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Full Paper

Effects of *Rhizoclonium hieroglyphicum* and *Spirogyra neglecta* combined freshwater algal extract on blood glucose, lipids and oxidative markers in diabetic rats

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Abstract: *Rhizoclonium hieroglyphicum* and *Spirogyra neglecta* are freshwater green macroalgae that exhibit antioxidant, anti-inflammatory and anti-diabetic properties. *R. hieroglyphicum* extract (RE) and *S. neglecta* extract (SE) were mixed to obtain a combined algal extract (RSE), and RSE was used as functional ingredient in an algal product (RSP). The total phenolic content of RE and SE was 8.58 and 107.02 mgGAE/g extract respectively. RSE and/or RSP, respectively, decreased glucose level by 38.53% and 41.28%, triglycerides by 2.96% and 36.84%, total cholesterol by 25.98% and 21.07% and malondialdehyde by 32.45% and 28.46% in diabetic rats but significantly increased superoxide dismutase, catalase and glutathione peroxidase activities. These findings indicate that RSE and RSP possess anti-hyperglycemic, anti-hypercholesterolemic and antioxidative stress effects.

Keywords: *Rhizoclonium hieroglyphicum*, *Spirogyra neglecta*, freshwater algae, anti-hyperglycemia, anti-hypercholesterolemia, antioxidative stress

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterised by high blood glucose levels (over 200 mg/dL). Asian individuals aged over 35 years suffering from overweight, obesity and dyslipidemia are at higher risk of developing T2DM [1]. The main cause of dyslipidemia is poor diet and lifestyle and lack of physical activity. In Thailand the incidence of T2DM is increasing and in 2016 10% of all Thai deaths were due to diabetes [2], evidence of a major public health problem. Public health intervention has focused on reducing the number of diabetic patients by stressing the importance of regular exercise, weight loss and a healthy diet.

Diabetes mellitus is associated with an increase in free radicals and oxidative stress, a cellular condition resulting from a physiological imbalance between antioxidants and free radicals [3]. The main antioxidant cellular enzymes are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). They provide natural antioxidant defence by scavenging active free radicals, maintaining a balance between oxidants and antioxidants. When the production of free radicals exceeds specific levels, elevated intake of antioxidant-rich foods or antioxidant supplements may enhance the body's potential to minimise the risk of free radical-related health problems [4].

Interestingly, some freshwater green macroalgae have nutritional and antioxidative properties. *Cladophora* sp. [5] and *Spirogyra varians* [6] have nutritional values and *Spirogyra neglecta* has antioxidative and anti-inflammatory properties [7]. *Rhizoclonium hieroglyphicum* (*Cladophora glomerata*) and *S. neglecta* belong to the division Chlorophyta. They are commonly consumed in northern and north-eastern Thailand and exhibit antioxidant [8, 9] and anti-inflammatory activities [7] as well as effectiveness against cancer [10] and gastric ulcers [7]. Ontawong et al. [11] and Srimaroeng et al. [12] found that *S. neglecta* and *C. glomerata* demonstrated antioxidant and anti-diabetic properties. Nevertheless, effects of *a combined R. hieroglyphicum* and *S. neglecta* algal extract on biomarkers in diabetic rats have not been evaluated. In this study we investigate using diabetic rats the effects of the combined algal extracts which may be beneficial as a nutraceutical product for diabetes mellitus.

MATERIALS AND METHODS

Chemicals and Reagents

Folin-Ciocalteu reagent, sodium carbonate, gallic acid, sodium fluoride, ethylene diamine tetraacetic acid (EDTA) and metformin were purchased from Merck (Germany). Streptozotocin was purchased from Sigma-Aldrich (Germany). Assay kits for glucose, triglyceride and cholesterol levels were purchased from Biotech (Thailand). Sodium dodecyl sulfate, thiobarbituric acid, acetic acid, tetrazolium salt, xanthine oxidase, potassium phosphate, methanol, hydrogen peroxide, potassium hydroxide, Purpald reagent, potassium periodate, Tris-hydrochloride, nicotinamide adenine dinucleotide phosphate oxidase (NADPH), glutathione, glutathione reductase, cumene hydroperoxide were purchased from Cayman (USA). Sorbitol and carrageenan were purchased from Ueno Fine Chemicals Industry and Chemipan Corporation (Thailand) respectively and were of food grade. .All other reagents were of analytical or chromatographic grade.

