

Antidiabetic Activity of *Caulerpa racemosa*: Role of Proinflammatory Mediators, Oxidative Stress, and Other Biomarkers



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ABSTRACT

A marine alga, *Caulerpa racemosa* (seaweed), exhibits few biological activities, such as antinociceptive/anti-inflammatory, antitumor, and growth regulator. This study aimed to determine the antidiabetic activity of this seaweed. High-performance thin-layer chromatography of *C. racemosa* ethanolic extract was performed to identify its active constituents. Antidiabetic activity of *C. racemosa* ethanolic extract (100 and 200 mg/kg) was evaluated using various biochemical paradigms against glipizide (5 mg/kg) in a streptozotocin-induced diabetes rat model. High-performance thin-layer chromatography revealed β -sitosterol as an active constituent and also indicated the presence of saponins and alkaloids. Treatment with *C. racemosa* ethanolic extract significantly reduced blood glucose levels in diabetic rats, and the degree of glucose reduction was comparable to that attained by glipizide treatment. The *C. racemosa* ethanolic extract treatment restored the impaired glycosylated hemoglobin level, liver glycogen level, glucose uptake by hemidiaphragm, and glucose transport by hepatic cells. Pretreatment with *C. racemosa* ethanolic extract also restored lipid abnormalities, elevated liver enzymes, elevated inflammatory markers, and depleted endogenous antioxidants. A superior effect was shown by *C. racemosa* ethanolic extract (200 mg/kg) over glipizide (5 mg/kg). Moreover, the restoration of the histoarchitecture of the pancreas by *C. racemosa* ethanolic extract (200 mg/kg) was comparable to that of the glipizide (5 mg/kg) treatment group. The present experimental findings demonstrate significant antidiabetic activity of *C. racemosa* ethanolic extract in diabetic rats using various biochemical paradigms. Further, *C. racemosa* ethanolic extract seems to be safe and does not affect vital organs adversely.

ABBREVIATIONS

ALP	Alkaline phosphatase
ALT	Alanine transaminase
AST	Aspartate Aminotransferase
CaR	<i>Caulerpa racemosa</i>
CaRE	<i>Caulerpa racemosa</i> ethanolic extract
CAT	catalase
CMC	carboxymethylcellulose
GSH	glutathione
HDL	high-density lipoproteins
HPTLC	high-performance thin-layer chromatography
IL-4	interleukin -4
LDL	low-density lipoprotein
MDA	malondialdehyde
NEFA	non-esterified fatty acids
SGOT	serum glutamate oxaloacetate transaminase
SGPT	serum glutamate pyruvate transaminase
STZ	streptozotocin
SOD	superoxide dismutase
TC	total cholesterol
TGA	triglyceride
VLDL	very-low-density lipoprotein

Introduction

Natural compounds derived from plant, microbial, and marine sources have been extensively used for therapeutic purposes since time immortal. However, their extensive use and increasing demand have resulted in insufficient plant-derived materials to satisfy market demands. Further, phytochemical research has focused more attention on discovering novel beneficial natural products. This situation has immensely contributed to stimulating the increasing interest in unconventional and unexplored sources of natural products. In this context, marine algae have attracted much attention over the past four decades [1]. Algae are omnipresent globally and are an important part of the aquatic ecosystem. Several seaweed species are used as therapeutics as well as food. The rheological properties of seaweeds have made them crucial as food additives. Apart from that, several bioactive compounds from marine algae are also known to exhibit various biological activities [2]. However, it has been observed from the literature that seaweeds are not investigated extensively for their antidiabetic activity.

Despite the presence of several hypoglycemic agents in the market, type 2 diabetes mellitus remains a major global health problem. Herbal products may act by several mechanisms to alleviate the diabetic symptoms, deriving multifaceted benefits [3]. Of all the herbal alternatives, the therapeutic properties of marine algae are the least explored. The hypoglycemic potential and bioactive compound present in seaweeds is a new unexplored potential area [4, 5].

These marine compounds either exhibit direct hypoglycemic activity or ameliorate complications associated with type 2 diabetes mellitus through other properties, viz. antioxidant, anti-inflammatory membrane stabilization, etc. Several animal studies have indicated an association between oxidative inflammation and the

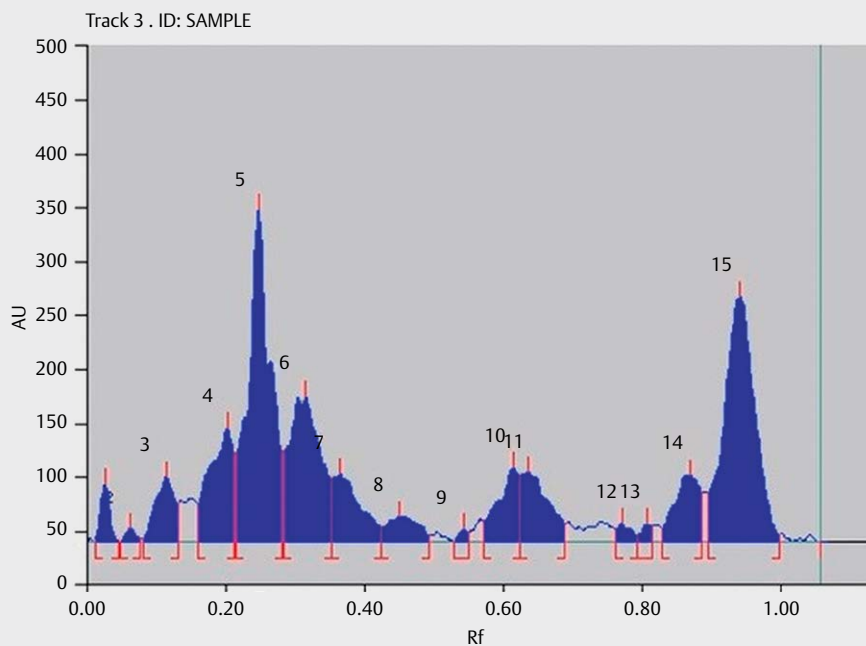
incidence of type 2 diabetes mellitus. Previous studies have shown that hyperglycemia and insulin resistance in diabetic patients lead to an elevated generation of reactive oxygen species [6, 7]. Furthermore, metabolic syndrome and insulin resistance are also associated with the generation of acute-phase inflammatory markers, showing the key role played by chronic low-grade inflammation in type 2 diabetes mellitus progression.

