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RANDOMISED DOUBLE BLIND PLACEBO CONTROLLED STUDY TO EVALUATE THE EFFECT OF CARDIPRO ON ENDOTHELIAL DYSFUNCTION AND BIOMARKERS IN PATIENTS WITH TYPE2 DIABETES MELLITUS.

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ABSTRACT

Background:
Diabetes mellitus is considered as an important risk factor of cardiovascular disease. Oxidative stress has been implicated as the underlying cause of both macrovascular and microvascular complications associated with type2 diabetes mellitus. Studies have reported that endothelial dysfunction occurs in patients with diabetes much earlier than clinical manifestations of diabetic vascular complications. Endothelial dysfunction is mainly due to accelerated nitric oxide (NO) degradation by reactive oxygen species. Therefore, therapies aimed at reducing oxidative stress may benefit patients with type2 diabetes mellitus and those at risk for developing diabetes.

Objective:
The objective of the study was to compare the effect of CardiPro (Polyherbal preparation) with placebo on endothelial dysfunction and the biomarkers of oxidative stress in patients with type 2 diabetes mellitus.

Materials and Methods:
Eligible patients were randomized to receive either, one capsule of CardiPro (polyherbal preparation) containing *Terminalia arjuna* 100mg, *Embilica officianalis*100mg, *Withania Somnifera* 100mg, *Boerhaavia diffusa* 50 mg and *Ocimum sanctum* 50mg or one capsule of Placebo thrice daily orally. Assessment of endothelial function was performed by salbutamol challenge test at baseline and after 12 weeks of treatment. Blood samples were collected at baseline and post treatment for estimation of malondialdehyde, nitric oxide, glutathione which are markers of oxidative stress. Safety parameters were assessed at baseline and after 12 weeks of treatment.

Results: Total 54 patients completed the study. Treatment with CardiPro produced significant improvement in endothelial function. CardiPro produced significant change in Reflection index (RI) with salbutamol challenge test from -2.41 ±1.72 at baseline to
-6.65 ±8.03, ($p<0.05$) after 12 weeks of treatment. CardiPro also showed significant decrease in malondialdehyde and increase in nitric oxide and glutathione levels compared to baseline. However in placebo group there was no significant changes observed in any of the parameters. Both the treatments were well tolerated.

Conclusion:
The findings of the present study showed that the polyherbal formulation (CardiPro) improved endothelial function compared to placebo in type2 diabetes mellitus patients.

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death globally. More people die annually from CVDs than from any other cause as reported by World Health Organization (WHO). Smoking, hypertension, high LDL cholesterol, low HDL cholesterol and diabetes mellitus are the five major risk factors for CVD.[1] Diabetes is associated with an increase risk of atherosclerosis, which may result in coronary artery disease.[2]Physiological impairments that link diabetes mellitus (DM) with a marked increase in atherosclerotic vascular disease,

include platelet hyper-reactivity, a tendency for negative arterial remodeling, impaired fibrinolysis, increased inflammation, and endothelial dysfunction. Endothelial dysfunction, present at disease onset, may be the cause of atherogenesis that is present throughout the course of DM and associated with late-stage adverse outcomes.[3,4] This endothelial dysfunction results from reduced bioavailability of the vasodilator nitric oxide (NO) mainly due to accelerated NO degradation by reactive oxygen species.[4]

A currently favoured hypothesis is that oxidative stress, through a single unifying mechanism of superoxide production, is the common pathogenic factor leading to insulin resistance, β-cell dysfunction, impaired glucose tolerance (IGT) and ultimately to type 2 DM (T2DM). Furthermore, this mechanism has been implicated as the underlying cause of both the macrovascular and microvascular complications associated with type2 diabetes mellitus. It follows that therapies aimed at reducing oxidative stress would benefit patients with type2 diabetes mellitus and those at risk for developing diabetes.[5-7]

