smooth muscle cells [21], attenuate guanylate cyclase activity induced by atrial natriuretic peptide and reduce the biosynthesis of prostacyclin [22]. Thus, glucocorticoids have the ability to suppress vasodilating processes by potentiate the constricting hormones present in the circulation. It significantly decreased the expression level of eNOS in human endothelial cells. It enhance the production of endothelin leads to enhance vasoreactivity of blood vessels as well as inhibition of cofactor tetrahydrobiopterin required for the synthesis of nitric oxide [23]. So, from all above study it may have conclude that cortisol is responsible for the endothelial dysfunction. Vascular deterioration is one of the complicating features of human and experimental diabetes and hyperglycaemia is the primary cause of diabetic micro and macrovascular complications [24].

4.3 Diabetes Mellitus

- In *in vivo* vascular reactivity study, Both LJALE & Metformin treated group showed improvement in relaxation response of acetylcholine (10^{-10} M to 10^{-4} M) in phenylephrine induced contraction in diabetic rat thoracic aorta (Fig.2). Significant increased ($p < 0.01$) in pD_2 and R_{max} of acetylcholine were found in LJALE (200 mg/kg) ($p < 0.5$), LJALE (500 mg/kg) ($p < 0.01$) and Metformin ($p < 0.01$) treated group compared to HFF-STZ diabetic rats (Table 1). These results indicate that LJALE attenuate the HFF-STZ induced endothelium dysfunction in rats.

4.4 Hypertension

- Both LJALE & Atorvastatin treated hypertensive group showed improvement in relaxation response of acetylcholine (10^{-10} M to 10^{-4} M) in phenylephrine induced contraction in hypertensive rat thoracic aorta. Significant increased ($p < 0.01$) in pD_2 and R_{max} of acetylcholine were found in LJALE (200 mg/kg) ($p < 0.5$), LJALE (500 mg/kg) ($p < 0.01$) and Atorvastatin ($p < 0.01$) treated group compared to fructose induced hypertensive rats (Fig. 3, Table 2).
- These results indicate that LJALE attenuate the fructose induced vascular dysfunction in rats.

4.5 Obesity:

- LJALE (200 mg/kg) treatment did not showed any significant effects on vascular reactivity of aorta. LJALE (500 mg/kg) treated group showed improvement in relaxation response of acetylcholine (10^{-10} M to 10^{-4} M) in phenylephrine induced contraction in obese rat thoracic aorta. Significant increased ($p < 0.05$) in pD_2 and R_{max} of acetylcholine were found in LJALE (500 mg/kg) and Orlistat (200 mg/kg) treated group compared to HFF obese rats (Fig.4, Table 3).
- These results indicate that LJALE attenuate the HFF induced endothelium dysfunction in rats.

❖ Nitric Oxide:

- Obese condition is linked causally with diminished Nitric oxide (NO) bioavailability. NO is emerging as a central regulator of energy metabolism & body composition. NO has the capacity to reduce fat mass by increasing mitochondrial biogenesis, regulating adipose tissue signalling and increasing the expression of genes that promote oxidation of energy substrates [25].

- In this study HFD obese rats showed significant ($P < 0.001$) decreased level of NO as compared to normal rats. Elevated free fatty acid lowers NO bioavailability in obese animals. While LJALE and Atorvastatin treated hypertensive animal reflect significant ($p < 0.001$) increased the Nitric oxide (Fig.5).

❖ **Peroxynitrite:**

- Peroxynitrite (ONOO^-) is a reaction product of superoxide (O_2^-) with the nitric oxide. Peroxynitrite may play critical role in promoting NO_s (Nitric Oxide Synthetase) uncoupling.
- During uncoupling condition NO_s is deprived of L arginine and tetrahydrobiopterin (BH_4), an important cofactor for a normal NO_s activity, Under these conditions, NO_s can become dysfunctional and uncoupled, leading to the generation of O_2^- and H_2O_2 instead of NO. Uncoupling occurs in endothelial dysfunction, decreased NO bioavailability, increased O_2^- production and formation of peroxynitrite.[26]
- HFD rats showed significant ($P < 0.001$) increased level of tissue peroxynitrite as compared to normal rats. While significant ($P < 0.001$) restoration of tissue peroxynitrite was found in LJALE and orlistat treated HFD obese animals (Fig. 6).
- It may reflect that LJALE has potent antioxidant property to combat against obesity and associated vascular complication.

5. Conclusion

The metabolic syndrome is a clustering of metabolic risk factors, including Diabetes Mellitus, Hypertension and Obesity. In summary, our results demonstrate several important new findings potentially related to the ongoing epidemic of metabolic syndrome. Diabetic animals showed increased level of $11\beta\text{-HSD1}$ compared to normal control animals. It may conclude that there may be overexpression $11\beta\text{-HSD1}$ enzyme. It plays a crucial role in the restoration of endothelial dysfunction. Prolonged obesity decrease responsiveness of Acetylcholine towards rat thoracic aorta indicates the endothelial dysfunction in obese rats. Finally we can conclude eNOS uncoupling and endothelial dysfunction which are the major causes of vascular complications. It is concluded that LJALE (500 mg/kg) showed better restoration of Nitric Oxide and peroxynitrite level in obese rats. It was clearly demonstrated from our in-vivo study that LJALE (500 mg/kg) revealed better restoration of endothelial dysfunction in disease control rats compared to LJALE (200 mg/kg). From all above statements we can conclude that *Lonicera japonica* protects SD rats against $11\beta\text{-HSD1}$ induced endothelium dysfunction in Metabolic Syndrome. LJALE (500 mg/kg) can become a curative therapy or can be added as an adjuvant therapy in treatment of Metabolic Syndrome.

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