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Research Article

In Vitro and *In Vivo* Antioxidant Activity of Methanolic Extract of Eucalyptus Globulus and Studies on Streptozotocin Induced Diabetes in Rats

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ABSTRACT

Eucalyptus globulus is commonly used in folk medicine in the management of diabetes mellitus in South America, Africa and Iran. The paper aims to give a comprehensive account of the effect of methanolic extract of *Eucalyptus globules* (EGE) on *in vitro* and *in vivo* antioxidant and antidiabetic activities. The *in vitro* antioxidant activity was investigated using different tests. In addition, HPLC TOF-MS analysis was carried out. Diabetes was induced by a single intraperitoneal injection of a freshly prepared streptozotocin (STZ) solution (50 mg/kg) to experimental Albino male rats. Then, diabetic rats were categorized into five groups. The first group received distilled water (control one). The second and the third groups were treated with EGE at doses of 150 mg/kg and 500 mg/kg, respectively. The fourth group received Glibil (3mg/kg) and the last one was not treated (untreated group). After 18 days of treatment, the level of plasma glucose, catalase (CAT) activity, protein levels were assayed in liver homogenates. The EGE enriched on flavonoids where quercetin-3-β-D-glucosidewas detected as a major compound. Moreover, it was found that EGE increased significantly the *in vitro* antioxidant activity and decreased the glucose levels and increased plasma antioxidant capacity, CAT activity levels. Also, it reduced the histological changes in the tissues of pancreas. These results suggest a scientific basis for the use of *Eucalyptus globules* probably as antioxidant power and antidiabetic treatment in traditional medicine.

INTRODUCTION

Diabetes mellitus (DM) is a hyperglycemia characterized by defects in the secretion of insulin, the action of insulin, or both [1]. Actually, its incidence has been gradually rising over the years which make it one of the three known major killers after cancer and cardio-cerebral vascular diseases [2].

The reactive oxygen species (ROS) is generated by hyperglycemia, lead to tissue damage, lipid peroxidation, auto-oxidation of glucose, inactivation of proteins, and glycation of non-enzymatic protein which are considered as intermediate mechanisms for hyperglycemia complications such as retinopathy, nephropathy, and coronary heart disease [3]. Also in diabetes, the alteration in the activities of antioxidant defense system especially enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and impaired glutathione (GSH) metabolism are detective [4, 5]. Presently, many synthetic hypoglycemic drugs are available but they can cause many side effects. Therefore, it is necessary to search for new drugs and interventions that can be used to treat and to control this metabolic disorder.

In the last few years, there has been a growing interest in plants which have formed the basis of folk medicine and are mainly used for alimentary purposes especially herb leaves [6]. In fact, for the treatment of diabetes, more than 1200 plants are reported to be used as traditional remedies [7]. Substances such as polyphenols, mainly flavonoids and phenolic acids, are synthesized by a wide array of plants, which exhibit antioxidant

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properties due to their hydrogen-donating, metal-chelating and anti-lipid peroxidation capacities [8] and in the treatment of non-insulino dependent diabetes mellitus (NIDDM) has been also reported as one of their possible use [9, 10, 7].

Eucalyptus globules Labill. (*Myrtaceae*) plays vital role in traditional medicine for the management of diabetes mellitus in South America, Africa and Iran [11]. In Algeria, the leaves of *Eucalyptus globules* were used as antibiotic, antiseptic, antibacterial, carminative, diaphoretic, expectorant, antipyretic, stimulating agents [12].

To the best of our knowledge, there are little studies regarding the *in vivo* antidiabetic of *Eucalyptus globulus*. Thus, in this paper, we explore the possibility of methanolic extract of *E. globulus* (EGE) to improve the antioxidant and antidiabetic activities where the *in vitro* antioxidant and antidiabetic activities of this extract in streptozotocin induced diabetes in rats was estimated as well as the phenolic compounds was identified using HPLC analysis.

MATERIALS AND METHODS

Plant Material

Leaves of *Eucalyptus globules* (Eucalyptus, kalitouss) were collected from Ras-El-Oued (Setif) region in Eastern Algeria (35° 56′ 59″ North, 5° 02′ 09″ East) during April, 2014. The plant was identified by Pr. Laouer Hocine from the Department of Ecology and Plant Biology, Faculty of Sciences of the Nature and life, University Ferhat Abbass, Setif-1, Algeria. A voucher specimen was deposited at the laboratory under the number EG19/003. The plant samples were air dried in shadow and powdered.

Extraction Procedure

Phenolic compounds were extracted from *Eucalyptus globules* leaves according to Markham [13] with slight modification. The powdered plant material (100g) was macerated in 1 liter of 85% aqueous methanol for 72h with frequent agitation. Then the residue obtained after filtration was re-extracted with 1 liter of 50 % aqueous methanol for 24 h. The resulting suspensions from the first and the second extractions were concentrated under reduced pressure using a rotary evaporator at 40 C° to obtain methanolic extract of *Eucalyptus globulus* (EGE). Then, the extract were stored at -20°C until use

Animals

The experimental male adult albino rats weighing 170–280 g obtained from Pasteur Institute (Algiers, Algeria) were acclimatized for one week, prior to experiments. They were housed in an air-conditioned animal room (25±2°C), with light/dark cycle photoperiod, and given free access to water and feeding *ad libitum*.

In Vitro Antioxidant Activity

Determination of Total Flavonoid Contents

Flavonoid content was determined using the method described by Bahorun *et al* [14]. In brief, 1 ml of extract were added to 1 ml of aluminium chloride ($AlCl_3$) solution (2%). After incubation of the mixture for 10 min at room temperature, the absorbance was measured at 430nm. Results were expressed as equivalent quercetin (mg quercetin /g dried extract).