Many herbs possess potent antioxidant, anti-inflammatory and cardio-protective properties and are used by patients with increased risk of cardiovascular morbidity and mortality. *Terminalia arjuna* has been reported to have beneficial role in heart failure and possess anti-ischemic properties. The beneficial role of Terminalia arjuna was suggested to be due to the presence of potent antioxidant constituents which causes improvement in endothelial dysfunction seen in coronary artery disease and heart failure.[8] *Emblica officinalis Gaertn* (Amla) is widely used in Indian medicine for the treatment of various diseases. There are studies which show significant antihyperglyceamic and lipid lowering effects of *Embilica officinalis* in diabetic patients.[9] *Withania somnifera* (Ashwagandha, WS) has been an important herb in Ayurvedic and indigenous medical system for over 3000 years. Numerous studies indicate that ashwagandha possesses antioxidant, antitumor, immunumodulatory and antidepressive rejuvenating properties.[10,11] *Ocimum Sanctum* also known as Tulsi, has been reported in Indian traditional systems of medicine and is found to possess hypocholesterolemic, hypotriglyceridemic and hypophospho-lipidemic effects.[12] *Boerhaavia diffusa* is one of the most popular herbal remedies in India. Many studies show the antioxidant activity of *Boerhaavia Diffusa*.[13]

The individual constituents of CardiPro a polyherbal preparation, are *Terminalia arjuna* 100mg, *Embilica officinalis* 100mg, *Withania Somnifera* 100mg, *Boerhaavia diffusa* 50 mg and *Ocimum sanctum* 50 mg. These ingredients are hypothesized to have an effect on endothelial dysfunction and also possess sustained antioxidant activity and thereby help in maintaining the level of glutathione and peroxidase levels and thus prevent the damaging effects of oxidized-LDL on aortic smooth muscles. [14]

The present study was designed to evaluate the effect of CardiPro on endothelial dysfunction in patients with type 2 diabetes mellitus on background therapy with standard antidiabetic medication and to further study its probable mechanism of action.

MATERIALS AND METHODS

This was a prospective, randomized, double blind study conducted in the department of clinical pharmacology and therapeutics, after approval of the Institutional Ethics Committee of Nizam's Institute of Medical Sciences (NIMS), Hyderabad, India. All subjects gave written Informed consent prior to participation in the study.

Patients of either gender, aged 30-60 years, fasting plasma glucose of 110-126 mg/dL, a glycosylated haemoglobin (HbA1c) between 6.5% and 8% and on stable dose of anti-diabetic treatment (metformin 1500-3000mg) for the past 8 weeks prior to the screening visit and endothelial function defined as ≤ 6% change in reflection index (RI) on post salbutamol challenge test were included in the study. Patients with severe uncontrolled hyperglycemia, uncontrolled hypertension, cardiac arrhythmia, impaired hepatic or renal function, history of malignancy or stroke, chronic alcoholism, any other serious disease requiring active treatment, on any other drugs known to alter endothelial function and treatment with any other herbal supplements, were excluded from the study. Pregnant and lactating women were also excluded.

All the eligible patients were randomized to receive either one capsule of CardiPro thrice daily or one capsule of identical placebo thrice daily for 12 weeks as per prior randomization schedule. Subjects were reviewed at 4, 8 and 12 weeks of therapy. At each visit they were evaluated for efficacy and safety. Pharmacodynamic evaluation for endothelial function was conducted at every visit. Blood sample was collected for evaluation of biomarkers before and at end of treatment.

The primary efficacy measure was a change in endothelial dysfunction assessed by change in reflection index at 12 weeks in both the treatment groups compared to baseline. Secondary efficacy measures included change in markers of oxidative stress and change in lipid profile after 12 weeks of treatment in both the groups.

Investigations including hematology, hepatic and renal biochemical parameters were assessed before and at the end of the study and as and when required in case of any adverse drug reaction (ADR). Subjects were enquired for the presence of ADR and the same was recorded in the case report form. Compliance was assessed by pill count method.

Procedure for Assessment of Endothelial Function

Salbutamol challenge test employing digital volume plethysmography was used to assess endothelial function as per the procedure described by Chowienczyk *et al* [15] and Naidu *et al*.[16] Patients were examined in supine position after 10 minutes of rest. A digital volume pulse (DVP) was obtained using pulse tracer of photo plethysmograph (Pulse Trace PCA2, PT200, Micro Medical, Gallingham, Kent, UK) transmitting infra red light at 940 nm, placed on the index finger of right hand. Signals from the plethysmograph were digitized using a 12 bit analogue to digital converter with a sampling frequency of 100 Hz. Digital volume pulse (DVP) waveforms were recorded over 20 second period and the height of the late systolic / early diastolic portion of the DVP was expressed as a percentage of the amplitude of the DVP to yield the reflection index (RI), as per the procedure described in detail by Millasseau *et al*.[17] Measurement of reflection index (RI) was obtained from DVP recording. The mean of three such recordings was considered as representative value.

