

Ameliorative Efficacy of the *Cassia auriculata* Root Against High-Fat-Diet + STZ-Induced Type-2 Diabetes in C57BL/6 Mice

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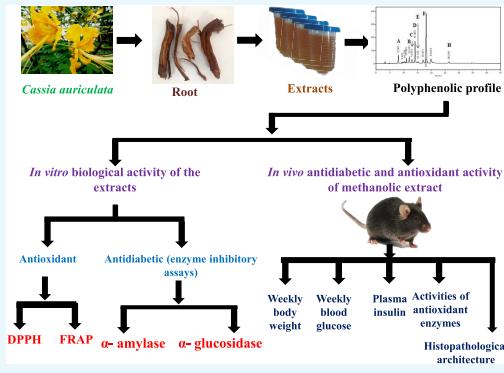
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ABSTRACT: Diabetes mellitus is a major metabolic disorder worldwide. Several herbs are being tested for the management of diabetes. *Cassia auriculata* is one of those herbs known for its nutritional value and health benefits. However, limited scientific evidence has been shown on the elucidation of its root bioactives as well as biological activity. This study attempted to identify and characterize phenolic compounds from the potent root extract and to evaluate its antioxidant as well as antidiabetic properties in both *in vitro* and *in vivo* models. The results revealed that the total polyphenol and flavonoid contents were highest in the methanolic extract. The methanolic extract of the *C. auriculata* root showed the highest antioxidant and antidiabetic activities *in vitro* than other extracts. These biological activities may be because the extract is rich in coumaric acid and –OH groups as revealed by high-performance liquid chromatography and Fourier-transform infrared spectroscopy analyses, respectively. Further, the antidiabetic activity of the methanolic extract was studied in a diet-induced type-2 diabetes mellitus (T2DM) C57BL/6 mouse model. A significant increase in fasting blood glucose and decreased plasma insulin levels in T2DM mice confirmed the development of the diabetic condition. In addition, the T2DM mice showed oxidative stress in the plasma as well as muscle tissue and significant alterations in the plasma biochemistry, *viz.*, lipid profile, liver, and renal function tests. However, the administration of the ethanolic extract of the *C. auriculata* root (150 mg/kg body weight) to T2DM mice normalized the condition comparable to that of control mice. Thus, the extract can be used as a potent antioxidant and antidiabetic agent in pharmaceutical companies.



1. INTRODUCTION

The naturally available herbs are being used as an alternative for allopathic drugs since ancient times in the ayurvedic system of medicine. The natural herbs gain importance over allopathy since the former have less or no side effects. The natural herbs *viz.*, *Curcuma longa*, *Withania somnifera*, *Acorus calamus*, *Tinospora cordifolia*, *Zingiber officinale*, *Asparagus racemosus*, *Garcinia cambogia*, *Moringa oleifera*, *Piper longa*, *Terminalia chebula*, etc., have been proven to act effectively against various deadly diseased conditions.¹ *Cassia auriculata* is one such herb known for its potency against various ailments. It is an evergreen shrub with attractive yellow flowers, belonging to the family Caesalpiniaceae. This shrub grows in different parts of Asia including India.² Various studies reported medicinal properties of different parts of the plant *C. auriculata*. For instance, tea from the leaves is used against chronic fever and as an anthelmintic,³ the methanolic extract of leaves and the aqueous extract of whole plant powder have antibacterial properties,⁴ the leaf extract also has anticancer properties,⁵ the ethanolic extract of roots shows hepatoprotective properties,⁶ and the aqueous extract of leaves reduces glucose and oxidative

stress markers in streptozotocin-induced diabetic rats.⁷ The aqueous extract of the flowers has antioxidant properties as it decreased oxidative stress in diabetic rats.⁸ The ethanolic extract of buds and flowers showed antidiabetic properties both *in vitro* and *in vivo*,⁹ the water extract of buds and flowers reduced blood sugar levels,² and the ethanolic extract of leaves and flowers managed the diabetic condition by acting as an insulin secretagogue agent.¹⁰ Further, it has been reported that the chloroform extract of aerial parts of the herb showed the antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi.¹¹ A comparative study among the different parts of the herb as reported by Uma Devi et al.¹² revealed that the flower and leaf extract has a potent antidiabetic property by reducing the serum glucose level and increasing plasma insulin

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levels. These biological properties of the herb *C. auriculata* may be because of the presence of bioactives. Hence, understanding the phytochemical constituents of the herb or its parts provides the knowledge of its biological properties. However, there are no studies on the quantification of different bioactives in the root of *C. auriculata* and its potential antidiabetic properties. Hence, this study aimed to investigate the presence of different phytochemical constituents in the root of *C. auriculata* both qualitatively and quantitatively and also to determine its biological properties such as antioxidant and antidiabetic activities in both *in vitro* and *in vivo* models.

2. RESULTS

2.1. Color and Yield of the Extracts of the Root of *C. auriculata* with Different Solvents. The different solvent extracts of the *C. auriculata* root showed different colors, *i.e.*, chloroform, ethanol, methanol, and aqueous extracts showed yellow, dark brown, light green, and light brown colors, respectively (Table 1). The methanolic extract showed the

retention times of the extracts in the chromatogram were compared with those of the accurate standards. The results of HPLC analysis of different extracts are represented in Table 3. The chloroform extract showed peaks for epicatechin, chlorogenic acid, and gallic acid. The ethanolic extract showed peaks similar to those of epicatechin, gallic acid, ferulic acid, syringic acid, whereas the methanolic extract of the *C. auriculata* root showed peaks matching with those of epicatechin, chlorogenic acid, gallic acid, ferulic acid, syringic acid, and coumaric acid standards. Further, in the aqueous extract, epicatechin, ferulic acid, and coumaric acid were absent and chlorogenic acid, gallic acid, syringic acid, tannic acid, resveratrol, caffeic acid, and protocatechuic acid were present (Figure 1). The highest amounts of gallic acid, syringic acid, and coumaric acid were found in chloroform, ethanol, and aqueous and methanolic extracts, respectively.

2.4. Fourier-Transform Infrared Spectroscopy (FTIR) Analysis of Different Solvent Extracts of the *C. auriculata* Root. The different functional groups in different solvent extracts of the *C. auriculata* root were determined by FTIR analysis. The FTIR spectra revealed that the four extracts studied showed a strong, broad stretching band at 3300 cm^{-1} , with methanolic and aqueous extracts showing broader bands than those of other extracts (chloroform extract, 3332 cm^{-1} ; ethanol extract, 3423 cm^{-1} ; aqueous extract 3242 cm^{-1} ; methanolic extract 3251 cm^{-1}). A broad stretching band at 3300 cm^{-1} indicates the presence of intermolecular $-\text{OH}$ groups (Figure 2). In addition, bands at 2924 , 2906 , 2949 and 2837 , and 2879 cm^{-1} in chloroform, ethanol, methanol, and aqueous extracts, respectively, indicate the presence of a C–H stretching alkane. Further, bands at 1614 cm^{-1} ($\text{C}=\text{C}$ stretching conjugated alkene), 1455 cm^{-1} (C–H bending alkane methylene group), 1214 cm^{-1} (C–N stretching amine), and 754 cm^{-1} (C–Cl stretching halo compound) for the chloroform extract; 3195 cm^{-1} (broad O–H stretching alcohol intramolecularly bonded) and 1035 cm^{-1} (C–O stretching primary alcohol) for the ethanol extract; 1652 , 1565 , 1480 cm^{-1} ($\text{C}=\text{N}$ stretching imine/oxime), 1411 cm^{-1} (O–H bending carboxylic acid), and 606 cm^{-1} (C–I stretching halo compound) for the methanolic extract; and 1550 cm^{-1} (N–O stretching nitro compound), 1413 cm^{-1} (C–H bending alkane methyl group), and 603 cm^{-1} (C–I stretching halo compound) for the aqueous extract were observed.

2.5. Antioxidant Activity. **2.5.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay.** The antioxidant ability of the different solvent extracts of *C. auriculata* was determined by calculating the IC_{50} values, wherein the samples with the least IC_{50} value show the robust scavenging activity against DPPH free radicals. Among the different solvent extracts from the *C. auriculata* root, the methanolic extract showed the maximum DPPH scavenging activity with the lowest IC_{50} value ($42.87 \pm 1.5\text{ }\mu\text{g/mL}$), followed by ethanol ($46.24 \pm 1.78\text{ }\mu\text{g/mL}$), aqueous ($57.04 \pm 2.75\text{ }\mu\text{g/mL}$), and chloroform extracts ($60.55 \pm 5.29\text{ }\mu\text{g/mL}$) (Table 4).