HPLC-TOF/MS Analysis

The HPLC analysis was carried out as described by Abay *et al* [15]. Agilent Technology of 1260 Infinity HPLC System was coupled with 6210 Time of Flight (TOF) LC/MS detector and ZORBAX SB-C18 (4.6 x 100 mm, 3.5µm) column. Mobile phases A and B were ultra-pure water with 0.1% formic acid and acetonitrile, respectively. Flow rate was 0.6mL min^{-1} and column temperature was 35°C. Injection volume was 10µL. The solvent program was as follow: 0-1 min 10% B; 1-20 min 50% B; 20-23 min 80% B; 23-25 min 10% B; 25-30 min 10% B. Ionization mode of HPLC-TOF/MS instrument was negative and operated with a nitrogen gas temperature of 325 °C, nitrogen gas flow of 10.0 L min^{-1}, nebulizer of 40 psi. Samples were filtered through a PTFE (0.45µm) filter by an injector to remove particulates.

DPPH Radical Scavenging Assay

DPPH radical scavenging activity of EGE was determined according the method described by Burits and Bucar [16]. 50µl of various concentrations of the extract were added to 5ml solution of the DPPH (0,004%). The absorbance was measured at 517nm after 30 minutes of incubation at ambient temperature and in the darkness. BHT was used as a reference standard. IC_{50} of extract concentration providing 50% inhibition was calculated from the plot of inhibition percentage against extract concentration. The equation that describes IC_{50} is as follows:

$$\text{Inhibition (\%)} = \frac{(\text{ABS control} - \text{ABS test})}{\text{ABS control}} \times 100$$

Where;
ABS control: Absorbance of control at the wavelength 517nm;
ABS test: Absorbance of the sample at the wavelength 517nm.

Ferrous Ion Chelating Activity

The Ferrous ion chelating activity was estimated using the method of Decker and Welch [17]. Briefly, 500 µl of different concentrations of plant extract or EDTA, 100 µl $FeCl_2$ samples (0.6 mM) and 900 µl of methanol were mixed. After 5 min, 100 µl of Ferrozine (5 mM) were added and the mixture was shaken well and then left to react for 10 min at room temperature. The absorbance of the complex Fe^{+2}-Ferrozine formed was then read at 562 nm. The percentage of inhibition of complex ferrozine–Fe^{2+} formation was calculated. EDTA was used as a reference. The equation can be written as:

$$\text{Chelating activity (\%)} = \frac{(\text{ABS control - ABS test})}{\text{ABS control}} \text{x } 100$$

Where;
ABS control: Absorbance of control at the wavelength 562nm;
ABS test: Absorbance of the sample at the wavelength 562nm.

β-Carotene Bleaching Assay

β-carotene is a free-radical and the mechanism of its bleaching resulting from the hydroperoxides formed from linoleic acid. The inhibition of the oxidative decomposition of β-carotene (discoloration) by the products of oxidation of the linoleic acid was determined according to the method described by Kartal *et al* [18]. In brief, 0.5 mg of β-carotene in 1ml of chloroform was mixed with 25µl of the linoleic acid and 200 mg of Tween 40. Chloroform was then completely evaporated by rotary evaporation. After that, 100 ml of distilled water saturated with oxygen were added with vigorous shaking. 350µl of solution of extract or antioxidants (BHT) dissolved in methanol (2 mg/ml) were added to 2.5ml of the preceding emulsion. The kinetics of discoloration of the emulsion in the presence and absence of antioxidant was followed at 490 nm with intervals of regular times during 24 hours of incubation at ambient temperature and in darkness. The percentage of inhibition of discoloration by extracts was measured as follows:

$$AA\% = \frac{ABS_{test}}{ABS_{BHT}} \text{ x } 100$$

Where;
AA%: Percentage inhibition (antioxidant activity)
ABS_{test}: Absorbance in the presence of the extract
ABS_{BHT}: Absorbance in the presence of positive control BHT.

Antidiabetic Activity

Induction of Experimental Diabetes

Diabetes was induced according to the method reported by Adolfo Andrade Cetto *et al* [19] with slight modifications. Diabetes was induced by single intraperitoneal injection of a freshly prepared streptozotocin (STZ) solution (50 mg/kg in cold sodium nitrate (0.9%) to overnight fasted rats. Rats with blood glucose levels ≥250mg/dl glucose were considered diabetic and were used for the study.

Experiment Design

Rats were randomly divided into five groups of six rats each with same average body weight.

Group 1 or control group: received 1 ml distilled water.

Group 2: received EGE at dose of 150mg/kg.

Group 3: was given EGE at dose of 500mg/kg.

Group 4: was given a standard oral hypoglycemic agent, glibenclamide or Glibil (3mg/kg)

Group 5: untreated group did not receive any thing.

The treatment was given every day via orogastric tube for 18 days.

Effect on Blood Glucose Level

Blood glucose level was measured after the injection of EGE at 0, 1, 2, 3, 24h and 10 days (day of sacrifice) using the glucose oxidase method by a reflective glucometer (ACCU-CHEK, Fast Clix, Germany).

Plasma Preparation

On day 18, all animals were sacrificed. The collection and centrifugation of blood were also done at 3000 rpm for 15 min in purpose to determine the plasma antioxidant activity and to analyze the hematological and biochemical parameters.