Seaweeds have traditionally been considered active agents for the treatment of inflammation, cancer, type 2 diabetes mellitus, bacterial infections, and obesity. CaR (sea grapes) is green seaweed that belongs to the Caulerpaceae family. CaR is extensively spread in tropical areas, particularly in Indo-Pacific Asia, where it is eaten either raw as a salad or cooked in vegetable soups. CaR has been considered a rich source of various bioactive components. Chemical analysis of CaR conducted previously has shown that CaR is rich in chemical constituents such as proteins, lipids, minerals, pigments, and vitamins [8]. Polysaccharides or polysaccharide extracts from CaR exhibit significant anticancer, anti-inflammatory, antibacterial, antiobesity, and antidiabetic properties [9]. Previous studies have also shown that CaR exhibits potent antioxidant activity [9]. Various extracts of CaR have also shown potent larvicidal activity [10]. No toxicity was seen in the sulfated polysaccharides detected in CaR and these reduced nociception and inflammatory activities are partly attributed to the activation of the hemoxigenase-1 pathway [11]. These data suggest that CaR not only exhibits versatile medicinal properties but it also is safe. Moreover, active components of CaR have the potential to activate various signaling pathways, such as immune signaling pathways, which can modulate the immune response for treatment of various diseases and disorders. With this background information, CaR was selected for this study. Phytochemical and acute toxicity studies were performed. Since the extracts were found to be safe, they were studied for hypoglycemic activity. Subsequently, its antidiabetic activity was evaluated in an STZ-induced diabetic rat model, along with its impact on proinflammatory markers, antioxidants, lipid, and serum biomarkers, and histoarchitecture of the pancreas to understand its underlying mechanisms of hypoglycemic activity [12, 13].

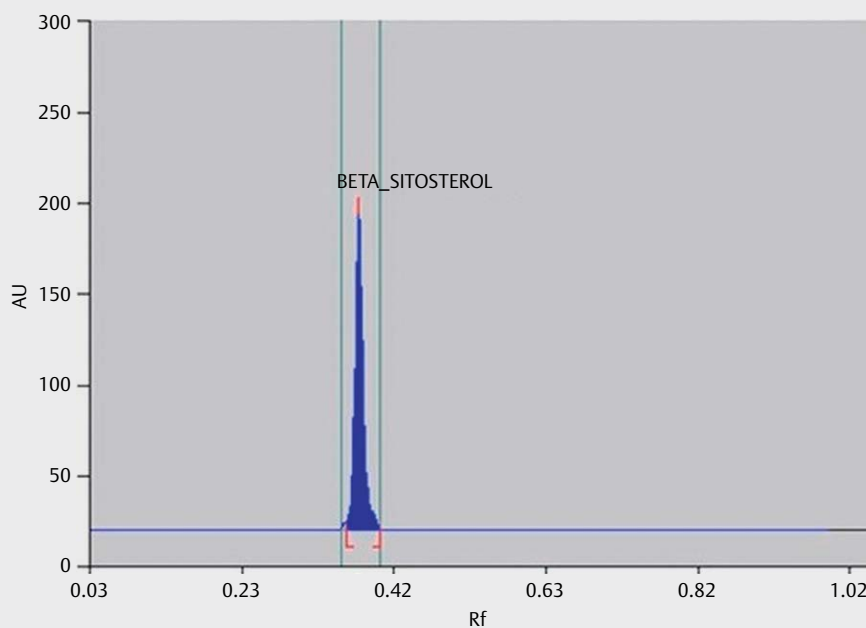
Results

The fingerprinting pattern of CaRE was studied using HPTLC. Around 15 different peaks were noted on the HPTLC fingerprint of CaRE (► **Figs. 1** and ► **2**). The plate was derivatized with Liebermann-Burchard reagent showed a pink colored compound, which was identified as β -sitosterol, (► **Fig. 3**) and others that saponins (like oleanolic acid, ursolic acid, and lupeol) as active constituents. Other plates sprayed with Dragendorff reagent showed only one compound, which presumably belonged to the alkaloid group.

In acute toxicity, the administration of CaRE at various doses did not lead to mortality until concentrations up to 2000 mg/kg body weight. Even at this dose, there were no gross behavioral changes or any adverse clinical symptoms, except mild sedation and analgesia. The rats were monitored once during first 30 min post-dosing and subsequently up to 24 h, followed by daily observation for 14 days.



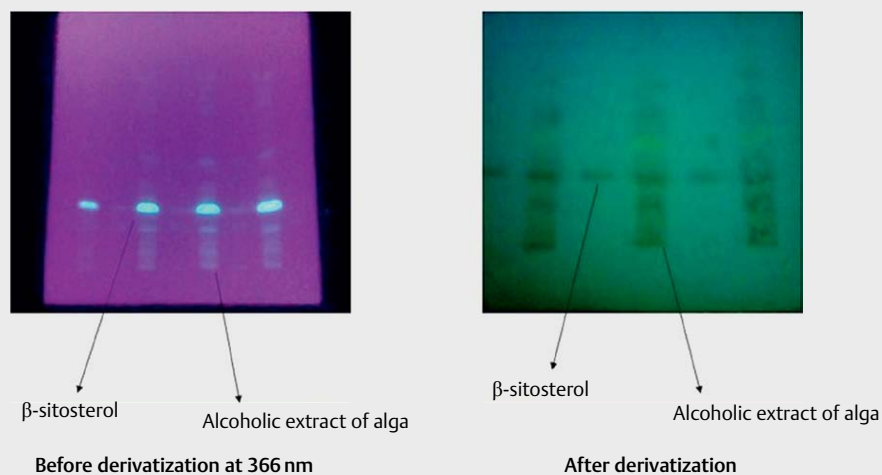
► **Fig. 1** HPTLC fingerprint of CaRE; solvent system petroleum ether: acetone (8:2 v/v) (Peak 6: Rf 0.38, peak area 5984.2).



► **Fig. 2** Peak display of standard β -sitosterol in CaRE.

A single-dose administration of the CaRE (200 mg/kg) on the 4th day after STZ administration led to a significant reduction in blood glucose levels at 1 and 6 h ($p < 0.01$). However, at 100 mg/kg dose, CaRE did not elicit any significant blood glucose reduction. Glipizide (5 mg/kg) treatment led to a greater reduction in blood glucose levels at 6 h compared to CaRE treatment (► **Table 1**).

STZ-induced diabetic rats showed a significant elevation in the levels of blood glucose. Oral administration of CaRE (100 and 200 mg/kg/day) and glipizide (5 mg/kg/day) to STZ-treated diabetic rats significantly reduced the levels of blood glucose. In groups treated with 200 mg/kg CaRE and 5 mg/kg glipizide, glucose levels reduced to almost normal levels. Treatment with both doses of



► **Fig. 3** HPTLC pattern of CaRE before and after derivatization at 366 nm.