Extract Preparation

R. hieroglyphicum and *S. neglecta* were collected from Nan province and Phrae province, Thailand, respectively. The freshwater algae were dried in a hot air oven at 55-60°C for 48 hr. Each of the dried algae was boiled in distilled water at 95-100°C for one hour. The algal extract solution

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was concentrated by rotary evaporation and lyophilised using a freeze dryer to produce the dried extract : *R. hieroglyphicum* extract (RE) and *S. neglecta* extract (SE), which were stored at 4°C prior to use.

A combined algal extract (RSE) was prepared from a mixture of RE and SE at 1:1 (wt/wt) ratio. An algal product (RSP), consisting of RSE (500 mg), sorbitol (70 mg) and carrageenan (15 mg), suitable as a functional ingredient in a drink, was also prepared.

Determination of Total Phenolic Content

The total phenolic content of RE and SE was determined using Folin-Ciocalteu method [13]. The algal extract (0.1 g) was dissolved in distilled water (1.0 mL) to make a sample solution, 0.2 mL of which was mixed with 1.0 mL of 10% Folin-Ciocalteu reagent and 0.8 mL of 7.5% sodium carbonate. The absorbance of the reaction mixture was measured spectrophotometrically at 765 nm using a spectrophotometer (Thermo Scientific^{TM}, Evolution 260 Bio, Finland) after 1 hr of incubation at room temperature. The total phenolic content was expressed as gallic acid equivalent (GAE) in mg/g of dried extract (mg GAE/g extract) using a calibration curve of gallic acid.

Animals

Male Wistar albino rats (120-150g) were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Thailand. The experimental procedures and protocols were approved by the Laboratory Animal Care and Use Committees at the Faculty of Medicine, Chiang Mai University (Certificate of Ethics no. 30/2561, dated 7 November 2018). Animals were housed in a room and maintained at 25 ± 1 ºC on a 12-hr light/dark cycle. The animals were given *ad libitum* access to water and food.

Animals were randomised and divided into six groups:

Group 1: normal control group (NC), fed with distilled water;
Group 2: normal control group (NP), fed with 500 mg/kg RSP;
Group 3: diabetic control group (DC), fed with distilled water;
Group 4: diabetic group (DP), fed with 500 mg/kg RSP;
Group 5: diabetic group (DE), fed with 500 mg/kg RSE;
Group 6: diabetic group (DM), fed with 50 mg/kg metformin.

Groups 1 and 2 were normal rats whereas diabetes was induced in groups 3-6 by intraperitoneal injection of streptozotocin (40 mg/kg) [14]. After 10 days, rats with fasting plasma glucose levels of 200 mg/dL or more were classified as diabetic and used in this study. All rats were orally administered the indicated samples daily for 8 weeks, after which the animals were euthanised and blood samples were collected for assessment of fasting plasma glucose, triglyceride, cholesterol and antioxidant enzymes (SOD, CAT and GPx).

Determination of Glucose, Triglyceride and Cholesterol Levels

The blood samples were collected from the tail vein (under light ether anaesthesia) into the microcentrifuge tube containing sodium fluoride anticoagulant for determination of glucose concentration and into a non-anticoagulant microcentrifuge tube for determination of triglyceride and cholesterol levels. They were measured using a commercial enzymatic colorimetric assay kit (Biotech, Thailand). The high-density lipoprotein (HDL) and low-density lipoprotein (LDL) levels in serum were determined by an automatic analyser (BS-380, Mindray Bio-Medical Electronics, China).