Histological Analysis

Animals were dissected and pancreas was removed. Tissues pieces were cleaned several times with saline and fixed in formalin 10% (Sigma-Aldrich) and then removed from it and embedded in paraffin wax. Finally, the tissues were cut into sections of 3µm each. The slides

were selected from each group and were examined under light microscope (Motic/ China), then the pointed field was taken by specialized camera (Anmo/Taiwan) connected with microscope.

In Vivo Antioxidant Capacity

Estimation of Plasma Antioxidant Capacity

Assessment of Plasma DPPH Radical-Scavenging Activity

Plasma ability to scavenge DPPH radical was measured according to the same *in vitro* method. Briefly, a volume of plasma was added to DPPH solution (0.004%). The solution was incubated for 30 min in the darkness followed by a centrifugation and the absorbance was then measured at 517 nm.

Preparation of Tissue Homogenates

Livers pieces were homogenized in KCl buffer (1.15M) using dounce homogenizer in ice-cold condition to obtain 10% (w/v) homogenate. After a centrifugation at 4000 rpm for 15 min, the supernatant collected was used for the determination of lipid peroxidation (MDA), catalase and total protein were assayed.

Determination of Total Protein

Protein concentration was measured according to the method of Gornall *et al* [20], by using the Biuret reagent and bovine serum albumin as a standard. In Brief, 1 ml biuret reagent was mixed with 25 µl sample or standard (albumin). After 10 min of incubation at 37 °C, the absorbance of mixture was measured at 540 nm. Total protein level was calculated as follows:

$$\text{Total protein (mg/ml)} = \frac{\text{ABS of sample}}{\text{ABS of standard}} \times n$$

Where n is standard concentration

Estimation of CAT Activity

CAT activity was measured according to the method of Clairborne [21]. So, 50µl of each tissue supernatant was added to 2950µl of 19mM H_2O_2 prepared in 0.1M phosphate buffer (pH 7.4). The breakdown of H_2O_2 was measured at 240nm at 1 min interval for 2 min using UV-visible spectrophotometer where the decomposition of hydrogen peroxide in the presence of CAT was followed by observing the decrease in absorption of peroxide solution in the ultraviolet (UV) region. Catalase activity was determined as µmol/min/mg protein.

Statistic Analysis

Statistical analysis was performed using Graph Pad Prism (version 5.01 for Windows). All *in vivo* experiments were reported as mean ± S.E.M and were analyzed by one one-wayANOVA followed by Dunnet's test. The *in vitro* data were expressed as the means ± standard deviation (SD) and were analyzed by student test. The $p < 0.05$ was considered to be statistically significant.

RESULTS

In Vitro Antioxidant Activity

Flavonoid Contents of *E.Globulus* Methanolic Extract

The Flavonoids contents in *E.globulus* methanolic extract measured in terms of QE/mg was found to be 90.86±0.02 mg QE/g extract.

HPLC Analysis

The HPLC separation profile revealed the presence of various peaks in the studied sample extract. 40 phenolic acids were detected in EGE where quercetin-3-β-D-glucoside was detected as a major compound (RT=9.58) as shown in Fig. 1, Table 1.

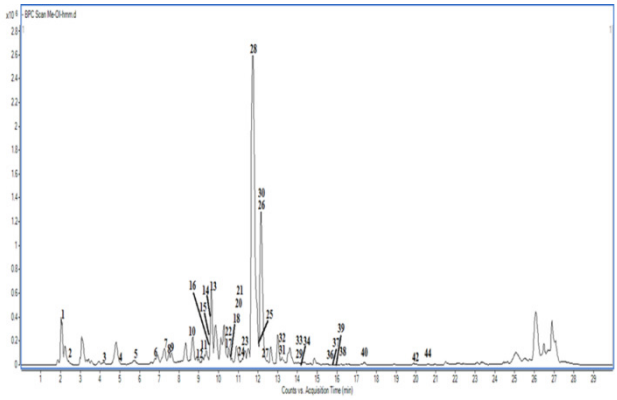

Figure 1: HPLC chromatograms of the methanolic extract of *E. globulus* (EGE) (1.Gallic acid; 2.Fumaric acid; 3.Gentisic acid; 4.Chlorogenic acid; 5.Catechin; 6.4-hydroxybenzoic acid; 7.protocatechuic acid; 8.caffeic acid; 9.Vanillic acid; 10.Syringic acid; 11.rutin; 12.4-hydroxybenzaldehyde; 13.Polydatine ; 14.Ellagic acid; 15.Scutellarin ; 16.Quercetin-3-β-D-glucoside; 17.sinapic acid; 18.naringin; 20.diosmin; 21.Taxifolin; 22.hesperidin; 23.Apigetrin; 24.Neohesperidin; 25.Myricetine; 26.Baicalin; 27.p-coumaric acid; 28.Fisetin; 29.Protocatechuic acid ethyl ester; 30.Morin; 31.Resveratrol; 32.Salicylic acid; 33.quercetin; 34.Silibinin; 36.Apigenin; 37.Naringenin; 38.kaempferol; 39.diosmetin; 40.Neochanin; 42.Wogonin; 44.Biochanin A).