► **Table 1** Acute and Sub-acute effects of CaRE on STZ-diabetic rats.

Acute effect of CaRE on STZ-diabetic rats				
Group and Treatment (mg/kg, p.o)	Blood glucose (mg %) at different time intervals (Mean ± SEM, n = 6)			
	0 h	1 h	3 h	6 h
Normal control	62.30 ± 6.30	62.83 ± 5.90	63.02 ± 7.10	63.11 ± 6.90
Diabetic control	492.33 ± 52.30	499.40 ± 57.60 ^{##}	433.50 ± 29.60 ^{##}	422.60 ± 44.10 ^{##}
CaRE (100)	445.20 ± 49.30	433.33 ± 47.80 ^{NS}	424.30 ± 42.30 ^{NS}	438.30 ± 54.30 ^{NS}
CaRE (200)	467.30 ± 49.60	366.16 ± 38.60 ^{**}	420.30 ± 52.80 [*]	132.20 ± 22.10 ^{**}
Glipizide (5)	467.00 ± 46.00	272.50 ± 21.30 [*]	268.82 ± 28.60 ^{**}	69.00 ± 8.90 ^{**}
Sub-acute effect of CaRE on STZ-diabetic rats				
Group and Treatment (mg/kg, p.o)	Blood glucose (mg %) at various time intervals (days) (Mean ± SEM, n = 6)			
	10	20	30	
Normal control	63.66 ± 2.90	62.16 ± 2.40	63.33 ± 2.40	
Diabetic control	389.66 ± 3.80 ^{##}	531.83 ± 3.30 ^{##}	449.16 ± 4.50 ^{##}	
CaRE (100)	355.50 ± 4.90 ^{**}	261.40 ± 3.50 ^{**}	108.66 ± 4.60 ^{**}	
CaRE (200)	115.16 ± 5.05 ^{**}	90.83 ± 3.50 ^{**}	94.33 ± 1.70 ^{**}	
Glipizide (5)	80.33 ± 3.50 ^{**}	125.63 ± 6.00 ^{**}	91.16 ± 3.60 ^{**}	

^{##} p < 0.01, when compared to normal control rats; ^{*} p < 0.05, when compared to diabetic control rats; ^{**} p < 0.01, when compared to diabetic control rats.

CaRE led to a significant reduction in levels of blood glucose and its hypoglycemic effect was comparable to that of 5 mg/kg glipizide on the 30th day (► **Table 1**).

In STZ diabetic rats, glucose uptake by the hemidiaphragm was markedly reduced. Administration of CaRE (100 and 200 mg/kg/day) significantly improved the glucose uptake via the hemidiaphragm ($p < 0.01$) (► **Table 2**). Glipizide-treated rats (5 mg/kg) did not exhibit increased glucose uptake via the hemidiaphragm. STZ treatment led to a significant reduction in hepatic levels of glycogen (► **Table 2**). Pretreatment with CaRE (200 mg/kg/day) signifi-

cantly prevented depletion of hepatic glycogen in STZ diabetic rats. The administration of glipizide (5 mg/kg/day) also significantly alleviated the liver glycogen reduction ($p < 0.01$) in STZ diabetic rats.

STZ-induced diabetic rats also exhibited a marked increase in the levels of HbA1c. Treatment with CaRE significantly reduced, dose-dependently, the elevated HbA1c levels (► **Table 2**). A similar reduction in the blood glucose level was also observed after glipizide (5 mg/kg) treatment. Treatment with both CaRE and glipizide produced significantly ($p < 0.01$) inhibited glucose transport in liver slices of STZ diabetic rats (► **Table 2**). STZ diabetic rats exhibited

► **Table 2** Effect of CaRE on various carbohydrate metabolism and lipid profile parameters in STZ-diabetic rats.

Effect of CaRE on various carbohydrate metabolism parameters in STZ-diabetic rats					
Group and Treatment (mg/kg, p.o.)	Glycosylated haemoglobin (Hb1Ac%)	Glucose uptake by diaphragm (mg/100 mg)	Liver glycogen (mg/gm)	Glucose transfer by liver (mg/gm)	
	(Mean ± SEM, n = 6)				
Normal control	5.00 ± 0.50	18.00 ± 2.10	41.00 ± 5.00	19.66 ± 1.10	
Diabetic control	12.00 ± 1.30 ^{##}	4.00 ± 0.60 ^{##}	13.00 ± 1.80 ^{##}	35.66 ± 3.80 ^{##}	
CaRE (100)	7.00 ± 0.70 ^{**}	9.20 ± 1.10 [*]	18.00 ± 2.30	12.35 ± 1.90 ^{**}	
CaRE (200)	6.00 ± 0.80 ^{**}	12.60 ± 1.80 ^{**}	28.00 ± 3.40 ^{**}	13.16 ± 1.05 ^{**}	
Glipizide (5)	8.00 ± 0.90 [*]	6.00 ± 0.50	34.00 ± 4.60 ^{**}	17.33 ± 1.50 ^{**}	
Effect of CaRE on lipid profile in STZ-diabetic rats					
Group and Treatment (mg/kg, p.o.)	Total cholesterol (mg/dL)	Triglycerides (mg/dL)	VLDL (mg/dL)	LDL (mg/dL)	HDL cholesterol (mg/dL)
Normal control	71.20 ± 5.29	98.60 ± 10.03	12.20 ± 2.03	37.32 ± 3.13	58.26 ± 61.91
Diabetic control	146.40 ± 10.10 ^{##}	212.60 ± 23.51 ^{##}	44.70 ± 6.88 ^{##}	177.70 ± 22.51 ^{##}	21.30 ± 1.17 ^{##}
CaRE (100)	92.00 ± 9.45 ^{**}	178.70 ± 23.63 ^{**}	28.52 ± 3.83 ^{**}	97.99 ± 10.13 ^{**}	39.80 ± 6.70 ^{**}
CaRE (200)	88.40 ± 9.58 ^{**}	146.60 ± 22.67 ^{**}	19.96 ± 2.89 ^{**}	82.24 ± 8.57 ^{**}	45.90 ± 4.17 ^{**}
Glipizide (5)	101.80 ± 12.50 [*]	189.80 ± 14.05 [*]	31.06 ± 4.85 [*]	130.36 ± 14.16 [*]	32.60 ± 5.68 [*]

^{##} p < 0.01, when compared to normal control rats; ^{*} p < 0.05, when compared to diabetic control rats; ^{**} p < 0.01, when compared to diabetic control rats.

significantly elevated levels of TGA, TC, VLDL, and LDL, and a decrease in the level of HDL in the serum ($p < 0.01$). Treatment with CaRE (100 and 200 mg/kg/day) significantly ameliorated the effects of STZ (► **Table 2**). Administration of glipizide also produced a similar effect on serum lipid levels.

