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ORIGINAL RESEARCH

Erythrina subumbrans (Hassk) Merr. (Fabaceae) Inhibits Insulin Resistance in the Adipose Tissue of High Fructose-Induced Wistar Rats

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Background: The twigs and roots of *Erythrina subumbrans* (Hassk). Merr. Was reported to possess antidiabetic activity by reducing the activity of α-glucosidase and α-amylase. TNF-α is a pro-inflammatory cytokine in obesity and diabetes mellitus (DM). It inhibits the action of insulin, causing insulin resistance. Adiponectin is an anti-inflammatory peptide synthesized in white adipose tissue (WAT) and its high levels are linked with a decreased risk of DM. However, information about the effect of *Erythrina subumbrans* (Hassk). Merr. on insulin resistance are still lacking.

Purpose: To obtain the effects of the ethanol extract of *E. subumbrans* (Hassk) Merr. leaves (EES) in improving insulin resistance conditions.

Methods: The leaves were collected at Ciamis, West Java, Indonesia, and were extracted using ethanol 96%. The effects of EES were studied in fructose-induced adult male Wistar rats by performing the insulin tolerance test (ITT) and assessing blood glucose, TNF-α, adiponectin, and FFA levels. The number of WAT and BAT of the adipose tissues was also studied. The total phenols and flavonoids in EES were determined by the spectrophotometric method and the presence of quercetin in EES was analyzed using the LC-MS method.

Results: EES significantly reduced % weight gain, TNF-α levels, and increased adiponectin levels in fructose-induced Wistar rats. EES significantly reduced the FFA levels of fructose-induced Wistar rats and significantly affected the formation of BAT similar to that of metformin. All rats in EES and metformin groups improved insulin resistance as proven by higher ITT values (3.01 ± 0.91 for EES 100 mg/kg BW; 3.01 ± 1.22 for EES 200 mg/kg BW; 5.86 ± 3.13 for EES 400 mg/kg BW; and 6.44 ± 2.58 for metformin) compared with the fructose-induced group without treatment (ITT = 2.62 ± 1.38). EES contains polyphenol compounds (2.7638 ± 0.0430 mg GAE/g extract), flavonoids (1.9626 ± 0.0152 mg QE/g extract), and quercetin 0.246 µg/mL at m/z 301.4744.

Conclusion: *Erythrina subumbrans* (Hassk). Merr. extract may have the potential to be further explored for its activity in improving insulin resistance conditions. However, further studies are needed to confirm its role in alleviating metabolic disorders.

Keywords: adiponectin, anti-diabetes, Erythrina, inflammation, insulin resistance, tumor necrosis factor

Introduction

Insulin resistance (IR) occurs when the insulin receptors on the surface of target tissues, particularly the liver, skeletal muscle, and adipose tissue, fail to respond to insulin stimulation. This condition impedes the removal of glucose, thus eventually resulting in an increase of insulin synthesis in the beta-cell of pancreatic Langerhans islet and hyperinsulinemia.[1] IR may be stimulated by numerous complex factors such as obesity, a sedentary lifestyle, physical inactivity, and a high-glycemic and fat diet, which affect the adipose tissue. Adipose tissue contributes to energy storage, metabolic regulation, and neuroendocrine and immune functions.[2,3] Globally, the incidence of IR among adolescents ranges from 15.5% to 46.5%.[4]

Received: 11 June 2024
Accepted: 17 August 2024
Published: 27 August 2024

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The development of IR may contribute to metabolic syndrome diseases, non-alcoholic fatty liver disease (NAFLD), type 2 diabetes mellitus (T2DM),[1] and the increase in the size of adipose cells (hypertrophy) due to inflammation and the infiltration of macrophages into the adipose cells. The adipocytes eventually undergo hypoxia due to the decrease of intracellular oxygen supply which leads to the development of inflammation and stimulates the release of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-8, and IL-1β from the M1 macrophages, in obesity patients.[5] Adipose tissue not only functions as the active tissue for cellular reactions, metabolic homeostasis, and important energy storage but is the largest endocrine organ as well. In patients with obesity, an excess of visceral fat accumulation may generate the dysfunction of adipose tissue due to hypoxia, which is followed by the reduction of the number of the M2 macrophages, thus resulting in a decrease in anti-inflammatory cytokines production (IL-3 and IL-4).[6] In addition, the hypertrophy of adipose cells induces the imbalance of the levels of anti-inflammatory and pro-inflammatory cytokines, thus increasing the free fatty acids (FFAs) and TNF-α levels, decreasing adiponectin, and raising the incidence of IR.[5] The level of adiponectin in obese patients with fatty liver disease is lower compared to those without fatty liver disease. It was reported that the soluble TNF-α level in obese patients with fatty liver disease is high.[7] The imbalance of anti-inflammatory and pro-inflammatory cytokines in obese individuals may provoke the incidence of fatty liver disease 4-fold higher than in non-obese patients.[8]