Patients were then administered 400µg of salbutamol by inhalation. After 15 minutes three measurements of RI were obtained again and the difference in mean RI before and after administration of salbutamol were used for assessing endothelial function. A change of ≤6% in RI post salbutamol was considered as endothelial dysfunction.

Biomarker evaluation*:* Malondialdehyde, [18] nitric oxide, [19] and glutathione [20] levels in serum samples of patients were estimated spectrophotometrically using standard methods. Lipid profile was assessed using standard techniques.

Safety Assessments:

Complete physical examination was conducted at every visit. Vital parameters including blood pressure, heart rate were recorded using Galaxy L&T multiparameter monitor and cardiac output was recorded using L&T Nivomon monitor at baseline and at end of treatment. Blood Samples were collected after an overnight fast of 12hrs for determination of haemoglobin, complete blood picture, HbA1c, blood urea and creatinine, liver function and lipid profile, which were measured using appropriate standard techniques. Further alterations in safety lab parameters and ADR reported were also evaluated.

Data Analysis

Data are expressed as mean ±SD. A paired t-test was performed within the group and unpaired t-test was performed between the groups. A p< 0.05 was considered to be statistically significant. All statistical analysis were performed using SAS software version 9.1.3 (USA).

Sample size Calculation**:** Assuming the proportion of subjects achieving improvement in endothelial function for herbal group is 72%, a sample of 60 subjects was calculated to provide 80% power to establish non-inferiority margin of 10% between both the groups. Based on these assumptions, a sample size of 27 evaluable subjects per group was required. Taking into account a follow up loss of about 10%, 30 subjects were required to be enrolled per group. The subjects had to be randomized into 1:1 ratio. Therefore total of 60 subjects had to be enrolled in the study to achieve 54 evaluable subjects.

RESULTS

A total of 60 subjects were enrolled in the study and 54 subjects completed the treatment course, four subjects were lost to follow-up; two other subjects relocated and hence were unable to continue the study. Demographic characteristics of the two study groups are shown in **Table I**.

Table I. Demographic characteristics of study groups (expressed as mean ±SD)

Parameter	CardiPro	Placebo
Total No.	27	27
Age	53.96 ± 8.13	50.38±8.24
Gender (Male/Female)	16/10	14/11
Weight (Kg)	67.98 ± 9.66	66.21±8.79
BMI(Kg/m^2)	25.67 ± 2.93	23.67±2.19

As seen from the above table, there was no significant difference between treatment groups in gender, age and body mass index.

Reflection index (RI) was used as a pharmacodynamic parameter to assess the endothelial function and malondialdehyde, nitric oxide and glutathione as markers of oxidative stress. The effect of the two treatments on these parameters is shown in **Table II.** CardiPro produced improvement of endothelial function, as indicated by a significant decrease in RI post salbutamol (p<0.05) when compared to baseline. There was significant reduction in malondialdehyde levels (p<0.01) when compared with baseline. Cardipro significantly increased the levels of biomarkers of oxidative stress such as nitric oxide (p<0.05) and glutathione (p<0.001). However there was no significant change in any of the above parameters from baseline in placebo group. There was significant change observed in absolute change in RI compared to placebo. **Figure1.** The mean percent reduction in malondialdehyde was 9% compared to placebo **Figure 2**. The mean percent increase in nitric oxide was recorded to be 43.32% and glutathione 39.95% compared to placebo **Figure 3 and Figure 4** respectively.

Table II. Effect of treatments on endothelial function and biomarkers of oxidative stress

Parameters	CardiPro (n=27)		Placebo (n=27)	
	Pretreatment	Post treatment	Pretreatment	Post treatment
Change in Reflection Index (%) Post Salbutamol challenge	-2.41 ±1.72	-6.65 ±8.03 ***,@**	-1.84 ±1.34	-0.76 ±2.60
MDA(nMol/ml)	2.87 ±1.12	2.62 ±1.12 #	3.38 ±0.68	3.45 ±0.62
NO (µMol/L)	26.32 ±11.00	37.08 ±25.61 ***,^**	25.28 ±10.61	23.88 ±9.22
Glutathione(µMol/L)	439.3 ±121.4	516.3 ±91.24 **$,@**	435.7 ±133.9	426.7 ±127.4

*-p<0.05 compared to baseline, #-p<0.01 compared to baseline and placebo
$-p<0.001 compared to baseline, ^-p<0.05 compared to placebo
@-p<0.01 compared to placebo

Figure 1 Absolute change in reflection index (RI) compared to placebo after 12 weeks of treatment.