2.5.2. Ferric Reducing Antioxidant Power (FRAP). In this study, the methanolic extract of *C. auriculata* showed the highest ferric reducing antioxidant power, followed by ethanol, chloroform, and aqueous extracts as shown in Table 4, and the inhibitory concentrations were found to be close to those of standards. The IC_{50} values of methanolic, ethanol, chloroform, and aqueous extracts were 47.13 ± 0.37 , 52.44 ± 0.46 , 67.59 ± 0.75 , and $95.68 \pm 1.50\text{ }\mu\text{g/mL}$, respectively, indicating that the

Table 1. Color and Yield of the Extracts of Roots of *C. auriculata* with Different Solvents

solvent	color	% yield (g/100 g)
chloroform	yellow	5
ethanol	dark brown	11.6
methanol	light green	13.8
aqueous	light brown	10.4

highest yield of 13.8% (w/w), followed by the ethanolic extract with a yield of 11.65%, the aqueous extract with a yield of 10.4%, and the chloroform extract with a yield of 5% (Table 1).

2.2. Estimation of Total Polyphenols and Flavonoids. The methanol extract of the root of *C. auriculata* has the highest total polyphenols, followed by aqueous, ethanol, and chloroform extracts (Table 2). Similarly, the methanol extract

Table 2. Total Polyphenols and Flavonoid Content of Different Solvent Extracts of *C. auriculata* Root^{A,B}

solvents	total polyphenols (mg/100 g of the sample)	flavonoid (mg/100 g of the sample)
chloroform	$1454.33 \pm 19.52^{\text{a}}$	$832.53 \pm 17.04^{\text{a}}$
ethanol	$6531.69 \pm 196.83^{\text{b}}$	$1340.79 \pm 1.14^{\text{c}}$
methanol	$8979.04 \pm 495.59^{\text{c}}$	$1647.12 \pm 20.71^{\text{d}}$
aqueous	$8301.26 \pm 269.29^{\text{c}}$	$1111.14 \pm 10.52^{\text{b}}$
significance	$P < 0.001$	$P < 0.001$

^ANote: All of the values are mean \pm SEM. ^BMean values with the same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly different as judged by Duncan's multiple range test.

of the root of *C. auriculata* possessed the highest amount of flavonoids, followed by ethanol, aqueous and chloroform extracts. This shows that methanol is a more potent solvent to extract the maximum amount of total polyphenols and flavonoids. The least amount of polyphenols and flavonoids was extracted from the chloroform extract among all of the other extracts.

2.3. High-Performance Liquid Chromatography (HPLC) Analysis. To know the different phenolic acids in the different solvent extracts of the *C. auriculata* root, the

Table 3. Polyphenolic Profile of Different Solvent Extracts of the *C. auriculata* Root^{A,B}

	chloroform	ethanol	methanol	aqueous
epicatechin (mg/100 g)	13.07 ± 0.39 ^c	3.15 ± 0.03 ^b	46.69 ± 0.27 ^d	ND
chlorogenic acid (mg/100 g)	0.60 ± 0.01 ^b	ND	44.24 ± 0.24 ^d	3.12 ± 0.04 ^c
gallic acid (mg/100 g)	27.68 ± 0.44 ^b	20.88 ± 0.39 ^a	39.85 ± 0.54 ^c	27.68 ± 0.44 ^b
ferulic acid (mg/100 g)	ND	46.22 ± 0.29 ^c	22.68 ± 0.04 ^b	ND
syringic acid (mg/100 g)	ND	70.98 ± 0.38 ^c	18.24 ± 0.28 ^b	79.57 ± 0.26 ^d
coumaric acid (mg/100 g)	ND	ND	189.47 ± 0.30 ^b	ND
tannic acid (mg/100 g)	ND	ND	ND	2.24 ± 0.01 ^b
resveratrol (mg/100 g)	ND	ND	ND	1.64 ± 0.04 ^b
caffeic acid (mg/100 g)	ND	ND	ND	18.05 ± 0.38 ^b
protocatechuic acid (mg/100 g)	ND	ND	ND	51.59 ± 0.40 ^b

^ANote: All of the values are mean ± SEM. ^BMean values with the same superscript letters in the given row are not significantly different, whereas those with different superscript letters are significantly different as judged by Duncan's multiple range test.

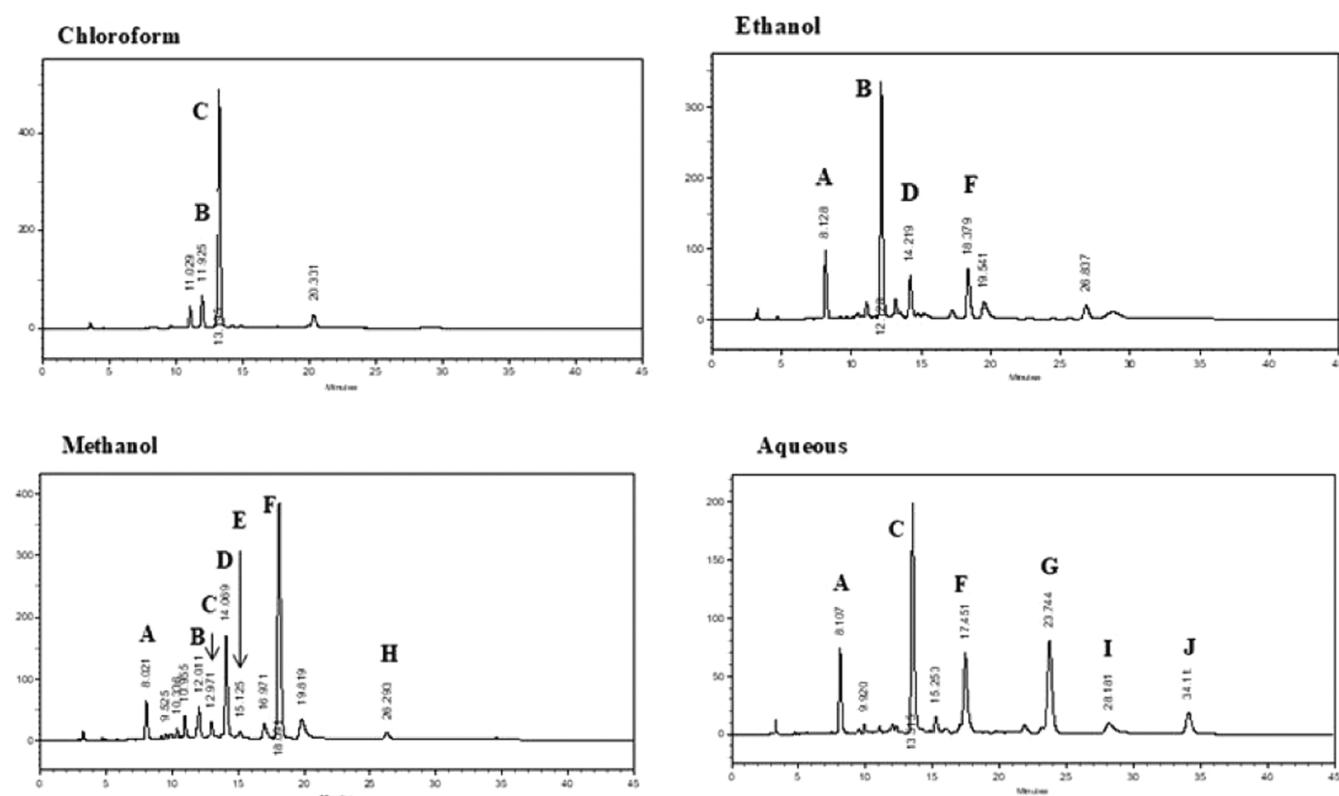


Figure 1. HPLC chromatograms of different solvent extracts of the *C. auriculata* root showing the polyphenols present. (A) Gallic acid; (B) epicatechin; (C) chlorogenic acid; (D) ferulic acid; (E) tannic acid; (F) syringic acid; (G) resveratrol; (H) coumaric acid; (I) caffeic acid; (J) protocatechuic acid.

methanol extract has more antioxidant potency than the other extracts of the root studied.

As the methanolic extract of the root showed the highest content of polyphenols and exhibited the highest antioxidant potential, it was selected for further *in vitro* and *in vivo* studies.

2.6. Antidiabetic Activity. **2.6.1. α -Amylase and α -Glucosidase Inhibition Assay.** The methanolic extract inhibited α -amylase activity in a dose-dependent manner. The IC_{50} value of the extract for inhibiting the α -amylase activity is represented in Figure 3, wherein the IC_{50} value is $44.75 \pm 1.31 \mu\text{g/mL}$ compared to that of the standard ($81.19 \pm 1.34 \mu\text{g/mL}$). Similarly, the methanolic extract inhibited the α -glucosidase activity, and the result is represented in Figure 3. The IC_{50} value of the extract for inhibiting α -glucosidase

activity is $66.86 \pm 1.72 \mu\text{g/mL}$ compared to that of its standard $94.83 \pm 9.83 \mu\text{g/mL}$.