When the homeostasis between the pro- and anti-inflammatory signals is disrupted, an inflammatory response may prevail. Flavonoids have been confirmed for their activities in modulating numerous phases of inflammatory processes, such as gene transcription and expression, the inhibition of certain enzyme activities, and the scavenging of reactive oxygen radicals.[9] Flavonoids are thought to play an important role in regulating carbohydrate hydrolysis, as well as insulin signaling and secretion.[10]

Pterocarpans and flavonoids have been reported to be present in the bark of *Erythrina subumbrans* (Hassk). Merr., and phytoconstituents isolated from the twigs and roots could inhibit α-glucosidase activity and/or α-amylase activity.[11] The extract of *E. subumbrans* (Hassk). Merr. leaves at a dose of 1000 mg/kg BW could significantly normalize blood glucose levels and decrease the serum MDA and 8-OHdG levels in high-fat diet (HFD)-induced DM rats.[10] It was confirmed that flavonoids improve the pathogenesis of DM and its complications through the regulation of glucose metabolism, hepatic enzyme activities, and a lipid profile.[12] Considering this, our study aims to investigate the effects of the ethanol extract of *Erythrina subumbrans* (Hassk) Merr. leaves (EES) to the levels of TNF-α, adiponectin, and free fatty acids in the adipose tissue of fructose-induced Wistar rats. The chemical properties of EES were also analyzed.

Material and Methods

Chemicals and Reagents

The chemicals and reagents used in this study were the Rat Tumor Necrosis Factor-Alpha (TNF-α) ELISA Kit (No. E0764Ra; https://www.bt-laboratory.com/index.php/Shop/Index/productShijiheDetail/p_id/3076.html), the Mouse Adiponectin (ADP) ELISA Kit (https://www.bt-laboratory.com/index.php/Shop/Index/productShijiheDetail/p_id/2248/cate/kit.html), the Mouse Free Fatty Acid ELISA Kit (MBS733261; https://www.mybiosource.com/ffa-mouse-elisa-kits/free-fatty-acid/733261), methanol 99.9%, for high-performance liquid chromatography (Thermo Scientific Chemicals; CAS No. 67–56-1), quercetin hydrate ($C_{15}H_{10}O_7 \cdot xH_2O$) MW 302.24 (Sigma-Aldrich; CAS No. 849061–97-8), technical grade ethanol 96% (Bratachem, Indonesia).

Plant Material and Preparation of the Extract

The leaves of the plant were collected at Sindang Kasih Village, Ciamis, West Java, Indonesia (Google map location at −7.46461602045191, 108.68529267363027), and the specimen was authenticated by Joko Kusmoro, a certified botanist, at Herbarium Jatinangor, Plant Taxonomy Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Padjadjaran University, Indonesia. The plant specimen was confirmed as *Erythrina subumbrans* (Hassk). Merr. (Fabaceae) (Herbarium 35/HB/2023) and matched those illustrated in Naturalis Biodiversity Center (NL)-Botany (https://www.gbif.org/occurrence/2517594190) and Flora of Java.[13]

The extraction technique was performed by following the Indonesian Herbal Pharmacopoeia (2017)[14] with a minor modification. The leaves were cleaned, washed, and dried in the laboratory for 5 days to a constant weight. The dried leaves (2.045 kg) were ground and sieved (mesh 4/18), and 1.3 kg of the coarse powder was soaked in 26 L of technical grade ethanol 96% for 3×24 h at 25 ± 2 °C. The extract (313.28 g) was filtered and the solvent was rotary-evaporated at 55 ± 5 °C to a thick consistency of *E. subumbrans* extract (abbreviated to EES).

Animals and Ethical Approval

Thirty adult male Wistar rats (200 ± 10 g), obtained from an animal breeding farm in Bandung, West Java, Indonesia, were randomly assigned to six groups (n = 5 per cage) in a room set at temperatures of 25 ± 2 °C, under a 12 h light, 12 h dark cycle, and 55% relative humidity. The cages were cleaned every three days. The rats were given ad libitum standard feed (containing carbohydrates 274 g, vegetable protein 706 g, and vegetable fat 20 g) and free access to fresh water during 7 days of acclimatization. The health condition and behavior of the rats were daily kept under observation during the adaptation and in vivo study. The animal handling procedure was approved by the Research Ethics Committee, Padjadjaran University, Indonesia (https://kep.unpad.ac.id/; approval documents number 506/UN6.KEP/EC/2022 and 903/UN6.KEP/EC/2023), which strictly follows The Guide for the Care and Use of Laboratory Animals (NRC 2011; eighth edition) (https://grants.nih.gov/grants/olaw/guide-for-The-care-and-use-of-laboratory-animals.pdf).[15]

Effect of EES on the Levels of Glucose, ITT, TNF-α, Adiponectin, and FFA of Fructose-Induced Wistar Rats