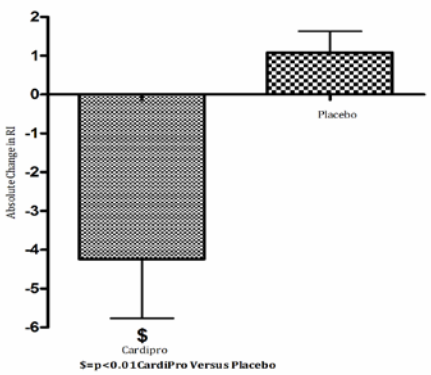

Figure 2 Mean percent change in Malondialdehyde (MDA) levels compared to placebo after 12 weeks of treatment.

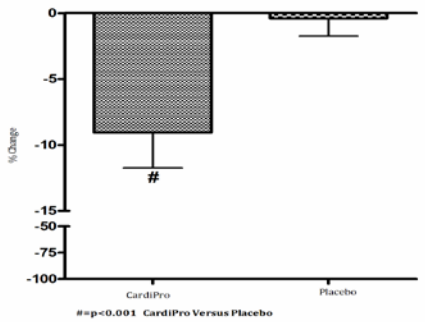

Figure 3 Mean percent change in nitric oxide levels compared to placebo after 12 weeks of treatment

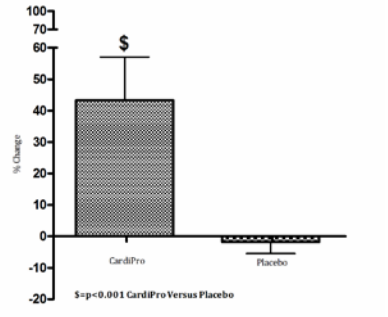

Figure 4 Mean percent change in glutathione levels compared to placebo after 12 weeks of treatment

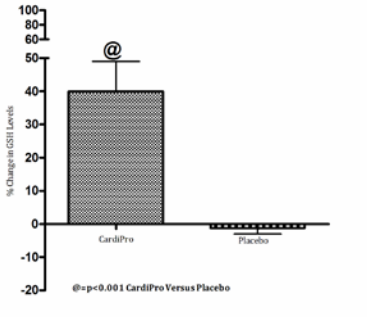

Table III. Changes in lipid profile after 12 weeks treatment with Placebo and Polyherbal (CardiPro)

Parameters	CardiPro(n=27)		Placebo (n=27)	
	Pretreatment	**Post treatment**	**Pretreatment**	**Post treatment**
TotalCholesterol (mg/dl)	187.62 ±28.42	162.60 ±18.55 #	183.60±28.69	196.4 ±21.88
HDL-C(mg/dl)	42.85 ±5.52	44.42 ±6.36 **$, @**	40.52 ±5.64	39.48 ±5.85
LDL-C (mg/dl)	131.58 ±39.69	111.65 ±22.65 #	152.0 ±39.51	156.0 ±36.87
Triglycerides (mg/dl)	186.6 ±45.40	144.70 ±41.71 #	197.16±33.74	199.84 ±29.99

$ = p<0.05 compared to baseline, **@=**p<0.01compared to placebo
 # = p<0.001 compared to baseline and placebo

The various constituents of CardiPro are reported to possess potent lipid lowering activity. In the present study also we have demonstrated the antilipidemic effect in diabetic patients with endothelial dysfunction. Treatment with CardiPro significantly reduced total cholesterol, LDL-C and triglycerides and significantly increased HDL levels compared to baseline, while there was no significant change in any of these parameters in placebo arm. **Table III**. The mean percent reduction compared to baseline in total cholesterol, LDL-C and triglycerides was 12.63%, 11.18% & 22.28% respectively and mean percent increase in HDL was 3.76% in cardipro group. When compared to placebo group the mean percent change in all lipid parameters were found to be significant.

Pharmacodynamic and Safety Assessment

There was no significant change observed in vital parameters (heart rate, blood pressure) measured by Galaxy L&T multiparameter monitor and cardiac output measured by L&T Nivomon monitor. Hematologic, hepatic and renal biochemical parameters were within normal limits with both treatments. Both the study medications were found to be safe, apart from two subjects complaint of diarrohea in CardiPro group and one subject with dyspepsia in placebo group.

No subjects discontinued the study because of these adverse events.