2.7. Effect of the *C. auriculata* Root Extract on Body Weight. In this present study, diabetes was induced with a combination of high fat diet and a low dose of streptozotocin (STZ) and the effect of the *C. auriculata* root extracts was evaluated. The change in the body weight of mice of different groups is presented in Figure 4. The mice were fed with the high-fat diet for 11 weeks; thus, a considerable increase in body weight was observed among the high-fat-diet-fed mice as compared to the control group. After the induction of diabetes, the weight of mice in the diabetic group significantly reduced, whereas the treatment with the *C. auriculata* root extract could help in regaining the weight much better than the metformin-treated group.

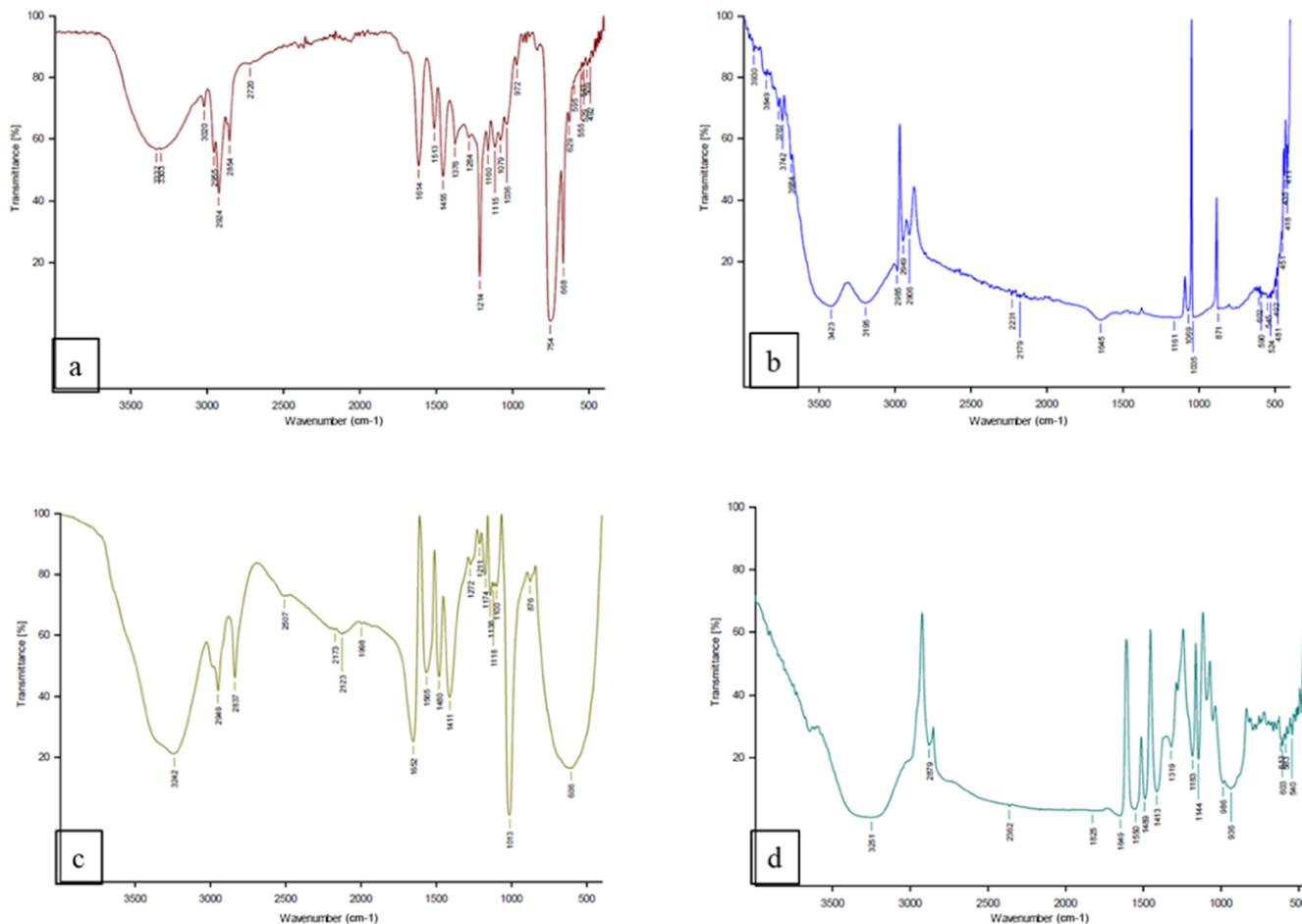


Figure 2. FTIR spectra of (a) chloroform, (b) ethanol, (c) methanol and (d) aqueous extracts of the *C. auriculata* root showing bands of different functional groups. Note the band between 3600 and 3000 cm^{-1} , suggesting the presence of $-\text{OH}$ groups in all of the four extracts, and significantly broader in the spectra of the methanolic extract.

Table 4. IC₅₀ Values of DPPH and FRAP Assays of Different Solvent Extracts of the *C. auriculata* Root^{A,B}

solvents	DPPH ($\mu\text{g/mL}$)	FRAP ($\mu\text{g/mL}$)
chloroform	$60.55 \pm 5.29^{\text{b}}$	$67.59 \pm 0.75^{\text{c}}$
ethanol	$46.24 \pm 1.78^{\text{a}}$	$52.44 \pm 0.46^{\text{b}}$
methanol	$42.87 \pm 1.51^{\text{a}}$	$47.13 \pm 0.37^{\text{a}}$
aqueous	$57.04 \pm 2.75^{\text{b}}$	$95.68 \pm 1.50^{\text{d}}$
standard	$43.29 \pm 1.17^{\text{a}}$	$50.76 \pm 0.43^{\text{b}}$
significance	$P < 0.05$	$P < 0.001$

^ANote: All of the values are mean \pm SEM. ^BMean values with the same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly different as judged by Duncan's multiple range test.

2.8. Effect of the Methanolic Extract the *C. auriculata* Root on Fasting Blood Glucose Levels. After 5 days of diabetes induction by STZ, all mice were tested for the blood glucose level, and the levels above 200 mg/dL confirmed the induction of diabetes in the mice. The blood glucose levels of all the four groups were monitored on a weekly basis. The control group showed normal blood glucose levels throughout the experiment in contrast to the diabetic group, which showed a gradual increase in the blood glucose level every week, a sign of uncontrolled diabetes (Figure 5). The treatment with metformin for 4 weeks could effectively reduce blood glucose in diabetic rats, and almost equal to the level of control mice at

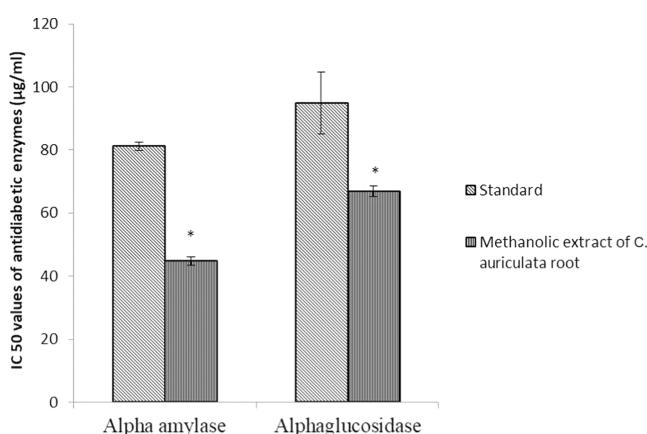


Figure 3. Vertical bar graphs showing the inhibitory potency of the methanolic extract of the *C. auriculata* root for the activities of α -amylase and α -glucosidase. Note: All of the values are mean \pm SEM. * Indicates that groups are significantly different as judged by Duncan's multiple range test.

the end of the experiment (8th week). Similarly, the treatment with the methanolic extract of the *C. auriculata* root also showed a significant reduction in the blood glucose level gradually, reduced to half when compared to the initial level after 3 weeks of treatment and further decreased to the level of metformin toward the end of the experiment.