The degree of lipid peroxidation was evaluated in terms of MDA formation. Increased MDA levels were observed in the liver and pancreas of STZ diabetic rats (► **Table 3**). In addition, a concomitant depletion of endogenous antioxidants enzymes, viz. CAT, GSH, and SOD, was observed in the liver and pancreas of STZ diabetic rats. Pretreatment with CaRE (100 and 200 mg/kg/day) significantly prevented MDA formation and restored the depleted levels of GSH, CAT, and SOD in the liver and pancreas of STZ diabetic rats (► **Table 3**). Treatment with 200 mg/kg CaRE led to a greater restoration of depleted antioxidants compared to treatment with glipizide in STZ diabetic rats.

STZ diabetic mice exhibited significantly elevated TNF- α and IL-4 levels in serum. Treatment with CaRE prevented the elevation of TNF- α and IL-4 levels in serum. The inhibitory effect of CaRE (200 mg/kg/day) on serum TNF- α and IL-4 levels was greater than that observed after glipizide (5 mg/kg/day) treatment (► **Table 4**).

STZ diabetic mice exhibited significantly elevated levels of serum liver biomarkers (AST, ALT, and ALP). Treatment with CaRE (100 and 200 mg/kg/day) for 30 days significantly prevented the elevation of these biomarkers. Glipizide (5 mg/kg/day) treatment also reduced the levels of serum biomarkers significantly; however, the degree of reduction was less compared to that obtained after CaRE treatment (► **Table 4**). The depleted serum levels of albumin and total protein were also restored significantly after treatment with CaRE and glipizide (► **Table 4**).

Control groups exhibited no abnormalities in the histoarchitecture of the pancreas (► **Fig. 4a**). However, STZ diabetic rats exhibited moderate to severe necrotic alterations in β -islets of Langerhans (► **Fig. 4b**). In a few mice, only a sporadic presence could be

observed. CaRE-treated (200 mg/kg/day) mice exhibited mild to moderate necrosis in β -islets (► **Fig. 4d**), which was comparable to that observed in glipizide-treated (5 mg/kg/day) groups (► **Fig. 4e**). Furthermore, CaRE (100 mg/kg/day) treatment led to mild improvement in the necrosis of β -islets (► **Fig. 4c**); however, the reduction in the number and size of β -islets was not significantly improved.

Discussion

Alcoholic extracts of marine algae CaRE were studied for fingerprinting patterns using HPTLC. We used several solvent systems, of which petether:acetone (8:2 v/v) was found to be most suitable for optimum resolution. Around 15 different peaks were detected and showed Rf at 0.05, 0.07, 0.12, 0.20, 0.38, 0.40, 0.44, 0.53, 0.60, 0.63, 0.78, 0.80, 0.89, and 0.99, which is represented in % area as 450.3, 256.1, 956.3, 1665.9, 7865.1, 5984.2, 4589.3, 658.1, 125.0, 357.3, 542.0, 170.3, 100.3, 564.2, and 9842.2 respectively (► **Figs. 1–3**). The plate was derivatized with Liberman-Burchard reagent, revealing a pink colored compound that comprised sosterol and saponins as active constituents. When a plate was sprayed using Dragandorff reagent, only one compound, probably an alkaloid, was identified.

In this study, we observed that CaRE elicited marked hypoglycemic activity compared to other extracts and was also found to have a safe toxicity profile. STZ is a selective pancreatic islets β cell cytotoxic agent and hence widely used to induce type 2 diabetes mellitus in rodents [14]. Intraperitoneal administration of STZ (55 mg/kg) to rats simulates conditions similar to type 2 diabetes mellitus, such as the elevation of blood glucose, glycosylated hemoglobin, and liver glycogen levels, along with a significant decrease in glucose uptake by the hemidiaphragm, increased glucose secretion by hepatic cells, and gluconeogenesis (deceased glucose transport for the formation of amino acids, citrate, etc.) [15]. Treat-

► **Table 3** Effects of CaRE on liver MDA and pancreatic MDA antioxidant enzymes in STZ-diabetic rats.

Effects of CaRE on liver MDA and antioxidant enzymes in STZ-diabetic rats				
Group and Treatment (mg/kg, p.o.)	Antioxidants enzymes (Mean ± SEM, n = 6)			
	MDA (nM of MDA/gm)	GSH (mg GSH/gm)	CAT (µM of H ₂ O ₂ /gm)	SOD (U/mg)
Normal control	7.20 ± 0.80	1.60 ± 0.30	36.32 ± 3.10	66.10 ± 8.20
Diabetic control	14.60 ± 2.40 ^{##}	1.10 ± 0.20 [#]	17.30 ± 1.30 [#]	35.00 ± 2.30 [#]
CaRE (100)	10.10 ± 1.20 *	1.40 ± 0.20 *	26.90 ± 1.30 *	46.80 ± 2.30 *
CaRE (200)	8.83 ± 1.00 **	1.50 ± 0.30 **	32.87 ± 4.70 **	56.50 ± 6.30 **
Glipizide (5)	11.20 ± 1.40	1.20 ± 0.20	24.30 ± 3.50	44.90 ± 6.80 *

Effects of CaRE on pancreatic MDA and antioxidant enzymes in STZ-diabetic rats				
Group and Treatment (mg/kg, p.o.)	Antioxidants enzymes (Mean ± SEM, n = 6)			
	MDA formation (nM of MDA/gm)	GSH (mg GSH/gm wet tissue)	CAT (µM of H ₂ O ₂ /gm)	SOD (U/mg of tissue)
Normal control	3.2 ± 0.4	1.6 ± 0.4	13.2 ± 2.2	20.2 ± 3.2
Diabetic control	8.6 ± 0.8 [#]	0.5 ± 0.2 [#]	6.5 ± 0.7 [#]	9.1 ± 1.3 [#]
CaRE (100)	6.3 ± 0.2	1.2 ± 0.2 *	9.5 ± 1.1 *	18.6 ± 1.9 *
CaRE (200)	4.3 ± 1.0 *	1.4 ± 0.3 **	11.4 ± 2.2 **	19.4 ± 5.3 **
Glipizide (5)	7.2 ± 0.6	0.6 ± 0.2	7.8 ± 2.6	13.5 ± 2.4

p < 0.01, when compared to normal control rats; * p < 0.05, when compared to diabetic control rats; ** p < 0.01, when compared to diabetic control rats.

► **Table 4** Effects of CaRE on serum nitric oxide (NO), cytokines and serum biomarkers in STZ-diabetic rats.