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Determination of Malondialdehyde Level

Malondialdehyde (MDA) is a product of lipid peroxidation. MDA level was determined using a thiobarbituric acid reactive substances (TBARS) assay kit (Cayman, USA) according to the manufacturer's protocol. The blood sample was collected in an anticoagulant (EDTA) and centrifuged at 1,000g for 10 min. The yellow plasma was used for testing. The sample (0.10 mL) was mixed with 8.1% sodium dodecyl sulphate solution (0.1 mL), 0.6% thiobarbituric acid (2.0 mL) and 20% acetic acid (2.0 mL). The mixture was incubated for 1 hr at 100ºC, placed in an ice bath to stop the reaction and centrifuged at 1,600g for 10 min. The absorbance of the supernatant was measured by a spectrophotometer at 540 nm.

Determination of Antioxidant Enzymes

SOD, CAT and GPx activities were determined in the plasma using specific assay kits (Cayman, USA).

Determination of SOD activity

Blood samples were collected without an anticoagulant and centrifuged at 2,000g for 15 min. The yellow plasma layer was removed by pipet and stored on ice. The plasma sample (10 μL) was added to 200 μL of 10 mM tetrazolium salt solution and 20 μL of xanthine oxidase (0.2 U/mL), mixed by shaking and incubated for 30 min. The sample absorbance at 460 nm was measured and the SOD activity was expressed as U/mL.

Determination of CAT activity

The plasma (20 μL) was mixed with 100 μL of phosphate buffer (pH 7.0) and 30 μL of methanol. The reaction was initiated by adding 8.82 M hydrogen peroxide solution (20 μL). The mixture was incubated with shaking for 20 min. at room temperature and 10M potassium hydroxide (30 μL), 136 mM Purpald reagent (30 μL) and 0.5M potassium periodate (10 μL) were added. The sample was incubated for 5 min. before the absorbance was read at 540 nm. The result was expressed as nmol/min./mL.

Determination of GPx activity

The blood sample was collected using an anticoagulant (EDTA) and centrifuged at 1,000g for 10 min. at 4ºC. The yellow plasma (20 μL) was then removed by pipet and added to 100 μL of Tris-HCl buffer (pH 7.6), 0.2 mM NADPH (Vol.?), 10 μL of 0.5 mM glutathione and 20 μL of glutathione reductase (1.0 U/mL).The reaction was initiated by adding 20 μL of 2 mM cumene hydroperoxide. The activity of the reaction was read at an absorbance of 340 nm. The result was expressed as nmol/min./mL.

Statistical Analyses

All data from the animal experiments are expressed as mean standard error of the mean (S.E.M.). Statistical comparisons were performed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. Differences were considered to be significant when $p \leq 0.05$.

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RESULTS AND DISCUSSION

The yield of RE and SE from the algae was 38.4% and 35.5% respectively. The total phenolic content of RE and SE was 8.58 and 107.02 mgGAE/g extract respectively. Previous studies reported that the phenolic compounds of RE and SE include gallic acid, eriodictyol, isoquercetin, kaempferol, quercetin, hydroquinone, rutin, catechin and tannic acid, with isoquercetin and catechin being the main phenolic components in *R. hieroglyphicum* and *S. neglecta* [9, 11, 15, 16].

In both algae extracts, isoquercetin is the major component of all phenolic compounds. Isoquercetin (quercetin-3-glycoside) prevents or slows the oxidation process in food digestion. Moreover, it decreases cholesterol and triglyceride levels while promoting glucose consumption in diabetic rats and rabbits [17, 18, 19]. It is sometimes included as a functional ingredient in nutraceutical products.

The percentage of weight gain and ratio of visceral fat weight to body weight in all experimental groups (normal and diabetic rats) are shown in Table 1. Diabetic rats were not obese in this study. Additionally, increases in weight and visceral fat in normal and diabetic groups were not significantly different at the end of the trial period.