Table 1: Chromatograph analysis of EGE

Name	RT	EGE(mg/kg)	Structure
1.Gallic acid	2,23	247,87	$C_7H_6O_5$
2.Fumaric acid	2,58	366,56	$C_4H_4O_4$
3.Gentisic acid	4,37	405,26	$C_7H_6O_4$
4.Chlorogenic acid	5,02	1358,64	$C_{16}H_{18}O_9$
5.Catechin	5,76	2142,84	$C_{15}H_{14}O_6$
6.4-hydroxybenzoic acid	6,80	4,85	$C_7H_6O_3$
7.protocatechuic acid	7,30	64,36	$C_7H_6O_4$
8.caffeic acid	7,57	tr	$C_9H_8O_4$
9.Vanillic acid	7,70	437,70	$C_8H_8O_4$
10.Syringic acid	8,62	192,92	$C_9H_{10}O_5$
11.rutin	9,29	23,03	$C_{27}H_{30}O_{16}$
12.4-hydroxybenzaldehyde	9,11	tr	$C_7H_6O_2$
13.Polydatine	9,77	14,96	$C_{20}H_{22}O_8$
14.Ellagic acid	9,76	154,58	$C_{14}H_6O_8$
15.Scutellarin	9,61	23,12	$C_{21}H_{18}O_{12}$
16.Quercetin-3-β-D-glucoside	9,58	7508,39	$C_{21}H_{20}O_{12}$
17.sinapic acid	10,57	tr	$C_{11}H_{12}O_5$
18.naringin	10,62	18,91	$C_27H_{32}O_{14}$
20.diosmin	10,61	187,02	$C_{28}H_{32}O_{15}$
21.Taxifolin	10,80	24,15	$C_{15}H_{12}O_7$
22.hesperidin	10,59	tr	$C_{28}H_{34}O_{15}$
23.Apigetrin	11,36	tr	$C_{21}H_{20}O_{10}$
24.Neohesperidin	11,18	tr	$C_{28}H_{36}O_{15}$
25.Myricetine	12,00	tr	$C_{15}H_{10}O_8$
26.Baicalin	12,21	11,95	$C_{21}H_{18}O_{11}$
27.p-coumaric acid	12,31	tr	$C_9H_8O_3$
28.Fisetin	11,95	tr	$C_{15}H_{10}O_6$
29.Protocatechuic acid ethyl ester	14,15	tr	$C_9H_{10}O_4$
30.Morin	12,23	54,86	$C_{15}H_{10}O_7$
31.Resveratrol	13,35	tr	$C_{14}H_{12}O_3$
32.Salicylic acid	13,35	tr	$C_7H_6O_3$
33.quercetin	14,15	tr	$C_{15}H_{10}O_7$
34.Silibinin	14,26	tr	$C_{25}H_{22}O_{10}$
35.Cinnamic acid	15,86	nd	$C_9H_8O_2$
36.Apigenin	15,89	tr	$C_{15}H_{10}O_5$
37.Naringenin	15,90	tr	$C_{15}H_{12}O_5$
38.kaempferol	16,24	tr	$C_{15}H_{10}O_6$
39.diosmetin	16,00	tr	$C_{16}H_{12}O_6$
40.Neochanin	17,49	tr	$C_{22}H_{22}O_9$
42.Wogonin	19,98	tr	$C_{16}H_{12}O_5$
44.Biochanin A	20,60	tr	$C_{16}H_{12}O_5$

DPPH Radical Scavenging Assay

The EGE was able to donate hydrogen atoms or electrons to DPPH radicals and this was determined by the decrease in its absorbance at 517nm. Table 1 revealed that EGE exhibited a strongest antioxidant activity (IC_{50} =0.086± 0.00 mg/ml) compared to quercetin as standard with IC_{50}= 0.0034±5.38 mg/ml.

Ferrous Ion Chelating Activity

The metal chelating, such as iron, is considered freely strong stimulation of the oxidation

reactions and the production of free radicals. As seen in Table 2, the EGE showed lower ability to chelate the ferrous ion (IC_{50} = 0.258±0.149 mg/ml) compared to EDTA value (IC_{50} = 0.0073±0.0001 mg / ml).

Table 2: Antioxidant activities of EGE

Extract / standard	AA%		
Assay	DPPH	Ferrous ion chelating	β-carotene
EGE	0.086±0,00***	0.258±0.149ns	84.14***
Quercetin	0.0034±5.38	-	-
EDTA	-	0.0073±0.0001	-
BHT	-	-	85.78±0.03

Values are expressed as means±SD (n=3). (ns: no significant difference; ***: p<0.001)

β-carotene /linoleic acid bleaching assay

In this assay the measurement of the inhibition of the organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation was used to determine the antioxidant capacity. Methanol extract showed a good inhibition percentage (84.14±0 %) compared to the BHT (85.78±0.03%), Table 2.

Antidiabetic Activity

Effect of EGE on Blood Glucose Level

The anti-hyperglycemic effect of the methanolic extract of the leaves of EGE (150 and 500mg/kg) and glibenclamide on blood glucose levels of diabetic and non-diabetic rats are shown in Table 3. EGE exhibited hypoglycemic effect in a dose dependent manner. It showed a gradual decrease in the blood sugar level from 0h to 10 days and seems to be similar to control group at 24 h for the two doses ($p \geq 0.05$) and at 10 days for the dose of 500mg/kg. The untreated rats showed an obvious increase of blood glucose level over the completed period compared to the normal control group.

Table 3: Glucose levels changes in the blood of treated and untreated animals.

Hours	Control[(a)]	150mg/kg	500mg/kg	Glibil	Untreated
0	1.19±0.13	4.83±0.01***	4.47±0.68***	4.13 ± 1.05**	HI***
1h	1.27 ± 0.15	5.45±1.05***	5.1±0.1***	4.31±0.87***	HI***
2h	1.32 ± 0.17	3.18±1.30***	3.71±0.70***	4.70 ±1 ***	HI***
3h	1.41 ± 0.10	2.56±1.50***	3.61±0.25***	4.6± 0.35***	HI***
24h	1.30 ± 0.12	1.07±0.05ns	1.48±0.38ns	4.29±0.55***	HI***
10 days after	1.11 ± 0.09	3.11±0.16***	1.70±0.82 ns	3.98± 1.74**	HI***

(a): dl/l. Value are mean ± SEM (n=6) (ns: not significant, **P<0.01,*** : P<0.001). (a): dl/l. Value are mean ± SEM (n=6) (ns: not significant, **P<0.01,*** : P<0.001) compared to control group. (HI): high than 6dl/l.