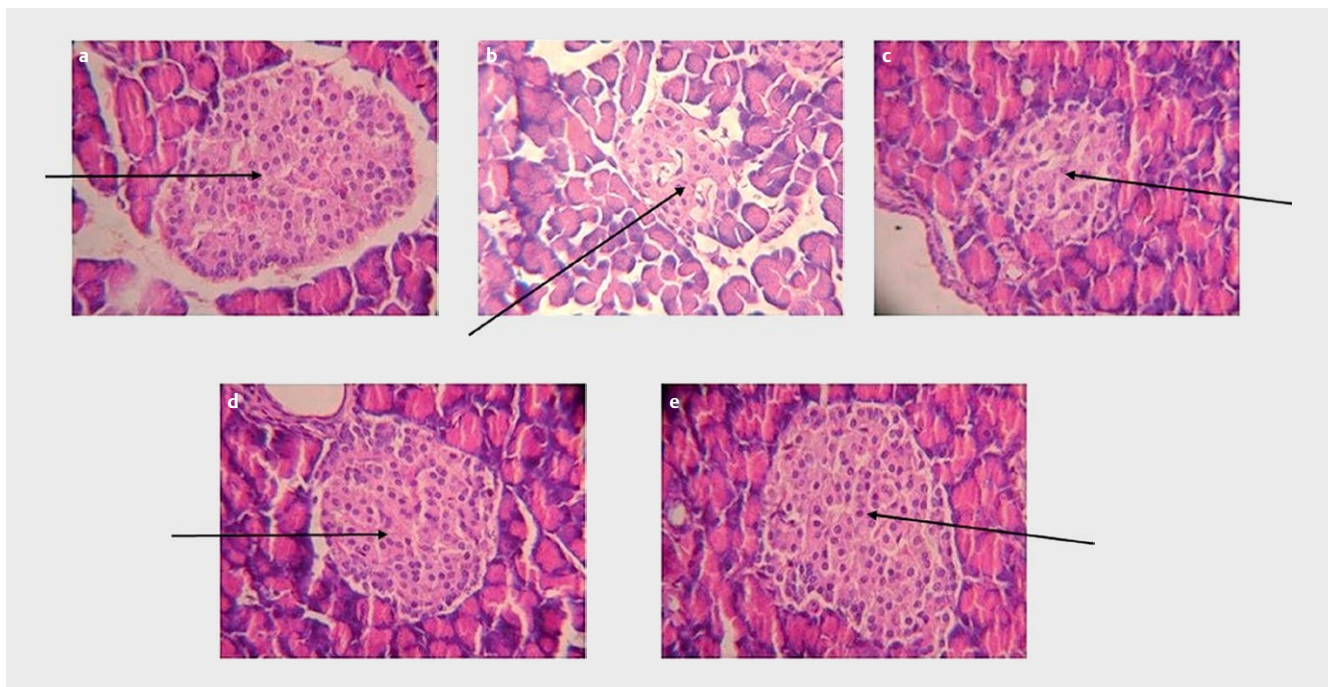
Effects of CaRE on serum nitric oxide (NO) and cytokines in STZ-diabetic rats				
Group and Treatment (mg/kg, p.o.)	NO (mcg/mg protein) (Mean ± SEM, n = 6)	Serum Cytokines (pg/mL) (Mean ± SEM, n = 6)		
		TNF-α	IL-4	
Normal control	39.00 ± 4.00	154.90 ± 65.41	182.00 ± 30.79	
Diabetic control	89.00 ± 9.00 ^{##}	387.60 ± 50.24 ^{##}	472.60 ± 60.16 ^{##}	
CaRE (100)	70.00 ± 8.00	291.30 ± 31.97	389.40 ± 44.74	
CaRE (200)	56.00 ± 8.00 *	182.00 ± 30.39 **	302.00 ± 31.16 *	
Glipizide (5)	49.00 ± 6.00 **	193.80 ± 32.03 **	231.30 ± 20.27 **	

Effect of CaRE on serum biomarkers in STZ-diabetic rats					
Serum Biomarkers	Group and Treatment (mg/kg, p.o.) (Mean ± SEM, n = 6)				
	Normal	Diabetic control	CaRE (100)	CaRE (200)	Glipizide (5)
AST (U/L)	22.2 ± 3.5	154.3 ± 16.5 ^{##}	82.6 ± 6.8 **	45.6 ± 4.5 **	111.3 ± 12.3 *
ALT (U/L)	12.3 ± 2.1	80.4 ± 12.3 ^{##}	37.2 ± 1.5 **	22.6 ± 5.2 **	52.3 ± 6.5
ALP (U/L)	10.3 ± 1.6	57.6 ± 9.3 ^{##}	26.3 ± 6.5 **	21.1 ± 2.3 **	33.1 ± 4.7 *
Serum creatinine (mg/dL)	0.8123 ± 0.0100	1.5570 ± 0.0600 ^{##}	0.8205 ± 0.0100 ⁺	0.9027 ± 0.0200 ⁺	0.9862 ± 0.0100 ⁺
Blood urea nitrogen (mg/dL)	20.60 ± 1.07	41.60 ± 1.72 ^{##}	22.20 ± 1.24 ^{**}	23.80 ± 1.06 ^{**}	30.20 ± 1.41 ⁺
Uric Acid (mg/dL)	1.97 ± 0.05	2.94 ± 0.08 ^{##}	2.11 ± 0.02 ⁺	2.12 ± 0.04 ⁺	2.43 ± 0.07 ⁺
Total protein (g/dL)	7.58 ± 0.23	3.97 ± 0.17 ^{##}	7.01 ± 0.33 ^{**}	7.26 ± 0.16 ^{**}	4.95 ± 0.08 ⁺
Albumin (g/dL)	5.44 ± 0.50	1.93 ± 0.10 ^{##}	4.15 ± 0.36 ^{**}	4.47 ± 0.26 ^{**}	2.44 ± 0.06

p < 0.01, when compared to normal control rats; * p < 0.05, when compared to diabetic control rats; ** p < 0.01, when compared to diabetic control rats.

ment of STZ diabetic rats with CaRE (100 and 200 mg/kg/day) and glipizide (5 mg/kg/day) for 30 days led to a sustained reduction in blood glucose and maximum reduction was seen on the 30th day, along with increased formation of and concomitant increase in glu-

cose uptake by skeletal muscles, liver glycogen (glycogenesis), and gluconeogenesis. The hypoglycemic activity 200 mg/kg CaRE was observed to be comparable to that of 5 mg/kg glipizide.