Table 1. Percentage of weight gain and visceral fat in normal and diabetic rats at 8th week

Group	%Weight gain	Visceral fat/body weight
NC	60.89 ± 4.35	0.0608 ± 0.0035
NP	58.58 ± 5.97	0.0598 ± 0.0055
DC	45.30 ± 10.75	0.0447 ± 0.0162
DP	55.28 ± 4.04	0.0569 ± 0.0043
DE	64.10 ± 5.06	0.0579 ± 0.0073
DM	55.21 ± 5.02	0.0590 ± 0.0024

Note: Data expressed as mean ± S.E.M. (n=6), p>0.05, NC=normal control rats, NP=normal control rats receiving 500mg/kg RSP, DC=diabetic control rats, DP=diabetic rats receiving 500mg/kg RSP, DE=diabetic rats receiving 500mg/kg RSE, DM= diabetic rats receiving 50mg/kg metformin

The normal rats that received RSP exhibited no significant increase in fasting glucose levels compared to the NC group at 8 weeks. The diabetic rat of the DP and DE groups experienced significant decreases in fasting glucose levels compared to the DC group. T2DM is characterised by fasting glucose >200 mg/dL (hyperglycaemia), insulin resistance and dyslipidemia, and metformin is a medicine used for lowering blood glucose concentrations. Figure 1A shows that the fasting glucose level decreases 41.28 and 38.53% in the DP and DE groups respectively, while the DM group has 42.03% less glucose than the DC group at the end of the experiment. The fasting glucose levels of the DP and DE groups indicate that RSP and RSE at a dosage of 500 mg/kg can decrease the glucose level as efficiently as metformin in the DM group.

Total cholesterol of the NP and NC groups (Figure 1B) is not significantly different (p>0.05). However, in diabetic groups it was found that the total cholesterol of DE and DP is less (25.98 and 21.07% respectively) than in the DC group. Moreover, triglycerides decrease in the DP group at a greater rate than DE (36.84% and 2.96% respectively) by the 8th week (Figures 1C). Ontawong et al. [11] found that 500 mg/kg of SE could decrease blood glucose and triglyceride levels by 35 and 45% respectively when compared to control diabetic rats. In addition, 500 mg/kg of RE had a potential

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anti-diabetic effect on normal rats in an oral glucose tolerance test [12]. The results of the present study show that RSE or RSP consisting of SE and RE in a 1:1 ratio can cause a decrease in blood glucose level at a similar extent as that by SE alone. Marine algae such as *Sargassum hystrix*, at a dose of 300 mg/kg, was also found to decrease the levels of triglycerides and cholesterol in diabetic rats [20]. *Sargassum oligocystum* also decreased fasting blood glucose and triglyceride levels [21] and Panlasigui et al. [22] reported that carrageenan reduced blood cholesterol and triglyceride levels in human subjects. This might explain why RSP containing carrageenan could reduce the triglyceride level better than RSE.