DISCUSSION

In the present study we have evaluated the effect of CardiPro versus placebo on endothelial function in patients with type 2 diabetes mellitus. The polyherbal preparation (CardiPro) showed beneficial effect on endothelial function along with a significant improvement in the biomarkers of oxidative stress such as nitric oxide and glutathione and decrease in the levels of malondialdehyde. Furthermore, CardiPro also significantly decreased total cholesterol, triglycerides, LDL-C and increased HDL, whereas placebo did not show any significant effect on endothelial function or other evaluated parameters.

Patients with diabetes have vascular complications and endothelial dysfunction is one of the early prognostic markers of atherosclerosis which may eventually result in cardiovascular disease.[21] Studies have reported that endothelial dysfunction occurs in patients with diabetes much earlier than clinical manifestations of diabetic vascular complications.[22,23] In our earlier study we have reported presence of endothelial dysfunction assessed by post salbutamol challenge with decrease in RI of <6% marker of endothelial dependent vasodilatation in diabetic patients.[15-17,24] Diabetes is associated with accelerated atherosclerosis and microvascular complications, which are the major causes of morbidity and mortality. Endothelial cell dysfunction is emerging as a key component in the pathophysiology of cardiovascular abnormalities associated with diabetes mellitus.[2]

Use of herbs for the treatment of cardiovascular disease in ayurveda, Chinese and unani systems of medicine has given a new lead to understand the pathophysiology of these diseases. Therefore, it is rational to use our natural resources for identifying and selecting inexpensive and safer approaches for the management of cardiovascular disease along with current therapy.[25] The bark of *Terminalia arjuna* is reported to possess cardioprotective property. A number of experimental and clinical studies have proved that dried bark powder of this plant have potent hypolipidemic, cardioprotective activity and also improved antioxidant status in patients with coronary artery disease.[26] Increased arterial stiffness, as measured by pulse wave velocity, is associated with cardiovascular risk factors and established coronary artery disease. Whilst arterial compliance is determined predominantly by structural factors, vascular endothelium is also involved. The vascular endothelium contributes to vascular tone and endothelial dysfunction has been implicated as an early functional alteration predating structural changes of the vasculature.[27] Bharani. A *et al*., [8] in their study showed *Terminalia arjuna* therapy for two weeks lead to significant reversal of impaired endothelial function in chronic smokers. Similarly in our present study, with CardiPro we have reported significant

improvement in endothelial function in diabetic subjects compared to placebo.

The roots of *Withania somnifera* (ashwagandha), is reported to have extensive therapeutic potential has immune-modulatory, antioxidant, hypoglycemic and anticancer activity. In the present study, the polyherbal formulation which also contains Withania somnifera as one of the constituent shows antioxidant action .

Conventional cardiac risk factors such as dyslipidemia, hypertension, smoking and type2 diabetes are associated with impaired endothelial function. The intact endothelium promotes vasodilatation principally via the release of NO-originally also called endothelium derived relaxing factor. Endothelium dependent vasodilators reduce pulse wave velocity suggesting nitric oxide plays a role in determining arterial distensability.[27] Free radical NO has emerged as a fundamental signalling device regulating virtually every critical cellular function and is a potent mediator of cellular damage in many conditions. Nitric oxide is produced in endothelial cells from the substrate L-arginine via endothelial nitric oxide syntahse (eNOS). Elevated asymmetric dimethylarginine levels cause coupling, a mechanism which leads to decreased NO bioavailability. The endothelial dysfunction associated with diabetes has been attributed to lack of bioavailable nitric oxide due to reduced ability to synthesize NO from L-arginine. New basic research insights provide possible mechanisms underlying the impaired NO bioavailability in type 2 diabetes.[28] In the present study, 12 weeks of treatment with polyherbal formulation significantly increased nitric oxide levels by 43.32% in type 2 diabetic patients compared to placebo. A study at Karachi found that fasting blood sugar and HbA1c levels were significantly high whereas serum NO levels were significantly low in diabetic normotensive and hypertensive patients compared to controls.[29] However researchers in Taiwan who assessed the NO level in aqueous humour in plasma observed no significant difference between any of the diabetic subgroups in plasma NO levels.[30] Prospective studies have established that reduction in NO bioavailability is a predictor of dyslipidemia as it is an endogenous and anti-atheroslcerotic molecule. By the dysfunction of the endothelial L-arginine-NO pathway, several cardiovascular risk factors impart their deleterious effects on the vascular wall including hypercholesterolemia.[28]