► **Fig. 4** : 4a – Effect of vehicle on histopathological changes in pancreas of normal rats; 4b – Effect of vehicle on histopathological changes in pancreas of diabetic rats; 4c – Effect of CaRE (100 mg/kg) on histopathological changes in pancreas of diabetic rats; 4d – Effect of CaRE (200 mg/kg) on histopathological changes in pancreas of diabetic rats; 4e – Effect of Glipizide (5 mg/kg) on histopathological changes in pancreas of diabetic rats.

One of the possible mechanisms linking hyperglycemia and vascular complications is the increase in non-enzymatic glycosylation. It has been shown in previous studies that during type 2 diabetes mellitus, excess blood glucose and hemoglobin react in a two-step process to form glycosylated hemoglobin (GHbA1c) [16]. This glycosylated HbA1c has been recommended as a standard of care to monitor type 2 diabetes mellitus. Therefore, we assessed the effect of CaRE treatment on the levels of glycosylated HbA1c. Treatment with CaRE and glipizide reduced the elevation of glycosylated hemoglobin significantly. The inhibitory effect of CaRE on GHbA1c in STZ diabetic rats was comparable to that induced by glipizide.

Oxidative stress plays a major role in type 2 diabetes mellitus pathogenesis. It is often responsible for the tissue damage associated with type 2 diabetes mellitus [17]. In diabetic patients, non-enzymatic protein glycation, oxidation of glucose, and increased lipid peroxidation leads to the generation of free radicals. This leads to the enzymatic degradation of cells and elevation in insulin resistance. Recent studies have shown that even the apolipoprotein component of LDL forms insoluble aggregates in the presence of hydroxyl radicals, leading to oxidative damage [18]. The main source of oxidative stress is the mitochondria. Modulation of insulin signaling by reactive oxygen or nitrogen species occurs via two ways. In response to insulin, these reactive oxygen/nitrogen species are produced for their physiological function and these reactive oxygen/nitrogen species negatively impact insulin signaling, which leads to insulin resistance [18]. The formation of free radicals may finally lead to the formation of MDA, a reactive aldehyde responsible for the formation of covalent protein adducts called advanced epoxidation end products.

Various studies have shown increased levels of MDA in diabetic patients, which has been associated with a poor prognosis of type 2 diabetes mellitus. A study was conducted with 120 diabetic patients, and it was found that MDA levels were high in type 2 diabetes mellitus patients and prediabetic patients compared to control group, suggesting that MDA levels are augmented during the progression of type 2 diabetes mellitus [19]. The MDA level has also been associated with insulin resistance. One study aimed to evaluate MDA levels in 96 prediabetic and 101 diabetic patients and establish its correlation to the Homeostatic Model Assessment of Insulin Resistance for the same subjects. It showed a statistically significant correlation between the level of MDA and insulin resistance in obese prediabetic patients [20]. Taking these results into consideration, we assessed if CaRE has potential to reduce the level of MDA. Superoxide dismutase catalyzes superoxide anions, which induce oxidative damage to cell membranes. GSH is a non-protein thiol found in all mammalian tissues. It is an efficient antioxidant that serves as an oxidative stress biomarker [21]. Depletion of SOD and GSH levels has been observed in type 2 diabetes mellitus patients [22].

The levels of antioxidant enzymes and lipid peroxidation were assessed in 90 type 2 diabetes mellitus patients and it was found that MDA levels were high in type 2 diabetes mellitus patients and levels of SOD and GSH were reduced [23]. Decreased levels of GSH might be one of the factors responsible for oxidative DNA damage in type 2 diabetes mellitus. The depletion of GSH and SOD may be attributed to diabetes-induced oxidative stress and free radical overproduction. CAT is another antioxidant enzyme found in peroxisomes and catalyzes H_2O_2 to H_2O and O_2 . These antioxidant enzymes work together to protect cells from free radicals induced by

oxidative stress [17, 24]. In the present study, we assessed if CaRE treatment has any effect on the levels of MDA, SOD, and GSH. CaRE treatment (30 days) prevented elevated MDA formation in the liver and pancreas of STZ diabetic rats, and we hypothesized that these effects might have helped restore the impaired endogenous antioxidant system and reinstate balanced antioxidant homeostasis in diabetic rats. Such antioxidant effects have likely contributed to its antidiabetic activity [25].

Various studies conducted on human and animal models have supported the fact that inflammation has a role in the initiation and progression of type 2 diabetes mellitus [26]. Cumulatively, evidence has suggested that the activation of inflammatory signaling pathways in the target cells of insulin action could be a contributing factor towards obesity, resistance to insulin, and related metabolic disorders, viz. type 2 diabetes mellitus [27].

Various studies have proven that the insulin resistance is associated with abnormal proinflammatory cytokine secretion and reduced anti-inflammatory cytokine generation [28]. Type 2 diabetes mellitus is often characterized by impairment of insulin function, which leads to metabolic syndrome [29]. TNF- α has been found to play a crucial role in insulin resistance. It also reduces glucose transporter type 4 expression [30]. Furthermore, activation of TNF- α induces the phosphorylation of the insulin receptor substrate-1, which works as an inhibitor of insulin receptors and down-regulates phosphatidylinositol-3 kinase activation [31, 32]. TNF- α levels have been observed to be elevated in type 2 diabetes mellitus patients of all age, disease duration, and ethnicity [33].

IL-4 contributes towards insulin resistance, which leads to increased glycogenolysis and decreased gluconeogenesis [34]. We observed a decrease in levels of proinflammatory mediators TNF- α and IL-4 after CaRE treatment, indicating that CaRE inhibits the secretion of these cytokines, thereby indicating improvement in insulin sensitivity. Our findings corroborate those of a previous study wherein improvement in insulin sensitivity was observed during long-term treatment with anti-TNF- α antibody infliximab in subjects who were insulin resistant [35].

Lipid abnormalities in diabetic patients, often termed as “diabetic dyslipidemia”, are characterized by high TC (Total cholesterol), LDL (low-density lipoprotein), and TG (triglycerides) levels, and low HDL (High-density lipoprotein) levels [36]. These abnormalities are common in prediabetic and type 2 diabetes mellitus patients. A recently published study suggested an association between a risk of type 2 diabetes mellitus and these lipid parameters [37]. Previously, hypertriglyceridemia and hypercholesterolemia have been reported in alloxan diabetic rats [38]. Various factors contribute towards the alteration in lipid metabolism in patients with type 2 diabetes mellitus, such as insulin deficiency or resistance, adipocytokines, and hyperglycemia. It has previously been shown that deficiency or insulin resistance results in the activation of intracellular hormone-sensitive lipase, leading to the increased release of NEFAs from the TGAs. This high circulating level of NEFA could increase the production of TGAs by the liver [36].