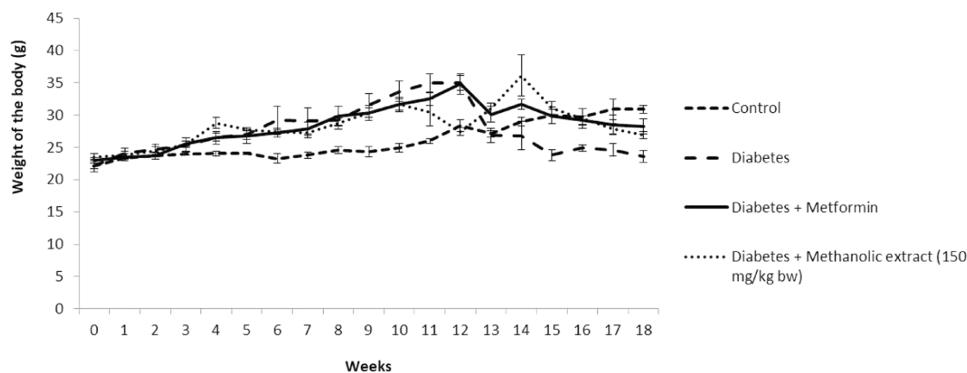


Figure 4. Line graph showing the change in the weight of the body of different experimental group mice throughout the experiment. Note a significant increase in the weight of the body till 11th week in the high-fat-diet-fed mice and a gradual decrease in those treated with metformin and the methanolic extract but not in diabetic mice.

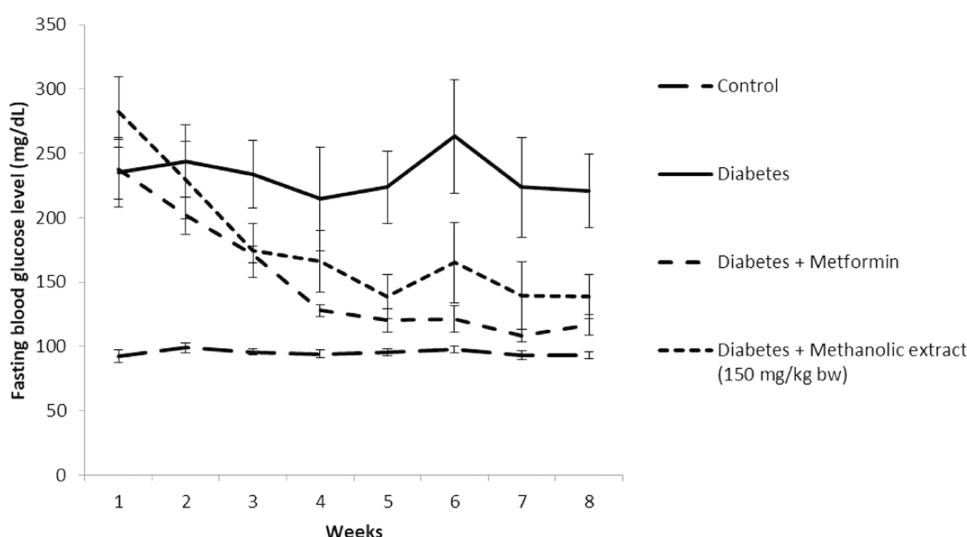


Figure 5. Line graph showing the fasting blood glucose level in different experimental group mice during the treatment period. Note a significantly elevated level of fasting blood glucose levels in the diabetic mice compared to control and the mice treated with the methanolic extract of *C. auriculata* and metformin treated diabetic mice.

Table 5. Effect of the Methanolic Extract of the *C. auriculata* Root on the Activities of Antioxidant Enzymes in the Serum and Muscle of Experimental Groups^{A,B}

	SOD (U/mg protein)		CAT (nmol/mg/min)	
	plasma	muscle	plasma	muscle
control	1875.24 ± 197.12 ^b	10.38 ± 0.62 ^b	0.25 ± 0.03 ^b	1.13 ± 0.12 ^a
diabetes	549.58 ± 69.39 ^a	6.21 ± 0.87 ^a	0.12 ± 0.01 ^a	0.48 ± 0.11 ^b
diabetes + metformin	2268.68 ± 77.31 ^{b,c}	8.78 ± 1.12 ^b	0.4 ± 0.08 ^c	2.4 ± 0.19 ^c
diabetes + methanolic extract (150 mg/kg bw)	2469.17 ± 252.67 ^c	11.17 ± 0.44 ^b	0.28 ± 0.03 ^{b,c}	1.62 ± 0.29 ^a
significance	P < 0.001	P < 0.05	P < 0.05	P < 0.003

^ANote: All of the values are mean ± SEM. ^BMean values with the same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly different as judged by Duncan's multiple range test.

2.9. Effect of Methanolic Extract of the *C. auriculata* Root on the Antioxidant Status in the Plasma and Muscles. The antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) were significantly decreased in diabetic mice compared with those of control animals in both the plasma and muscles (Table 5). However, the activities of SOD and CAT were increased even above the control level in the methanolic extract of the *C. auriculata* root-and metformin-treated mice.

2.10. Effect of Methanolic Extract of *C. auriculata* Root on Oxidative Stress Markers in Plasma and Muscle. There was a significantly higher level of reactive oxygen species (ROS) and nitric oxide (NO) in both the plasma and muscles in the diabetic rats compared to those of control mice (Table 6). However, the treatment of diabetic mice with metformin could effectively lower the ROS and NO levels to the levels of controls and the methanolic extract of the *C. auriculata* root reduced these levels better than metformin. Further, the metformin and the *C. auriculata* root extract

Table 6. Effect of the Methanolic Extract of the *C. auriculata* Root on the Concentration of Oxidative Stress Markers in the Serum and Muscle of Experimental Groups^{A,B}

	ROS ($\mu\text{mol of DCF formed/min/mg protein}$)		NO (ng/mg protein)	
	plasma	muscle	plasma	muscle
control	2601.77 \pm 182.88 ^b	2595.56 \pm 95.98 ^a	3.86 \pm 0.17 ^b	2.59 \pm 0.25 ^a
diabetes	3347.69 \pm 15.77 ^c	3230.25 \pm 44.56 ^c	6.52 \pm 0.56 ^c	5.19 \pm 0.47 ^b
diabetes + metformin	2783.77 \pm 106.92 ^b	2842.57 \pm 51.11 ^b	3.85 \pm 106.92 ^b	1.98 \pm 0.32 ^a
diabetes + methanolic extract (150 mg/kg bw)	1496.69 \pm 54.55 ^a	2726.95 \pm 42.97 ^{a,b}	2.88 \pm 54.55 ^a	2.73 \pm 0.23 ^a
significance	P < 0.001	P < 0.001	P < 0.001	P < 0.001

^ANote: All of the values are mean \pm SEM. ^BMean values with the same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly different as judged by Duncan's multiple range test.

reduced the ROS and NO levels and showed similarity with the control mice.

2.11. Effect of the *C. auriculata* Root Extract on Plasma Insulin Levels. The plasma insulin levels of the different mice groups are represented in Table 7. There was a

Table 7. Effect of the Methanolic Extract of the *C. auriculata* Root on the Serum Concentration of Insulin of Experimental Groups^{A,B}

	insulin ($\mu\text{L U/mL}$)
control	0.65 \pm 0.02 ^a
diabetes	0.22 \pm 0.01 ^b
diabetes + metformin	0.68 \pm 0.03 ^a
diabetes + methanolic extract	0.71 \pm 0.02 ^a
significance	P < 0.001

^ANote: All of the values are mean \pm SEM. ^BMean values with the same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly different as judged by Duncan's multiple range test.

significantly lower level of plasma insulin in diabetic mice compared to that of the control group ($P < 0.001$), whereas the levels were similar to those of control mice in the *C. auriculata* root extract and metformin-treated mice.

2.12. Effect of the Methanolic Extract of the *C. auriculata* Root on the Lipid Profile. There was a significant increase in the plasma levels of triglycerides (TG), low-density lipoprotein (LDL), total cholesterol (TC), and very low density lipoprotein (VLDL) with a concomitant decrease in the plasma levels of high-density lipoprotein (HDL) in diabetic mice, whereas these were normalized to the level of control mice in the *C. auriculata* root extract and metformin-treated mice. In addition, the atherogenic index, a new index to determine the alteration in the lipid profile, was also found to be higher in diabetic mice compared to the mice of other groups studied (Table 8).

2.13. Effect of the Methanolic Extract of the *C. auriculata* Root on Plasma Markers of Hepatic Damage.

The treatment with the *C. auriculata* root extract maintained the normal hepatic condition as there was a significant decrease in the activities of plasma markers of hepatic damage, i.e., aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) in extract-treated diabetic mice compared to diabetic mice (Figure 6). The diabetic group mice showed a significant rise in the activities of plasma ALP, AST, and ALT, indicating liver damage.