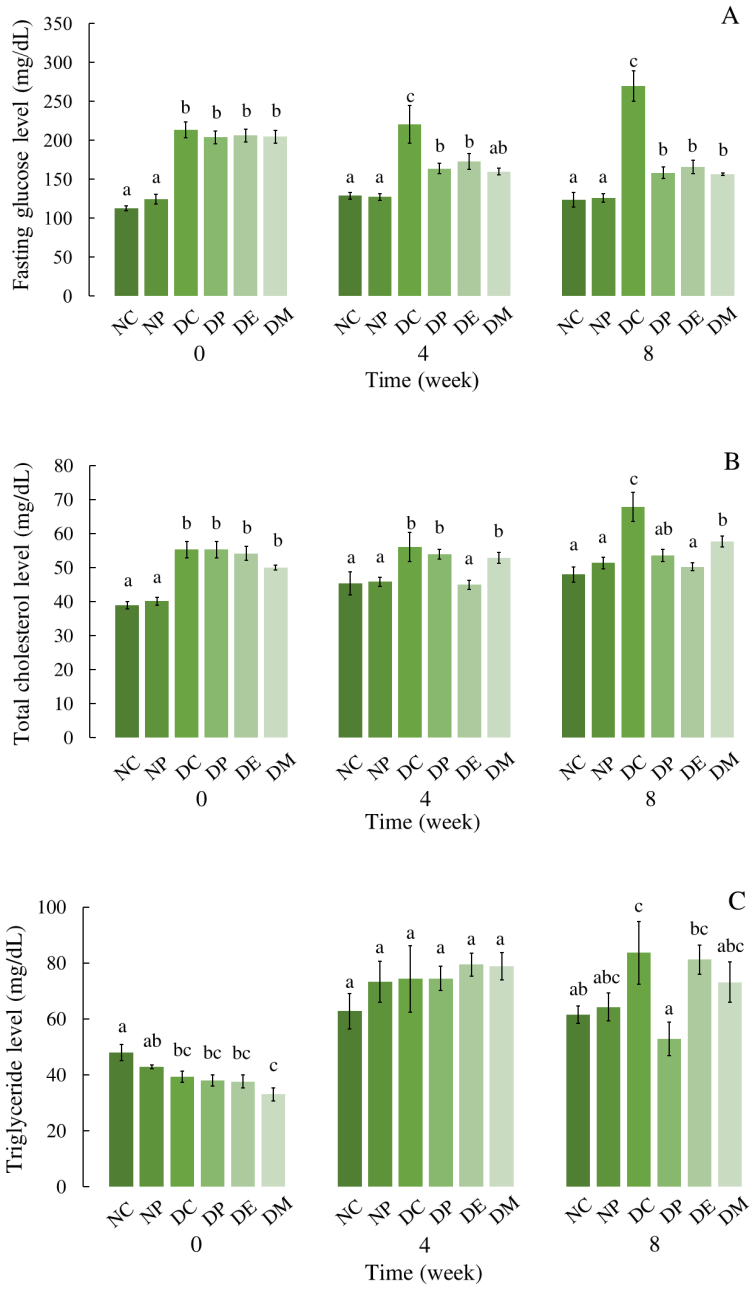

Figure 1. Glucose (A), total cholesterol (B) and triglyceride levels (C) in normal and diabetic rats after 0, 4 and 8 weeks of exposure to algal extract and customised algal product. Different letters indicate statistically significant differences between groups (p<0.05) according to Duncan's multiple range tests. NC=normal control rats, NP=normal control rats receiving 500 mg/kg RSP, DC=diabetic control rats, DP=diabetic rats receiving 500 mg/kg RSP, DE=diabetic rats receiving 500 mg/kg RSE, DM= diabetic rats receiving 50 mg/kg metformin

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Total cholesterol of the DP and DE groups is significantly decreased to normal levels by the 8th week. However, the levels of HDL across all groups are not significantly different. In addition, LDL in all diabetic groups is lower than in normal groups (Table 2). High triglyceride levels combined with high LDL cholesterol or low HDL cholesterol are linked with fatty accumulation on the artery walls, which increases the risk of heart attack, stroke, cardiovascular and heart diseases [23]. These results demonstrate that RSE and RSP seem to possess anti-hypercholesterol activity.

Table 2. Effects of RSE and RSP on total cholesterol, HDL and LDL levels at 8th week

Group	Total cholesterol (mg/dL)	HDL (mg/dL)	LDL (mg/dL)
NC	48.03 ± 2.25^{a}	60.80 ± 4.47^{a}	13.00 ± 0.71^{ab}
NP	51.36 ± 1.74^{a}	50.80 ± 4.32^{a}	15.40 ± 0.93^{a}
DC	67.90 ± 4.31^{c}	55.40 ± 3.17^{a}	10.00 ± 0.84^{c}
DP	53.59 ± 1.84^{ab}	56.20 ± 3.92^{a}	11.60 ± 0.87^{bc}
DE	50.26 ± 1.20^{a}	49.40 ± 3.70^{a}	11.40 ± 0.98^{bc}
DM	57.64 ± 1.64^{b}	59.60 ± 3.61^{a}	11.80 ± 0.92^{bc}