Diabetic dyslipidemia is a crucial risk factor of diabetes-associated cardiovascular disorders. Therefore, the treatment of dyslipidemia is an important strategy for the management of type 2 diabetes mellitus. We found that CaRE showed hypolipidemic activity in rats, further suggesting that CaRE could not only lower glucose

levels but could also manage other complications associated with type 2 diabetes mellitus. The exact underlying mechanism(s) for CaRE's hypolipidemic activity observed in the present experiments is not known. However, it is presumed that CaRE may directly down-regulate NADH and NADPH. The other mechanisms responsible for the hypolipidemic effect of CaRE include (1) inhibition of lipase or/and an inhibitory effect on lipolytic hormones on the fat depots and (2) cholesterol biosynthesis or absorption of lipids from the gastrointestinal tract or uptake process.

The hypolipidemic activity displayed by CaRE is beneficial and may be useful to reduce cardiovascular complications in diabetic conditions and may help to ameliorate the risk of acute myocardial infarction as claimed by the NCEP guidelines (2002) [39]. Increased hepatic serum markers, like transaminases, including ALP, have been documented in experimental models of type 2 diabetes mellitus [40]. The increased transaminases indicate impaired hepatic function largely due to cellular damage to hepatocytes. Furthermore, the increased transaminases may serve as a substrate to amino acids, thereby enhancing gluconeogenesis as well as ketogenesis. Such observations have also been clinically documented in diabetic patients [41]. In the present study, CaRE (100 and 200 mg/kg) and glipizide treatment in STZ diabetic rats inhibited the increased serum transaminases, which may be attributed to their hypoglycemic activity and/or modulation of carbohydrate, lipid, and protein metabolism. Alternatively, the decreased serum transaminases may be attributed to decreased oxidative stress and, consequently, reduced cytotoxicity to hepatocytes. Oxidative stress due to reactive lipid peroxidation recruits inflammatory cells and can be a potential triggering factor for the increased serum transaminases and enhanced formation of proinflammatory cytokines (IL-4 and TNF- α) in type 2 diabetes mellitus [42].

STZ treatment produced atrophy of β -islets of Langerhans associated with severe necrosis represented by pyknotic nuclei and acidophilic cytoplasm in the necrotic cells along with vascular degenerative alteration, resulting in the reduction of size and number of β -islets significantly. Only a sporadic presence was observed in few rats. The treatment of STZ diabetic rats with CaRE prevented the degree of necrosis dose-dependently. The improvement of necrosis of β -islets after treatment with 200 mg/kg CaRE was comparable to that induced by treatment with 5 mg/kg glipizide. Furthermore, the size and number of β -islets also improved after treatment with 200 mg/kg CaRE and 5 mg/kg glipizide. The reduction in necrosis and improvement in size and numbers of β -islet of Langerhans can be attributed to the amelioration of oxidative stress, which also leads to stabilization of their membranes.

The HPTLC analysis of CaRE revealed the presence of β -sitosterol as an active constituent. HPTLC analysis also showed the presence of alkaloid and saponin classes of compounds. It has been reported that β -sitosterol exerts an inhibitory effect on glycated hemoglobin, thiobarbituric acid-reactive substances, blood glucose, and nitric oxide, with upregulation in serum insulin levels and pancreatic antioxidant levels [43]. In agreement with these findings, Bumrela et al. have also confirmed the antioxidant potential of β -sitosterol both *in vivo* and *in vitro* [44]. HPTLC analysis also demonstrated the presence of saponins, which could be oleanolic acid, ursolic acid, and lupeol. Both oleanolic acid and ursolic acid showed significant protection against chemically induced liver injury in an-

imal models. Oleanolic acid and ursolic acid have also been long recognized to exert antihyperlipidemic and anti-inflammatory effects [45]. Various *in vitro* and animal studies demonstrated that lupeol possesses a potential cholesterol-lowering activity [46]. Further, caulerpin, a bisindole alkaloid, has also been isolated by De Souza et al. Further, caulerpin, a bisindole alkaloid, has also been isolated by Lucena et al. from CaRE and found to be a potent anti-inflammatory agent in various models of inflammation [47].

The antidiabetic and antioxidant activities exhibited by the phytoconstituents isolated (identified by HPTLC analysis) from the ethanolic extract of CaRE are in agreement with the various biological activities reported in the literature. The present experimental findings of CaRE demonstrated significant antidiabetic activity in STZ diabetic rats. The various biochemical paradigms observed in STZ diabetes included decreased glucose and lipid metabolism, reduction in levels of proinflammatory mediators, and oxidative stress alleviation. Histopathological studies suggested its antidiabetic activity is derived from the antioxidant activity. Downregulation of proinflammatory mediators improves the histoarchitecture of β -islets. Such effects may be attributed to the presence of saponins, β -sitosterol, and other phytochemicals. Further, saponins and β -sitosterol found in the extract may be responsible for such beneficial therapeutic effects. Hypolipidemic activity may be beneficial for patients with type 2 diabetes mellitus. CaRE seems to be safe and doesn't affect vital organs adversely. CaRE needs to be further assessed through human clinical trials to assess its therapeutic efficacy in type 2 diabetes mellitus.

Materials and Methods

Plant material

CaR seaweed was collected from the coastal area of Okha port in the Gujarat state of India. The collected seaweed sample was authenticated by the Department of Botany of a seaweed-based organization, Carrag Seaveg Pvt. Ltd., Bhavnagar, Gujarat, India. A voucher herbarium specimen, number CSPL/GA/CR07-09, has been preserved at Carrag Seaveg.

Drugs and chemicals

Glipizide powder (purity: 99.9%) was obtained from USV Pharma. STZ was procured from Sigma-Aldrich. Commercial kits used for biochemical analysis were: glucose reagent kit GOD/POD end point (Biolab), serum glutamate oxaloacetate transaminase (SGOT/AST) determination kit (Accurex Biomedical Pvt. Ltd.), serum glutamate pyruvate transaminase (SGPT/ALT) determination kit (Accurex Biomedical Pvt. Ltd.), alkaline phosphatase determination kit (Accurex Biomedical Pvt. Ltd.), lipid profile kit (Nirmal Labs), Keto-Test GK test strips for urine glucose determination (Diascreen), renal function parameters kit (Nirmal Labs), and reagent strips for urinalysis (Hypoguard). The control, test item, and reference drug were dissolved in 1% CMC.

Preparation of extract

Samples of dried seaweed were prepared by cutting or crushing the seaweed into small pieces and then grinding into a coarse powder. Coarse powders (50 g) were extracted with 500 mL of absolute

ethanol (95%) using a Soxhlet extractor at 500 °C. Occasional shaking was carried out for a period of 72 h. This crude extract was filtered off and subsequently evaporated to dryness at 45 °C. The concentrated dry mass was used for further investigations. The extract obtained was in ethanol.