Table 4: Biochemical and hematological parameters in blood of treated and untreated animals

	Control	150mg/kg	500mg/kg	Glibil	Untreated
RCB 10^6/mm³	9.22 ± 0.9	8.95±1.36 ns	9.06±2.75 ns	8.13±0.21**	7.53±0.85***
PLT 10^3/mm³	230± 0.08	204± 1.87ns	207.4±1.32 ns	185.6±0.72**	175± 2.88***
GB 10^3/mm³	12.1 ± 0.14	11.5±1.03ns	11.96±1.94ns	11.4 ± 0.28ns	8.3 ± 0.64**
HGB g/dl	14.3 ± 0.02	13.98±2.04 ns	14 ± 0.88 ns	13 ± 0.52 ns	11.7 ± 0.8*
Lipidic metabolism					
Triglycerides g/l	1.26 ± 0.28	1.27± 0.46 ns	1.28 ±0.15 ns	1.46±0.25 ***	1.67 ±0.25 ***
Total cholesterol g/l	0.53 ± 0.02	0.54 ±0.08 ns	0.59 ± 0.06**	0.61±0.06 ***	0.67±0.09 ***
LDL g/l	0.05 ± 0.01	0.05 ±0.03 ns	0.065±0.03ns	0.07±0.03 ns	0.09± 0.02**
HDL g/l	0.64± 0.03	0.65 ±0.10 ns	0.66 ±0.08 ns	0.73±0.05 **	0.82± 0.06 ***
Protein					
Albumin g/l	37.6 ± 0.72	38 ± 0.6 ns	37.66±0.95ns	39.2±0.04 *	44.6± 0.32**
Hepatic Bilan					
AST UI/l	128.06±0.21	132.32±1.13**	129.93±1.86 ns	130.3±0.4 ns	135.4±0.69***
ALT UI/l	51.48 ± 0.93	57.26 ±1.72**	53.43 ± 0.71ns	61.6±0.69 ***	79.3± 0.46 ***

Values are mean ± SEM (n=6) (ns : no significant difference ; ,*p < 0.05, ** p < 0.01, *** p < 0.001).

Effect of EGE on Hematological Parameters

Untreated or diabetic rats showed a high significant decrease ($P < 0.001$) of RBC count ($7.53\pm0.58 \times 10^6/mm^3$) when compared to normal control rats ($9.22\pm0.9 \times 10^6/mm^3$). The same result was observed concerning the hemoglobin content and PLT. All the treatment with two doses of extracts showed a remarkable elevation of RBC count in diabetic rats (Table 4).

Effect of EGE on Blood Biochemical Parameters

In the present study, it has been found that high levels of cholesterol (0.67±0.09g/l and 1.67±0.25g/l) and triglycerides (1.67±0.25g/l) were noticed for the untreated rats (Table 4). Also, ALT level increased significantly ($P < 0.001$) in rats untreated group (79.3±0.46 UI/l) compared to control group (51.48±0.93UI/l). However, the both doses of *E.globulus* decreased the all tested biochemical parameters.

Pancreas Histopathology

Present observations on the pancreas sections showed progressive damage associated with the severity of hyperglycemia. As can be seen from Fig. 2, no histological changes were shown in pancreatic tissue of control group, whereas, the pancreas in untreated group, were disappeared. Streaky inflammation by lymphocytes in the Islets region was showed in pancreas of the EGE treated animals.