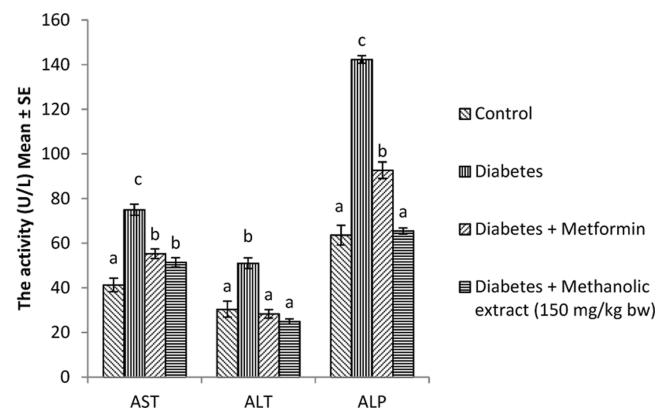


Figure 6. Vertical bar graphs showing the activities of AST, ALT, and ALP in different experimental group mice. AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase. Note: All of the values are mean \pm SEM. Mean values with the same superscript letters in the given parameter are not significantly different, whereas those with different superscript letters are significantly different as judged by Duncan's multiple range test.

2.14. Effect of the Methanolic Extract of the *C. auriculata* Root on Plasma Markers of Renal Damage. The diabetic group mice showed a significant upsurge ($P < 0.05$) of urea, BUN, uric acid, and creatinine levels in the plasma compared to the control groups (Figure 7a,b). However, diabetic mice treated with the *C. auriculata* root extract and metformin showed significantly lower plasma urea,

Table 8. Effect of the Methanolic Extract of the *C. auriculata* Root on the Lipid Profile of Experimental Groups^{A,B}

	total cholesterol (mg/dL)	triglycerides (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)	AI
control	170.46 \pm 8.22 ^a	92.75 \pm 2.28 ^a	49.79 \pm 2.15 ^b	78.17 \pm 2.86 ^a	18.55 \pm 0.46 ^a	0.28 \pm 0.03 ^a
diabetes	276.11 \pm 8.99 ^c	219.56 \pm 17.94 ^b	30.67 \pm 5.81 ^a	153.80 \pm 4.32 ^c	43.91 \pm 3.59 ^b	0.87 \pm 0.13 ^b
diabetes + metformin	199.79 \pm 5.36 ^b	96.84 \pm 1.79 ^a	45.07 \pm 4.67 ^b	95.05 \pm 2.46 ^b	19.97 \pm 0.66 ^a	0.31 \pm 0.05 ^a
diabetes + methanolic extract	169.49 \pm 5.39 ^a	90.30 \pm 7.62 ^a	44.75 \pm 2.48 ^b	82.54 \pm 3.59 ^a	17.89 \pm 1.19 ^a	0.29 \pm 0.04 ^a
significance	P < 0.001	P < 0.001	P < 0.05	P < 0.001	P < 0.001	P < 0.001

^ANote: All of the values are mean \pm SEM. ^BMean values with the same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly different as judged by Duncan's multiple range test.

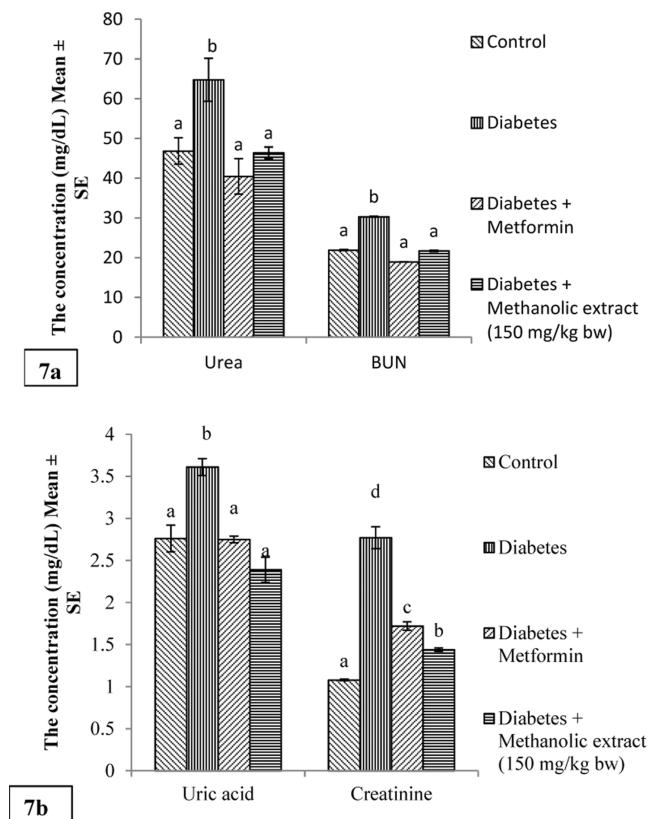


Figure 7. (a and b) Vertical bar graphs showing the plasma concentration of urea and BUN (a) and uric acid and creatinine (b) in different experimental group mice. Note: All of the values are mean \pm SEM. Mean values with the same superscript letters are not significantly different, whereas those with different superscript letters are significantly different as judged by Duncan's multiple range test.

BUN, uric acid, and creatinine levels, indicating the attenuation of alteration in the plasma levels of renal markers caused by diabetes.

2.15. Effect of the Methanolic Extract of the *C. auriculata* Root on the Histomorphology of Muscles.

The histological architecture in the diabetic group mice was altered compared to that of the normal architecture in the control mice. The diabetic mice muscle histomorphology showed the presence of mononucleated inflammatory cells and muscles replaced by the adipose tissue with nonhomogeneous fiber size distribution against homogeneous fiber size distribution in the control mice (Figure 8a,b). In addition, in mice treated with metformin and the methanolic extract of the *C. auriculata* root, homogenous fiber distribution similar to that of control mice was observed (Figure 8c,d). This suggests that the ameliorative efficacy of the methanolic extract against high-fat-diet plus STZ-induced diabetic alterations in muscle histomorphology.

3. DISCUSSION

The herbs are used for medicinal purposes because of their less or no side effects. The bioactives present in the herbs may be the reason for their health-benefiting properties. However, the phytocomponents of the *C. auriculata* root are not well known/understood. Hence, this study investigated the total polyphenols as well as flavonoids present in different solvent extracts and the antidiabetic activity of the methanolic extract of the root both in *in vitro* and *in vivo* models. The methanolic

extract of the *C. auriculata* root showed the highest amount of total polyphenols, followed by aqueous, ethanol, and chloroform extracts in the order mentioned. The HPLC profile of polyphenols showed that coumaric acid in the methanolic extract, gallic acid in the chloroform extract, and syringic acid in ethanolic and aqueous extracts are present in the highest concentrations compared to other polyphenols present in respective solvent extracts. Further, the flavonoid content was also found to be highest in the methanolic extract compared to other extracts studied. The methanolic extract was followed by ethanolic, aqueous, and chloroform extracts. Furthermore, the results of this study revealed that the antioxidant potency of the methanolic extract was high, with the least IC₅₀ value ($42.87 \pm 1.51 \mu\text{g/mL}$), followed by ethanol, aqueous, and chloroform extracts compared to the standard. The potent methanolic extract, among four extracts studied, showed antidiabetic properties both *in vitro* and *in vivo*.

The methanolic extract reduced the hyperglycemia in the high-fat-diet-fed + STZ-injected type-2 diabetic C57BL/6 mice (T2D mice), suggesting the hypoglycemic/antidiabetic property. The administration of a high-fat diet + STZ injection resulted in a decrease in the body weight, an increase in the fasting blood glucose level, the concentration of ROS and NO in the plasma and muscles, with a concomitant decrease in the plasma insulin and activities of antioxidant enzymes. The above results are further authenticated by the alteration in the normal architecture of the muscle tissue in the diabetic mice compared to other control mice. Further, there was a significant increase in the activities of plasma AST, ALT, and ALP as well as the plasma concentration of urea, uric acid, and BUN in the high-fat-diet-fed diabetic mice compared to control mice. The levels of plasma TC, TG, LDL, VLDL, and the atherogenic index were found to be elevated significantly, with a significant decrease in the HDL level in T2D mice compared to control. However, the administration of 150 mg/kg body weight of the methanolic extract of the *C. auriculata* root for 8 weeks could normalize the condition to the level of control. This indicates that the *C. auriculata* root extract act as an antidiabetic agent.