Oxidative stress induced by reactive oxygen species (ROS) also plays an important role in the etiology of atherosclerosis and coronary heart disease.[31] Economides *et al*.,[32] has suggested that oxidative stress is one of the mechanisms involved in endothelial dysfunction. Wide spread attention has been focused on involvement of oxygen free radicals in pathogenesis of diabetes. Cellular enzymatic (SOD) and non enzymatic antioxidants (GSH) act as primary line of defense to cope with the deleterious effect of these radical species. Jyoti S *et al*.,[33] in their study on hypoglycemic and antioxidant effect of *Ocimum sanctum* noticed that decreased malondialdehyde content 42.4% with concomitant increase in both enzymatic 50.2% and non enzymatic 85.5% antioxidant defense system on treatment with tulsi leaves. Pand *et al*.,[34] also reported significantly increased activity of the two antioxidant enzymes in the liver (SOD & catalase) following treatment with aqueous extract of *Ocimum sanctum*. Thus *Ocimum sanctum* may be linked and its action may be mediated through modulation of cellular antioxidant defense system. Further, invitro and animal studies have indicated that *Embilica officinalis* (amla) have potent antioxidant effect against several test systems such as superoxide radicals, hydroxyl radicals scavenging action and in systemic augmentation of antioxidant enzymes in animals.[35] In an earlier study Antony B. *et al*.,[36]showed the beneficial effects of amla on atherosclerosis and dyslipidemia. In a recent study Amir khan *et al*.,[37] have investigated the efficacy of antioxidant agent tocotrienols and *Boerhaavia diffusa* by analysing the lipid parameters, antioxidant enzymes as well as invitro oxidizability of low density lipoproteins (LDL). After 4 weeks of administration the test medication significantly reduced the overall oxidative burden and effectively ameliorated the above altered parameters. Thus indicating a strong hypolipidemic/antiatherogenic and antioxidant effect of tocotrienols and *Boerhaavia diffusa*. These results are in agreement with our study where we have demonstrated significant reduction in malondialdehyde along with increase in glutathione levels on treatment with CardiPro.

Hypercholesterolemia is a major risk factor for the development of atherosclerosis and is associated with coronary and peripheral vascular disease. Several lines of evidences show that the improvement of coronary artery disease (CAD) is associated with lowering hypercholesterolemia. To treat hypercholesterolemia, extensive interventions are recommended including diet contol, exercise and the use of hypocholesterolemic drugs. However some patients cannot tolerate the adverse events from these drugs, such as liver damage which necessitates the use of other safer and efficacious alternative medications.[38]

Twelve weeks treatment with CardiPro produced significant improvement in lipid profile in the present study.

Muhammed Shoaib Akhtar *et al*.,[9] evaluated the antihyperglycemic and lipid lowering properties of *Embilica officinalis* (amla) in normal and diabetic human volunteers. Significant decrease were observed in total cholesterol and triglycerides and improvement in HDL-C in normal and diabetic volunteers receiving 2 or 3gm *Embilica officinalis* powder per day. Antony B *et al*.,[36] in their study of *Embilica officinalis* also reported significant reduction in TC,LDL and TG whereas there was significant elevation of HDL. The results of their study at doses of 500mg/ day and 1000mg/ day brought significant reduction in the level of risk factors arising from dyslipidemia and inflammation.[35] The exact mechanism by which the amla exerts beneficial effect is presently not clear. Amla, like statin is credited with HMG CoA reductase inhibitory activity. Thus exerting beneficial effects on parameters.[39] In a recent study Udaya Kumar R *et al*.,[40] evaluated the hypoglyceamic and hypolipidemic effects of extracts of *Withania somnifera* root and leaves in alloxan induced diabetic rats and found them to be effective. Similarly Sarkar A *et al*.,[12] have observed that *Ocimum sanctum* (tulsi) leaves exert hypocholesterolemic, hypotriglyceridemic and hypophospholipidemic effects in rabbits. Further Mani *et al*.,[41] have also reported significant reduction in lipid profile in serum and tissue lipids in normal and diabetic rats treated with tulsi powder.

CONCLUSION

In conclusion, type 2 diabetes mellitus is associated with endothelial dysfunction which is mainly due to reduced nitric oxide bioavailability and increased oxidative stress. Treatment with CardiPro (polyherbal preparation) significantly improved endothelial function. CardiPro showed a favourable effect on endothelial dysfunction with increase in nitric oxide, glutathione and decreased levels of malondialdehyde, marker of oxidative stress. Both treatments were well tolerated. The present study was conducted in a limited number of participants, hence studies in more number of subjects are needed to explore the beneficial role of these ingredients of CardiPro on endothelial dysfunction.

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