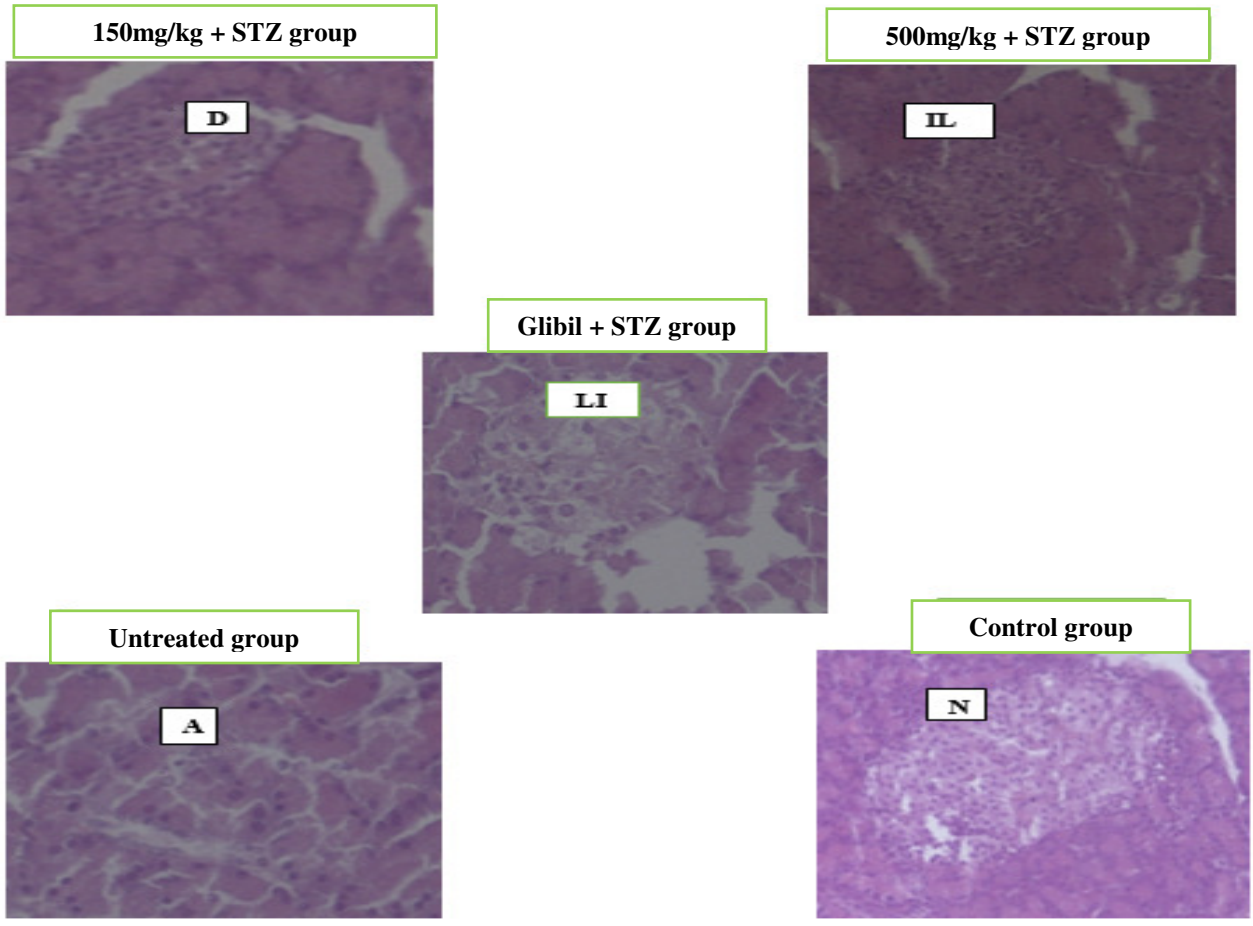

Figure 2: Photomicrographs of pancreatic islets in rats treated with EGE after 18 days of the treatment. Severe islet cell necrosis is observed, X20. (150mg/kg +STZ group): dystrophic Islands of Langerhans (IL). (500mg/kg + STZ group): only one IL. (Glibil + STZ group): IL infiltrated by lymphocytes.(Untreated group): Absence of IL. (Control group): Normal IL.

***In Vivo* Antioxiadant Activity**

Plasma Antioxidant Capacity

Plasma Antioxidant Capacity Using DPPH Radical

In this study, the administration *per os* of EGE at doses of 150 and 500 mg/Kg in rats showed significant increase in plasma antioxidant capacity with percentage inhibition of 18.78±0.16 and 26.64±0.42%, respectively. Glibil treated group and the untreated group with inhibition percentage of 15.43±0.85% and 7.25±0.66%, respectively showed no significant difference when compared to control group (14.19 ±1.57 %), Fig. 3.

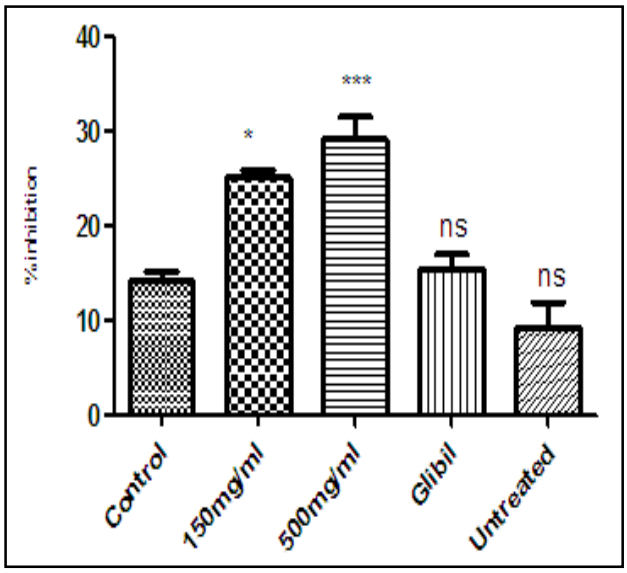

Figure 3: Comparison between different groups in plasma antioxidant capacity using DPPH radical. Data were presented as % means ± SEM (n = 6). (ns: no significant difference ; * $p < 0.05$; *** $p < 0.001$) compared to control group.

Total Protein Level

As seen in Fig. 4, the treatment with ME of *E.globulus* at the doses of 150 and 500mg/kg did not change total protein level in liver (20.46±0.65 mg/ml; 21.62±0.46mg/ml) when compared to control group (21.60± 1.19 mg/ml).

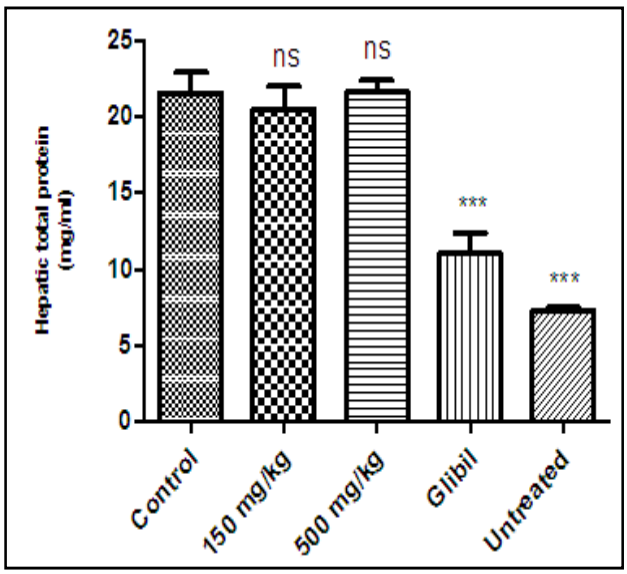

Figure 4: Effect of methanolic extract of *E.globulus* on total protein level in the liver of rats. Values are means ± SEM (n=6). (ns: no significant difference; **: P<0.01; *** : P<0.001).