Several studies have reported that the health-promoting properties of medicinal herbs are due to the presence of different bioactives.¹³ Hence, the identification and characterization of bioactives or phytoconstituents of herbs have become a major topic of various studies. In this investigation, the root of *C. auriculata* was subjected to different solvent extractions in the order of increasing polarity and it was found that the methanolic extract could extract the highest amount of total polyphenols compared to other solvents. Indeed, studies have shown that polar solvents could extract the maximum amount of polyphenols.¹⁴ Further, the flavonoids were also found to be highest in the methanolic extract. In addition to the estimation of total polyphenols from different solvent extracts, the different polyphenols present were also determined in this study. The different solvent extracts were subjected to HPLC analysis, and it was found that methanolic, chloroform, and ethanol and aqueous extracts were rich in coumaric acid, gallic acid, and syringic acid, respectively. In addition, researchers are reporting alternate natural sources of polyphenols with high bioavailability;¹⁵ indeed, the results of our study showed that the *C. auriculata* root is also an alternate source for polyphenols.

The imbalance between the antioxidant and pro-oxidant levels results in oxidative stress, and antioxidants scavenge the

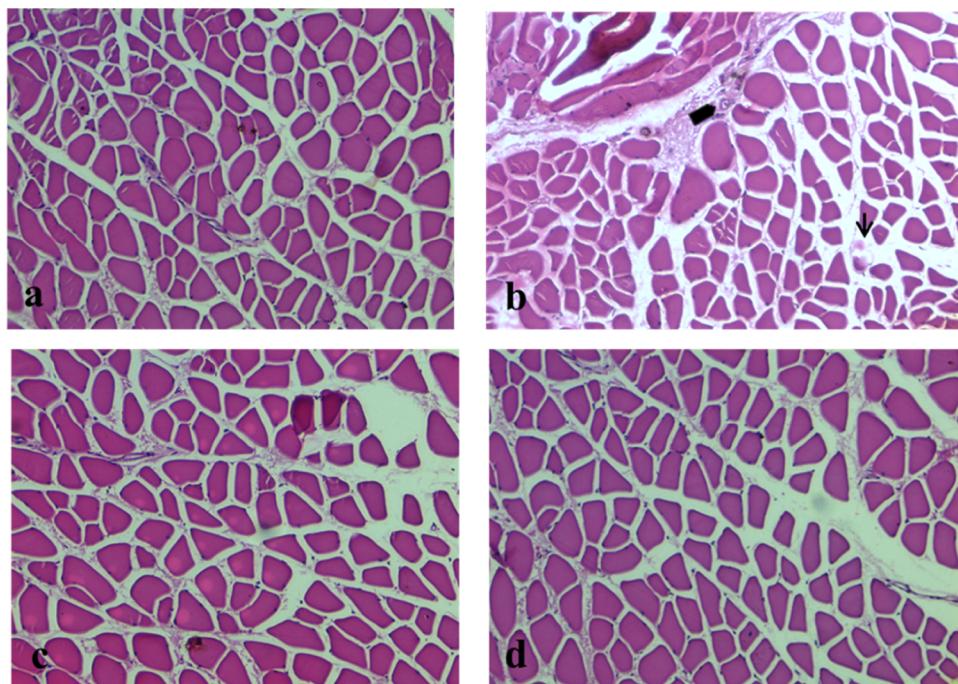


Figure 8. Photomicrographs of muscle tissues of (a) control, (b) diabetic, (c) diabetic + metformin, (d) diabetic + the methanolic-extract-treated mice. Note the presence of mononucleated inflammatory cells (up-pointing bullet) and muscles replaced by the adipose tissue with (down-pointing arrow) with nonhomogenous fiber size distribution against homogeneous fiber size distribution in control mice and metformin- as well as the methanolic extract of *C. auriculata*- treated diabetic mice muscle section.

free radicals to reduce the oxidative stress. In this study, to determine the antioxidant potential of different solvent extracts of the root, DPPH and FRAP assays were performed. The results of the DPPH assay revealed that the methanolic extract showed better antioxidant activity compared to other extracts and standard. This was further confirmed by the FRAP assay, which showed a similar pattern of the result. The earlier studies had shown that *C. auriculata* leaves¹⁰ and flowers^{2,8} have antioxidant properties. However, the methanolic extract in this study showed the least IC₅₀ value of about $42.87 \pm 1.51 \mu\text{g}/\text{mL}$ for the DPPH assay and $47.13 \pm 0.37 \mu\text{g}/\text{mL}$ for the FRAP assay. This may be because of the highest polyphenol content, as polyphenols are known for their antioxidant potential.^{16,17} It is further confirmed by FTIR analyses, the results of which revealed that the methanolic extract has a broader peak at 3300 cm^{-1} , range which indicates the presence of $-\text{OH}$ groups.

Hyperglycemia is the hallmark of the diabetic condition.¹⁸ Hyperglycemia for prolonged periods results in diabetes and its associated secondary complications like diabetic nephropathy, neuropathy, retinopathy, cardiovascular disease, etc. Hence, maintaining the near-normoglycemic condition is a dire need in a diabetic patient. The blood glucose level increases soon after the digestion of food and the absorption of monomers; hence, the inhibition of oligo/polysaccharide-hydrolyzing enzymes, *viz.*, α -amylase and α -glucosidase, could delay the hydrolysis of polysaccharides. In this study, the methanolic extract of the *C. auriculata* root, the potent extracts among all the extracts studied, suppressed the activities of hydrolyzing enzymes, suggesting potency in reducing the hyperglycemic condition.

Based on the results of *in vitro* antioxidant and *in vitro* antidiabetic properties of the methanolic extract of the *C. auriculata* root, a study was designed to evaluate the same *in*

vivo in a diabetic mice model. In this study, a high-fat diet was fed with a small single dose of streptozotocin to induce type-2 diabetes in C57BL/6 mice. A high-fat diet for 11 weeks induces insulin resistance and a small dose of streptozotocin decreases the plasma insulin levels and, in turn, increases blood glucose, providing a diabetic model to study the efficacy of herbs. The T2D mice showed a significant increase in fasting blood glucose and decreased plasma insulin levels which indicates that the mice were diabetic and developed insulin resistance. The decrease in the plasma insulin despite a high-fat diet maybe because of the fact that in a diabetic condition, the prolonged insulin resistance with compensatory hyperinsulinaemia sends negative signals to the pancreatic β -cell to stop insulin production as there are increased levels of plasma insulin. A similar mechanism is mimicked in this animal model where a high-fat diet for 11 weeks induces insulin resistance and a small dose of STZ injection decreases the insulin level immediately but maintains the insulin resistance to develop a type-2 diabetic model. These results are in agreement with earlier results.¹⁹ However, the diabetic mice treated with metformin and the methanolic extract of the *C. auriculata* root showed the above parameters similar to those of control mice, indicating the amelioration or management of the diabetic condition.

The body weight of all the T2D mice was increased over control mice in this study. After STZ induction, the body weight of diabetic mice decreased compared to other group mice. This may be because in the diabetic condition, the muscle protein is degraded to provide amino acids as substrates for gluconeogenesis²⁰ as the decreased level of insulin due to STZ-induced diabetes cannot transfer glucose into the cells and utilize the same. However, it has been mentioned earlier that insulin and glucose levels were normalized to the level of control mice in metformin and

extract-treated mice and hence the body weight is not reduced as there may be increased glucose uptake and thereby reduced gluconeogenesis in treated diabetic mice.

The plasma lipid profile of T2D mice was altered in this study compared to other groups mice. These results are similar to those of earlier studies where Holmes et al.²¹ reported that high-fat-diet-fed rats showed increased plasma TG and TC levels. Studies reported that diabetes increases hepatic TG.²² The increased amount of TG leads to the accumulation of fats and causes nonalcoholic fatty liver disease.²³ Increased levels of LDL-C and TG have been proven to contribute to the development of hypertension and cardiovascular diseases.^{24,25} Indeed, in this study, there is a significant increase in the plasma levels of TG and LDL, which might lead to cardiovascular diseases. In recent years, the lipid profile alteration has been determined by the atherogenic index. The atherogenic index of plasma is a logarithmically transformed ratio of TG and HDL-C. It is considered as an indicator of dyslipidemia and associated diseases.²⁶ The atherogenic index of diabetic mice in this study is significantly high compared to control, metformin, and extract-treated mice, indicating a significant damage/alteration in the diabetic mice, which may lead to cardiovascular diseases. This is because the atherogenic index is linked with cardiovascular diseases.²⁷

It is well established that as a secondary complication, diabetes is associated with dysfunction or damage in other organs. In this study, the activities of plasma AST, ALT, and ALP were elevated in the T2D mice than in control group mice. The elevated AST, ALT, and ALP activities indicate liver dysfunction. Earlier studies have reported that in the diabetic condition the liver function biomarkers such as ALT, AST, and ALP elevate,⁹ with which our study is also in line. However, the diabetic mice administered with metformin and the methanolic extract of the *C. auriculata* root showed the plasma AST, ALT, and ALP activities comparable to those of control mice.