High-performance thin-layer chromatography analysis of *Caulerpa racemosa* ethanolic extract

CaRE (10 mg) was dissolved in 10 mL of chloroform (final concentration: 1000 ng/ μ L) and analyzed using a CAMAG LINOMATE V automatic sample applicator on a 10 \times 10 cm HPTLC plate coated with 250 μ m layers of Silica gel G 60 F254 (Merck) and solvent system. Petether:acetone [(8:2) (v/v)] was used as the mobile phase. A Scanner-3 (CAMAG) was used to perform densitometer scans at 366 nm. The plates were derivatized in Liebermann-Burchard reagent.

Animals

Adult female mice (*Mus musculus* Swiss; 18–20 g) and male mice (*Rattus norvegicus* Wistar; 200–250 g) (age: 10–12 weeks) were used for the study. They were housed under standard conditions (room temperature: 24 \pm 3 °C, relative humidity: 45–55%, 12:12 dark/light cycle). They were fed ad libitum and given filtered tap water. Standard pellet diet was provided. The experimental protocol was reviewed and approved (CPCSEA/IAEC/SPTM/P-14/2014) by the Institutional Animal Ethics Committee for Purpose of Control and Supervision of Experimental Animals.

Acute toxicity studies

OECD guideline 423 (OECD 2001) in Swiss albino mice was followed for the acute toxicity study. The mice were orally administered CaRE (300–2000 mg/kg) solutions in 1% CMC. The mice were then kept under observation for 72 h.

Experimental induction of type 2 diabetes mellitus

STZ (55 mg/kg) solution in 0.1 M cold citrate buffer (pH 4.5) was administered intraperitoneally. Blank citrate buffer was used for the control mice. Initially, Wistar rats were screened for pre-dose glucose levels and then STZ was administered for inducing type 2 diabetes mellitus. After 72 h, blood was withdrawn from all animals and blood glucose levels were analyzed again. Mice with fasting blood glucose levels above 300 mg/dL and exhibiting polydipsia and polyphagia were selected for the 30-day anti-diabetic study.

Antidiabetic activity evaluation in streptozotocin-induced diabetic rats

Acute dose-response

Overnight fasted STZ diabetic rats were divided into groups of six rats each as follows: group I comprised normal control rats orally administered with 1% CMC solution (10 mL/kg),

group II comprised diabetic control rats [diabetic rats orally administered 1% CMC solution (10 mL/kg)], group III comprised diabetic rats orally administered CaRE (100 mg/kg) in 1% CMC, group IV comprised diabetic rats orally administered CaRE (200 mg/kg) in 1% CMC, and group V comprised diabetic rats orally administered glipizide (5 mg/kg) in 1% CMC. Fasting blood glucose levels

were estimated from retro-orbital sinus prior to and at 1, 3, and 6 h after treatment [48].

Subacute (30 day) response at double dose

The same animals of the single-dose short-term study were continued at two different doses of CaRE (100 and 200 mg/kg) or glipizide (5 mg/kg) orally daily for 30 days. Blood glucose levels were assayed on the 10th, 20th, and 30th days after drug/CaRE treatment. Animals were sacrificed by euthanasia on the 31st day and the liver, pancreas, and diaphragm were extracted, washed with cold saline, and preserved under -20°C until used for various biochemical investigations.

Carbohydrate metabolism

Determination of glycosylated hemoglobin (HbA1c)

The glucose level in the blood plasma was evaluated using glucose estimation kits. Glycosylated hemoglobin (HbA1c) was assessed using a previously proposed method Parker et al. [49].

Estimation of liver glycogen

The liver glycogen level was estimated using a previously proposed method [50]. The supernatant was mixed with 95 % ethanol in a 1:5 ratio and incubated overnight for glycogen precipitation. Then, the glycogen precipitate was dissolved in distilled water (2 mL). The standard and blank solutions were prepared using glucose (0.5 mg/mL) and distilled water, respectively. Next, anthrone reagent (5 mL) was added to all tubes, which were then incubated in a boiling water bath for 15 min. Then, absorbance was measured at 620 nm. Results are expressed as mg glycogen/g tissue [51].

Glucose uptake and glucose transport

The mice were sacrificed under ether anesthesia and their livers and diaphragm were extracted. The excised tissues were immediately placed in ice-cold perfusion solution [containing KCl (0.04 %), NaCl (0.687 %), MgSO_4 (0.014 %), NaHPO_4 (0.014 %), CaCl_2 (0.028 %), NaHCO_3 (0.21 %)]. In another batch of perfusate, glucose at a concentration of 400 mg % was taken. Next, the tissue slices were incubated at 37°C for 1.5 h with appropriate aeration. Thereafter, the slices were washed with water and oven-dried at 800°C for 4–5 h. The glucose uptake was measured as mg glucose/100 mg dry weight of the diaphragm, while glucose levels in perfusate were measured as mg glucose/g of dry weight of the liver [52].

Lipid profile

Serum levels of TC, TGA, HDL, LDL, and VLDL were assayed using respective analytical kits (Nirmal Labs).

Antioxidant, inflammatory, and serum biomarkers

The liver and pancreas were extracted as described previously, washed with ice-cold saline, and weighed. Tissue homogenates (10 % w/v) were prepared in cold 0.1 M phosphate buffer solution (pH 7.4) and used to determine MDA formation [53]. Cell debris was removed by centrifugation ($10000 \times g$ at 4°C for 20 min) and the supernatant was assayed to determine nitrite levels [54] and enzyme antioxidants, viz. superoxide dismutase [55], CAT [56] and reduced GSH [57]. The inflammatory biomarkers, including cy-

tokines, TNF- α and IL-4, serum markers (AST, ALT), and ALP, were assayed on the 31st day using commercial kits.

Histoarchitecture of the pancreas

Pancreatic tissue was extracted and preserved in 10 % formalin. After fixation, tissues were embedded in paraffin. Then, the 3–4 μm thick sections were cut and stained using eosin and hematoxylin. The stained specimens were then examined under a light microscope ($40\times$).

Statistical analysis

The results are expressed as the mean \pm SEM. Statistical analysis was performed through ANOVA followed by Dunnett's test. CaRE- and glipizide-treated groups were compared with the corresponding normal or diabetic control groups. Statistical significance was indicated by $p < 0.05$. SPSS and Graph Pad Prism Version 08 were used for statistical analysis.

Conflict of Interest

The authors declare that they have no conflict of interest.

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