Estimation of Catalase Activity

As shown in Fig. 5, the activity of CAT in livers of rats treated with *E.globulus* decreased significantly when compared to untreated group which showed the highest catalase activity (0.0071±0.0006 µmol/min/mg protein). Moreover, the Glibil and *E.globulus* treated groups showed a similar catalase activity to that of control group.

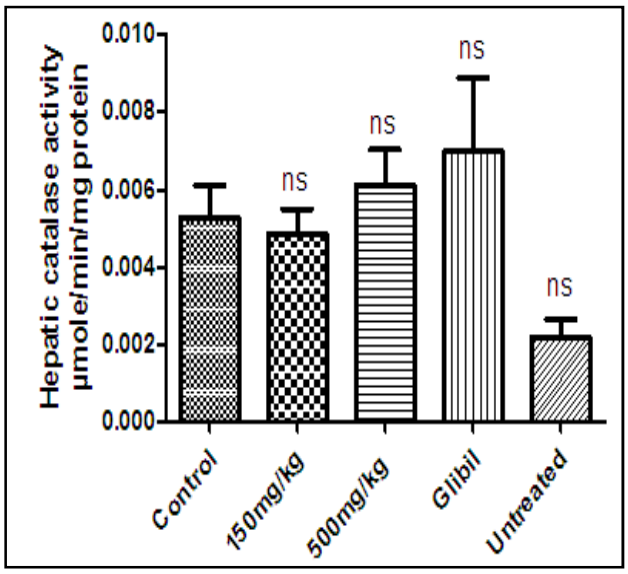

Figure 5: Effect of methanolic extract of *E.globulus* on catalase activity level in the liver of rats. Values are means ± SEM (n=6). (ns: no significant difference; **: P<0.01; *** : P<0.001).

DISCUSSION

One of the important causes of diabetes complexity has been submitted to the higher oxidative stress [22]. However, abnormalities in glucose and lipid metabolism, inadequate pancreatic beta-cells for insulin secretion and resistance to insulin activity are involved in the gender and pathogenesis of T2D [23]. Thus, our paper presents a novel view of evaluating the *in vitro* antioxidant capacity of methanolic extract of *E. globulus* (EGE) using many methods. In addition, the HPLC analysis was carried out and the antidiabetic activity of EGE in sterptocytozin induced diabetic in rats and *in vivo* antioxidant activity in liver of rats were evaluated.

The variety and different apportionement of phenolic compounds in the plant may explain the different ranges obtained for the total phenolic contents [24]. The leaves had the highest values of phenolic compounds in many plants and this was in accordance with this study.

The data obtained is quercetin and catechins which were the dominant flavonoids in *E. globules* .Whereas, Dezsi *et al*. [25] reported only the presence of chlorogenic acid, a phenolic acid in the *E. globulus* extract. The samples of flavonoids and flavonols indicated large

quantitative differences where hyperoside, isoquercitrin, rutin and quercitrin were found in this extract with high amount.

There are several ways of testing antioxidant activity of natural products, but it is believed that radical scavenging via hydrogen atom donation is the predominant mode [26].

An important implication of these findings is that the EGE exhibited a strong antioxidant activity through established tests. Concerning DPPH scavenging activity, results showed that an increase in EGE concentration resulted in an increase in free radical-scavenging activity. Whereas the extract, free radical-scavenging activities were compared to BHT. These results suggested that the protective effects are probably due to its flavonoids contents.

Pro-oxidative metal ions in living systems may be stabilized by chelating agents via complexing them [27, 28]. Moreover, some phenolic compounds and flavonoids exhibit antioxidant activity by the chelating of metal ions [29] and form a stable complex with transition metals (Fe^{3+}, Al^{3+}, Cu^{2+}, Zn^{2+}); the stoichiometry of the complex and the site of chelation depend on the nature of the flavonoid principally the presence of the catechol part [30].

Our data indicated that the studied plant is potential sources of secondary metabolites where EGE extract interfered with the formation of ferrous and ferrozine complex, suggesting that the extract exhibited observable chelating activity.

The antioxidant activity of sample was reflected in its ability of the bleaching of β-carotene inhibition. In this assay, the results showed the lipid peroxidation inhibition in response to EGE. In General, the presence of O-dihydroxyl group in aromatic ring of phenolic compounds, give them stronger antioxidant activity than monophenolics [31]. For instance, caffeic acid reacted better than *p*-coumaric acid with oxygen centered radicals.

Therefore, antioxidative activities observed in this plant extract could be the synergistic action of more than two compounds that present in the plant. It has been mentioned that further natural antioxidative molecules creates an powerful defense system against free radical attack and work synergistically with each other to create a wide spectrum of antioxidative activities [32].

Secretion of insulin in response to the higher glucose levels in plasma is quickly [33]. Streptozotocin (STZ) is a glucose analogue that is selectively toxic to β cells in the pancreatic islets and specifically damages pancreatic β cells [34]. This compound has been used widely to produce an experimental model of diabetes mellitus in animals [35].

In this study, the administration *per os* of the two doses of EGE showed a decrease in the level of plasma glucose. Zafar and Naeem [36] analyzed the mean plasma glucose levels of rats after administration of STZ at variable intervals of time and found that the plasma glucose levels were significantly higher and increased in groups increasing with time.