Similarly, kidney dysfunction was also observed only in diabetic mice in this study as reported by previous studies. For instance, Nambirajan et al.⁹ showed significantly increased levels of urea and creatinine, indicating kidney damage in diabetic rats. The prolonged hyperglycemia in the diabetic condition is known to damage the kidney and leads to diabetic nephropathy. As an indication of kidney dysfunction, there was a significant increase in the plasma concentration of urea, BUN, uric acid, and creatinine in diabetic mice compared to control and metformin- and extract-treated diabetic mice.

In addition to the elevated fasting blood glucose levels, decreased plasma insulin levels, and alterations in the lipid profile as characteristic features of diabetes, oxidative stress is also associated with diabetes. Hyperglycemia is known to generate reactive oxygen species, which in turn cause oxidative stress.²⁸ In the present study, there was a significant increase in the levels of oxidative stress markers, i.e., ROS and NO, in both the plasma and muscles with a concomitant decrease in the activities of antioxidant enzymes SOD and CAT in the T2D mice compared to control mice. However, mice treated with metformin and the methanolic extract of the *C. auriculata* root showed an increase in the activities of SOD and CAT in the plasma and muscles, confirming the antioxidant property of the methanolic extract of the *C. auriculata* root. The oxidative stress in the muscle tissue is further supported by the deleterious alteration in the histological architecture of the muscle in diabetic mice as there was a deposition of fat and

infiltration of leukocytes, indicating inflammation or damage to the tissue.

Based on the results obtained, a possible mechanism of action is derived as follows. It is well established that feeding with a high-fat diet develops insulin resistance,²⁹ a model for the type-2-diabetic studies. However, recently, an alternate model wherein a high-fat diet fed for 2–3 months with a single dose (60 mg/kg body weight) of STZ would develop a suitable type-2 diabetic model.¹⁰ The high-fat diet alters lipid metabolism and develops insulin resistance. The intra-peritoneal injection of STZ further alters the insulin level and glucose absorption, resulting in a suitable condition for type-2 diabetes. In this study, a significant increase in fasting blood glucose and a decrease in plasma insulin indicate the induction of insulin resistance after a high-fat diet + STZ injection. This further resulted in the development of the diabetic condition. As a secondary complication of diabetes, there was a significant damage/disruption in the liver and kidney function. Insulin plays a vital role in glucose absorption through glucose transporter (Glut). Glut 4 is an insulin-dependent glut transporters present in the muscle and adipose tissue. Because of insulin resistance and decreased plasma insulin levels, the glut transporters present in muscle cannot function normally, hence altering the function or physiology of the muscle. Altered physiology in muscle further resulted in oxidative stress as evident by increased concentration of ROS and NO with a significant decrease in the activities of SOD and CAT in the muscle. Further, the polyphenol-rich methanolic extract of the *C. auriculata* root could manage the alterations due to high-fat diet + STZ-induced diabetes similar to that of metformin. This may be because of its rich –OH groups and an efficacy to increase the insulin sensitization as the plasma insulin levels were similar to the levels of control mice in extract-treated mice.

To conclude, the methanolic extract of the *C. auriculata* root had the highest amount of polyphenols and flavonoids and was rich in –OH groups with potent antioxidant and antidiabetic properties *in vitro*. The methanolic extract also managed the type-2 diabetic condition to the level of controls. The mechanism of action of the extract may be by increasing the insulin secretion as well as sensitivity in extract-treated diabetic mice.

4. MATERIALS AND METHODS

4.1. Chemicals. All the analytical and laboratory-grade chemicals were supplied by Rankem (Bangalore, India), whereas HPLC-grade chemicals, standards, and the enzyme-linked immunosorbent assay (ELISA) kit for insulin were procured from Sigma-Aldrich Chemicals Private Limited (Bangalore, India). Kits for biochemical analyses of plasma were procured from Agappe Diagnostic Limited (Bangalore, India).

4.2. Procurement and Processing of the *C. auriculata* Root. The root of the *C. auriculata* plant was collected from Chandravana botanical garden, maintained by Mysore Medical College, Mysuru. After the collection of the plant, it was authenticated by a botany expert, and the voucher number of the herbarium submitted is UOMBOT20CA17. The root was rinsed thoroughly in distilled water and alcohol, shade-dried, coarsely powdered in a hammer mill, and stored in a sealed container at –20 °C until further use.

4.3. Defatting of the Samples. The processed *C. auriculata* root was defatted by the cold extraction method.

Briefly, 10 g of the coarsely powdered *C. auriculata* root sample was taken in a conical flask with a stopper and extracted with 100 mL of hexane at room temperature for 8 h. Then, the extract was filtered using Whatman No. 1 filter paper, and the residual defatted powder was evaporated and then stored in airtight container at -20°C .

4.4. Solvent Extraction of Total Polyphenols. The total polyphenols were extracted using different solvents with increasing polarity, *viz.*, chloroform, ethanol, methanol, and water. Briefly, 10 g of defatted root sample was taken in a stopper conical flask along with 100 mL of respective solvents. The conical flasks were kept at room temperature in a rotary shaker for 8 h. Later, the extracts were filtered using Whatman No. 1 filter paper, evaporated using a rotary evaporator, and stored at -20°C until further analyses.

4.5. Estimation of Total Polyphenols and Flavonoids. The estimation of total polyphenols, and flavonoids was performed according to the methods of McDonald et al.³⁰ and Chang et al.³¹ respectively, for different solvent extracts of the *C. auriculata* root.

4.6. HPLC Analysis of Polyphenols in Different Solvent Extracts of the *C. auriculata* Root. The polyphenol extracts of defatted samples of the *C. auriculata* root were subjected to HPLC analysis using the protocol of Govardhan Singh et al.³² in a Shimadzu LC-10AVP (Gradient System PDA, RID, UV) system on a reversed-phase C18 column ($250 \times 4.6 \text{ mm}^2$, Phenomenex). Different polyphenols in the different extracts of the *C. auriculata* root were identified and quantified by comparing with those of the standard peaks.

4.7. FTIR Analysis of Polyphenolic Extracts of the *C. auriculata* Root. The different solvent extracts of the *C. auriculata* root were subjected to FTIR analysis to determine the functional groups present. Briefly, FTIR spectra were recorded at a resolution of 2 cm^{-1} in the range of 400–4000 cm^{-1} .

4.8. Biological Activities of Polyphenol-Rich Extracts of the *C. auriculata* Root. **4.8.1. In Vitro Antioxidant Activities.** **4.8.1.1. DPPH Free Radical Scavenging Activity.** Free radical scavenging activity was investigated using DPPH assay as described by Rakholiya et al.³³ In brief, 150 μL of different concentrations of ascorbic acid (standard) and solvent extracts was taken in different test tubes; 2 mL of 0.1 mM DPPH in methanol was added; the total volume was made up to 3 mL with methanol, and the solution was mixed thoroughly and incubated for 30 min in dark; and the absorbance was recorded at 517 nm against the blank. The percentage of radical scavenging activity was calculated using the following formula: DPPH radical scavenging ability (%) = $\left[\left((\text{Absref} - \text{Abssam}) / \text{Absref} \right) \times 100 \right]$, and the samples were compared by calculating IC_{50} values.

4.8.1.2. FRAP. Ferric reducing antioxidant power was measured following the method of Jeong et al.³⁴ About 8.5 mL of the reaction mixture consisting of 1 mL of different concentrations of the standard (ascorbic acid) and solvent extracts was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6), and 2.5 mL of 1% of potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$] was thoroughly mixed and incubated for 30 min at 50°C . About 2.5 mL of 10% (v/v) trichloroacetic acid was added, and the solution was centrifuged at 3000 rpm for 10 min. Last, 2.5 mL of the supernatant was taken and diluted with the same amount of distilled water and 0.5 mL of 0.1% (w/v) ferric chloride. The absorbance was measured at 700 nm, and an

increase in the absorbance of the reaction mixture indicates increasing reducing power.