Note: Data expressed as mean ± S.E.M. (n=6). Different letters within each column indicate statistically significant differences (p<0.05) according to Duncan's multiple range tests. NC=normal control rats, NP=normal control rats and received 500 mg/kg RSP, DC=diabetic control rats, DP=diabetic rats receiving 500 mg/kg RSP, DE=diabetic rats receiving 500mg/kg RSE, DM=diabetic rats receiving 50 mg/kg metformin

MDA is the end-product of lipid peroxidation and is the most frequently used biomarker of oxidative stress in many diseases despite its wide variation in healthy people [24]. Furthermore, high levels of MDA lead to high oxidative stress and oxidative damages are involved in both physiological and major pathological processes [25]. The present study shows the MDA level of the DC group is greater than the treated groups (DP, DE and DM), the MDA levels of the latter being significantly lower than that of the former (Figure 2). Thus, RSE, RSP seem to prevent oxidative stress in diabetic rats.

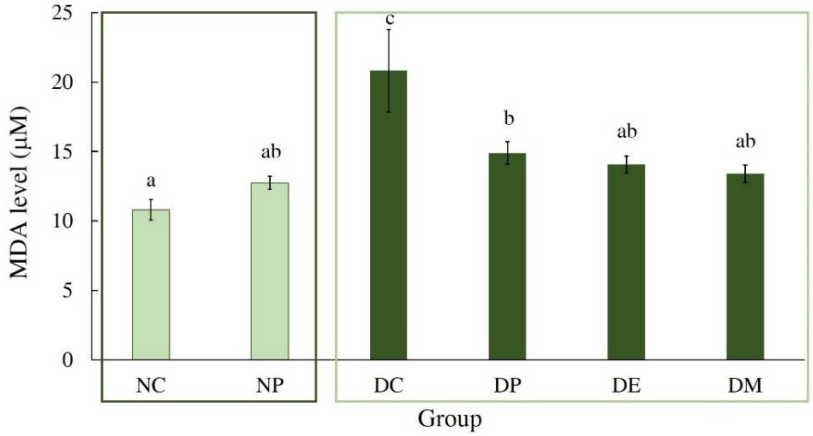

Figure 2. MDA levels of normal and diabetic rat groups. Different letters indicate statistically significant differences between groups (p<0.05) according to Duncan's multiple range tests. NC=normal control rats, NP=normal control rats receiving 500 mg/kg RSP, DC=diabetic control rats, DP=diabetic rats receiving 500 mg/kg RSP, DE=diabetic rats receiving 500 mg/kg RSE, DM= diabetic rats receiving 50mg/kg metformin

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Free radicals are formed disproportionately in diabetic rats by glucose oxidation, non-enzymatic glycation of proteins and subsequent oxidative degradation of glycated proteins. A high level of free radicals and simultaneous decline of antioxidant defence mechanisms lead to damage to cellular organelles and enzymes, increased lipid peroxidation and development of insulin resistance [26]. Figure 3 shows that the antioxidant enzymes SOD, CAT and GPx are significantly less active in DC rats. The primary product in the course of oxygen metabolism is superoxide radical ($O_2^{\cdot-}$), which is