Also, Houacine *et al* [37] indicated that the administration of *E. globulus* had significant dose-dependent reduction in blood glucose of hyperglycemic rats ($P < 0.05$) and could occur partly by stimulating insulin production from the islets of pancreas, or it could stimulate insulin production and glucose utilization similar to glibenclamide due to the attendance of certain hypoglycemic bioactive components in *E. globulus* extract.

A number of studies revealed the direct effect of quercetin, which is one of the compounds of the studied plant, on insulin-secreting β cells [38]. Alam *et al* [39] reported that the treatment of quercetinin streptozotocin (STZ)-induced diabetic mice resulted in the reduction of hyperglycemia-stimulating GLUT4 and glucokinase, increased glucose uptake, and decreased glycogenolysis and gluconeogenesis in liver.

Mansi and Lahham [40] indicted that various hematological and immunological parameters altered during the course of diabetes. Thus, the purpose of the present study was to scrutinize the influence of oral administration of EGE on the levels of hematological parameters including hemoglobin (Hb), white blood cells (WBC), platelets (PLT), and red blood cells (RBC). As conclusion, the administration of the EGE resulted in increased parameters levels near to normal level.

In this study, the HPLC analysis confirmed the presence of many phenolic and flavonoids compounds from EGE. Thus, these bioactive principles, at least in-part, may be responsible for the observed effect of the studied plant. Furthermore, Punithavathi *et al* [41] demonstrated that the oral treatment with gallic acid (10 and 20mg/kg) daily for a period of 21days showed a significant ($P<0.05$) protective

actions on all studied the biochemical parameters.

The most hepatocellular injury markers are aminotransferase (ALT) and γ-glutamyltranspeptidase (GGT). The augmentation activity of these indicators is associated with resistance of insulin, type 2 diabetes, and metabolic syndrome [42]. In accordance with these findings, streptozotocin treatment has a significant role in the alteration of liver functions since the activity of AST and ALT were significantly higher than those of normal values.

Intraperitoneal (IP) injection of quercetin, which is one of the flavonoids identified in the studied plant into STZ-induced diabetic rats lead to: glucose tolerance, hepatic glucokinase activity are increased; hyperglycemia, and plasma cholesterol and triglycerides are reduced [43]. This finding corroborated with our results which indicated that EGE was more effective in comparison with glibenclamide in attenuating the increased serum parameters resulting from damage of STZ-induced diabetic rats.

DPPH radical scavenging activity is very useful test for the study of natural antioxidants. In the present study, EGE have good antioxidant capacity to increase plasma DPPH radical scavenging capacity rather than the drug Glibil. It was reported that the relationship between DPPH assay and plant extract are related to the contents of phenolic compounds in plant extracts [44]. In our study, EGE showed effectiveness on DPPH radical.

In diabetic rats, acute hyperglycemia, pancreas tissues damages were observed. Where EGE decreased hyperglycemia and improved the regeneration of tissues. These are in accordance with the reports which provide preliminary biochemical and histological support to the medicinal uses of plants extracts as gallic acid in the management and/or control of diabetes mellitus and the protective effects on histopathology of pancreas [41].

Our studies have showed that diabetes was affected systems of the maintaining cellular homeostasis for instance enzymatic and non-enzymatic. Particularly the activities of, SOD, CAT, GSH-Px and also the lipid peroxidation level were changed in STZ treated animals. The tissue damage degree induced by free radicals depends on the poise between free radical generation and the endogenous antioxidant defense mechanism [45].

Liver function histology help in the diagnosis of any abnormal/normal condition of liver where the leakage of cellular enzymes into plasma was the indicator of hepatic tissue damage [46].

The treatment with EGE of diabetic rats produced alteration in liver protein level compared with control animals, whereas, total proteins were very low in liver of untreated diabetic rats. The result showed that plant extract was more effective than glibenclamide in restoring and protecting liver tissues towards normal.

Hydrogen peroxide performs a significant role in the immune system and acts either directly or indirectly as a messenger molecule in the inflammation events and cells signaling pathways [47]. In present study, the activities of CAT in liver tissue of the STZ-diabetic rats were near to those of control group. Chiang *et al*. [48] reported that the increase in the antioxidant activity including the increase of antioxidant activity enzymes, for example superoxide dismutase and catalase. In addition, it was also determined the correlation between the alteration in the antioxidant enzymes activities and their respective mRNA expression in the phenolic acids-supplemented rats.

CAT are implicated in the H_2O_2 elimination [49] and SOD deals by dismutate superoxide radicals to give H_2O_2after that deals by GPx [50]. In diabetic rats, the cumulation of lipid peroxides and the increase oxidative stress are related to the function of all three enzymes [51]. Thus, in the mechanisms of abnormal tissue function observed in diabetes has involved the normalization in enzymatic antioxidant defense system and variations in their activities [52].

CONCLUSION

From the research that has been carried out, we could conclude that methanolic extract of *Eucalyptus globulus* enriched on polyphenol compounds and flavonoids which were identified by HPLC TOF-MS analysis. In addition, the administration of EGE at doses 150mg/kg and 500mg/kg caused an increase in plasma antioxidant activity, decrease in glucose levels, increasing the CAT activities. It also reduced the histological changes in the tissues of pancreas. Finally, the EGE has significant potential as a natural antioxidant to promote health and to reduce the diabetes mellitus.

COMPETING INTEREST STATEMENT
The authors declare no conflict of interest.

ADDITIONAL INFORMATION
No additional information is available for this paper.

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