4.8.2. In Vitro Antidiabetic Activities. **4.8.2.1. α -Amylase Inhibitory Assay.** The α -amylase inhibitory effect of different solvent extracts of the *C. auriculata* root was analysed by the method described by Hemalatha et al.³⁵ with slight changes. Briefly, 0.1 M phosphate buffer (pH 6.9) containing 6.7 mM sodium chloride was prepared, and porcine pancreatic α -amylase and 1% (w/v) starch solution was prepared using the above buffer. Different concentrations of extracts were taken, to which 500 μL of starch was added and the mixture was incubated at 37°C for 10 min. About 50 μL of porcine pancreatic amylase (0.15 unit/mL) was added to initiate the reaction and was incubated for 30 min at 37°C . Then, 1 mL of dinitro salicylic acid reagent (1 g of DNSA, 30 g of Rochelle salt was dissolved in 20 mL of 2 N sodium hydroxide and made up to 100 mL with distilled water) was added to all test tubes and incubated for 5 min in boiling water to terminate the reaction. Test tubes were cooled to room temperature and diluted with 5 mL of water, and the absorbance was measured at 540 nm. Acarbose (1 mg/mL) was used as a positive control. The α -amylase inhibitory activity of the extract was calculated using the following formula:

$$\text{inhibition (\%)} = \left[1 - \frac{\text{absorbance of the sample}}{\text{absorbance of the control}} \right] \times 100$$

Based on the percentage of inhibition of the enzyme activity, IC_{50} values were calculated and the inhibitory effect of the extract was compared with that of the standard.

4.8.2.2. α -Glucosidase Inhibition Assay. The methanolic extract of the *C. auriculata* root was tested for its inhibitory activity against rat intestinal α -glucosidase using 4-nitrophenyl- α -D-glucopyranoside as a substrate according to the method reported by Hemalatha et al.³⁵ The reaction mixture containing 0.6 mL of 0.05 M phosphate buffer (pH 6.8), 0.25 mL of 3 mM 4-nitrophenyl- α -D-glucopyranoside, and 0.1 mL of the sample at various concentrations was prepared and incubated for 5 min at 37°C . About 0.25 mL of the α -glucosidase solution (0.15 U/mL) was added to initiate the reaction; it was incubated at 37°C for 40 min. Then, 0.25 mL of 0.67 M sodium carbonate solution was added to terminate the reaction. The absorbance was recorded at 405 nm. Acarbose was used as a positive control. The percentage of α -glucosidase inhibition was calculated by the following formula:

$$\begin{aligned} \alpha - \text{glucosidase inhibition (\%)} \\ = \left[1 - \frac{\text{absorbance of the sample}}{\text{absorbance of the control}} \right] \times 100 \end{aligned}$$

Based on the percentage of inhibition of the enzyme activity, the IC_{50} value of the methanolic extract was calculated, and the inhibitory effect of the extract was compared with that of the standard.

4.9. Animal Maintenance. Six week-old male C57BL/6 mice were used in this experiment and maintained at $22 \pm 2^{\circ}\text{C}$ temperature, $55 \pm 5\%$ humidity, and 12-h light/dark cycle. The animals were given food and water *ad libitum*. The animals were maintained at the Animal House Facility, Vipragen Biosciences Private Limited, Mysore, and acclimatized for 1 week before the commencement of the experiment. All the protocols and procedures were approved by the Institutional Animal Ethical Committee (VIP-IAEC-206-2020), and CPCSEA guidelines were followed throughout the experiment.

4.10. Experimental Design. After acclimatization, animals were divided into two sets: the first set ($n = 5$) is control (group 1) with AIN 93 diet (Table 9) and the second set ($n =$

Table 9. Composition and Energy of AIN 93M and High-Fat Diets

ingredients	AIN 93M diet (g)	energy (kcal)	high-fat diet (g)	energy (kcal)
casein	180	690.66	180	690.66
sucrose	100	389.5	100	389.5
corn starch	550	141.21	270	69.48
oil	70	18.01	0	0
cellulose	50	0	50	0
minerals	35	0	35	0
vitamins	10	0	10	0
choline	2	0	2	0
L-cystine	3	0	3	0
lard	0	0	350	3150
total	1000	1239.38	1000	4299.64

15) received a high-fat diet (Table 9) for 8 weeks, followed by a single small dose of streptozotocin (60 mg/kg body weight) dissolved in 50 mM citrate buffer (pH 4.5) and 5% of sucrose solution for 24 h. After 5 days, fasting blood glucose (FBG) levels were measured and it was confirmed that second group mice were with FBG levels ≥ 200 mg/dL (11.1 mmol/L). Then, the second set of mice were divided into three groups, viz., group 2: diabetic (positive control), group 3: diabetic + metformin (102.74 mg/kg bw) (standard group) (the human dose: 500 mg is converted into mouse dose using "DoseCal" calculator of Janhavi et al.³⁶), and group 4: diabetic + the methanolic extract of the *C. auriculata* root (150 mg/kg bw) (treatment group). The treatment of metformin and the methanolic extract of root of *C. auriculata* was given for 8 weeks, and the mice were maintained with the AIN 93 diet. After the last treatment, mice were fasted overnight, euthanized and blood and muscle samples were collected. The blood was centrifuged at 3500 rpm for 10 min, and the plasma obtained as the supernatant was separated. The muscle sample was stored in 10% formalin for the histological study. The remaining muscle and plasma were stored at -20°C until further use.

4.11. Biochemical Analyses. All the plasma biochemical analyses (lipid profiling, liver, and renal function tests) were performed using kits of Agappe Diagnostic Limited, Kerala, India.

4.12. Oxidative Stress Marker in the Plasma and Muscles. The ROS and NO in the plasma and muscles were estimated according to Black and Brandt³⁷ and Green et al.,³⁸ respectively, with slight modifications, i.e., the muscle tissue was homogenized in 0.32 M sucrose.

The ROS generation was measured by incubating the reaction aliquot consisting of 0.1 M Tris-HCl buffer, the sample, and DCF-DA for 1 h at room temperature in the dark. The reaction was read with an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The values are expressed as μmol DCF formed/min/mg protein.

For the measurement of NO generation, the sample was incubated with the Griess reagent for 10 min at room temperature in dark and read at 540 nm. The values are expressed as ng of NO generated/mg protein.

4.13. Activities of Antioxidant Enzymes in the Plasma and Muscles. The activities of antioxidant enzymes, SOD³⁹ and CAT,⁴⁰ in the plasma and muscles were determined according to the standard protocols with slight modifications, i.e., the muscle tissue was homogenized in 0.32 M sucrose.

For the determination of the activity of SOD, to 0.1 M Tris-HCl buffer of pH 8.2, the enzyme sample and 0.2 mM pyrogallol were added and monitored at 420 nm for 2 min for the autoxidation of pyrogallol with and without the presence of sample in the reaction mixture. The quantity of enzyme used to inhibit 50% of autoxidation of pyrogallol is considered as 1 unit. The specific activity was expressed as U/mg protein.

The activity of catalase was determined. Briefly, to the 1 mL reaction mixture, 0.1 M phosphate buffer (pH 7.0), the enzyme sample, and 8.8 mM H₂O₂ were added. The reaction was monitored at 240 nm for 3 min, and the change in absorbance was recorded for every 30 s. The activity of catalase was expressed as nmol/min/mg protein.

4.14. Histopathology. The muscle tissue was removed from the animals and fixed in buffered formaldehyde (10% v/v). After dehydrating with a graded ethanol solution, the tissue was embedded in paraffin wax. Sections of 5 μm thickness were taken in a microtome, mounted on glass slides, deparaffinized in xylene and stained for hematoxylin and eosin (H&E). The slides were observed under a light microscope (Olympus Optical Co., Ltd., Germany) and analyzed for the recovery of damages caused due to high blood glucose by the treatment of metformin and the methanolic extract of root of *C. auriculata*.

4.15. Statistical Analysis. All results are expressed as mean \pm standard error (SE). One-way analysis of variance (ANOVA) was employed, followed by the posthoc Duncan test to identify significant differences ($P < 0.05$) using the SPSS software 17.0. The significant difference ($P < 0.05$) between the standard and the methanolic extract of the *C. auriculata* root in *in vitro* anti-diabetic assay was determined by Student's *t*-test.

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Author Contributions

#B.S., P.J., and S.M. contributed equally to the manuscript. S.D. and M.S.P.: conceptualized the concept and proposed the project; B.S., P.J., S.M., and P.V.: executed the experiments; S.D. and P.J.: compiled and interpreted the results; S.D. and P.J.: prepared the manuscript; and M.S.P., S.D., and M.S.N.: did a critical revision of the manuscript and finalized it.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ALP, alkaline phosphatase; ALT, alanine transferase; AST, aspartate transferase; BUN, blood urea nitrogen; CAT, catalase; CPCSEA, Committee for the Purpose of Control and Supervision of Experiments on Animals; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; FRAP, ferrous reducing antioxidant power; FTIR, Fourier-transform infrared spectroscopy; HDL, high-density lipoprotein; HPLC, high-pressure liquid chromatography; LDL, low-density lipoprotein; NO, nitric oxide; ROS, reactive oxygen species; SOD, superoxide dismutase; STZ, streptozotocin; TC, total cholesterol; TG, triglyceride; T2DM, type-2 diabetic mice; VLDL, very low density lipoprotein

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