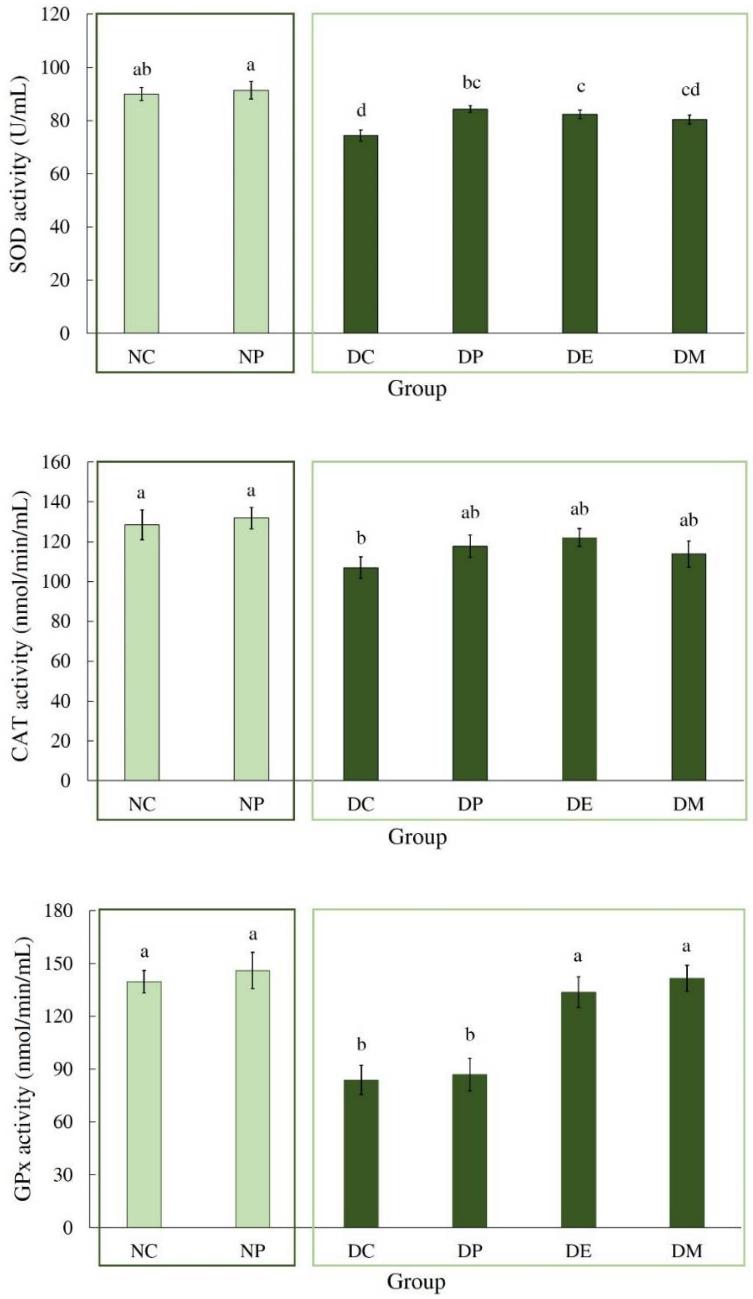

Figure 3. Antioxidant enzyme (SOD, CAT and GPx) activities. Different letters indicate statistically significant differences (p<0.05) according to Duncan's multiple range tests. NC=normal control rats, NP=normal control rats receiving 500mg/kg RSP, DC=diabetic control rats, DP=diabetic rats receiving 500 mg/kg RSP, DE=diabetic rats receiving 500 mg/kg RSE, DM=diabetic rats receiving 50 mg/kg metformin

highly reactive. Superoxide is converted to a far less reactive product, hydrogen peroxide (H_2O_2), by a family of metalloenzymes known as SOD [19]. The SOD and GPx activities in the DP and DE groups were found to be significantly greater than those in the DC group, demonstrating that RSP and RSE can reduce the amount of superoxide radicals in diabetic rats.

CONCLUSIONS

RSE and RSP decrease glucose, triglyceride and total cholesterol levels as well as protect against oxidative stress in diabetic rats, as demonstrated by a decreased MDA concentration and elevated antioxidant enzyme activities, especially SOD and GPx. Thus, both RSE and RSP seem to be beneficial and effective as a functional ingredient in nutraceutical products for diabetes mellitus.

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