The in vivo studies in Wistar rats were performed at the Pharmacology Laboratory of Bhakti Kencana University, Bandung, West Java, Indonesia, as follows: (1) the normal group (treated with standard feed); (2) the fructose-induced group (treated with fructose 60% w/v); (3) the control group (treated with metformin 45 mg/kg BW); (4) the test-1 group (treated with EES 100 mg/kg BW); (5) test-2 group (treated with EES 200 mg/kg BW); (6) test-3 group (treated with EES 400 mg/kg BW). The control and EES groups were induced using fructose 60% w/v (2.5 mL/200 g BW), by oral route using a rodent 18-gauge rounded tipped for 7 weeks. The rats were observed for their body weight (BW) at D0, D15, D30, D45, and D60, and blood glucose at D0, D15, D30, D45, and D60, and their blood glucose, TNF-α, adiponectin, and free fatty acid (FFA) levels were measured at day 60 (D60). An adequate volume of blood was collected from the retro-orbital sinus using a capillary glass tube.[16] Eventually, on the same day (D60), the rats were intraperitoneally injected with insulin 0.1 U/kg BW to measure the insulin tolerance test (ITT). Blood was collected from their tail vein and was measured for their blood glucose levels at 0, 15, 30, 45, and 60 min, post-insulin injection. The blood was centrifuged for 10 min to separate the serum.

Subsequently, the rats were sacrificed using carbon dioxide euthanasia for 2 minutes by trained personnel. Death was confirmed by determining cardiac and respiratory arrest.[17] The adipose tissue of the rats was collected by separating the fat layer on the retroperitoneal (located behind the abdominal or peritoneal cavity), epididymal (a narrow, tightly coiled tube that is attached to the testicles), and perirenal (surrounding the kidneys in the retroperitoneal space) in tubes containing formaldehyde 10% following a previous procedure,[18] and the collected adipose tissue was washed with phosphate-buffered saline (PBS) to remove blood and was analyzed for the total number and morphology of white adipocytes and brown adipocytes. The histopathology was carried out at the Veterinary BBPSI, Bogor, West Java, Indonesia (https://veteriner.bsip.pertanian.go.id/).

Total Phenols and Total Flavonoids in EES

The determination of the total phenols was carried out by following a colorimetric method described previously.[19,20] Briefly, 0.5 mL EES was added with 2.5 mL of 10% Folin-Ciocâlteu phenol reagent (F9252 Sigma-Aldrich, Sigma-Aldrich Singapore) and 2 mL of 7.5% sodium bicarbonate, and after 30 minutes of reaction in a water bath at 45 °C, the absorbance of the mixture was measured at 720 nm. The standard curve was created by plotting the concentration versus the absorbance of gallic acid and was used to determine the total phenols in mg equivalence to gallic acid (GAE).

The total flavonoids were determined by a colorimetric assay adapting a previous procedure.[21] The standard curve was created by plotting the concentration versus the absorbance of quercetin and was used to determine the total flavonoids in mg equivalence to quercetin (QE).

Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis of EES

The liquid chromatography-tandem mass spectrometric (LC-MS) analysis of quercetin in the EES extract was carried out by adapting a previous procedure announced elsewhere.[22] A standard solution of quercetin was prepared by dissolving 5 mg of accurately weighed quercetin hydrate in 25 mL of methanol for high-performance liquid chromatography. The solution was sonicated for 10 minutes to homogeneity and further diluted with the same solvent to obtain various concentrations of quercetin 0.1; 0.3; 0.6; 0.9; 1.2; and 10 µg/mL. 1 µL of the solutions were injected into the Shimadzu 8045 model CBM-20A HPLC System Controller, equipped with binary gradient LC-20ADXR pumps, in tandem with a triple quadrupole mass spectrometer system with a SIL-20ACXR auto-rack sampler. The chromatography separation was performed on a C18 column with the mobile phase consisting of methanol and formic acid. The flow rate was set at 0.50 mL/minute, rinsing volume of 500 µL, needle stroke of 52 mm, rinsing speed at 35 µL/second, sampling speed at 15 µL/second, maximum oven temperature at 90 °C, and cooler temperature at 15 °C. Scanning was done in the range of 100–500 m/z. The transitions *m/z* 301 > 151 and 301 > 178 for quercetin were monitored.[23] Eventually, 1 µL of EES 200 mg/mL was injected into the same system and the chromatograms and spectrums of quercetin and EES were recorded.

Statistical Analysis

IBM SPSS for Windows was employed to analyze the data. Significant differences between groups were analyzed using one-way analysis of variance (ANOVA) continued with the post-hoc Tukey's test. All data are presented as the mean ± SD; $P < 0.05$ indicates a significant result.

Results

Effects of EES on the Body Weight, Blood Glucose, and ITT of Fructose-Induced Wistar Rats

At the end of the study (D60), rats in all groups demonstrated an increase in body weight (BW); however, treating the rats with metformin and EES 400 mg/kg BW resulted in a significantly lower % weight gain compared to the rats in the fructose-induced group (Table 1). Conversely, at the end of the study (D60), rats in all groups did not demonstrate an increase in blood glucose; it is interesting to know that inducing the rats with 60% w/v fructose for 60 days did not result in hyperglycemia (Table 2). The 60-day feed with fructose 60% w/v has induced the occurrence of insulin resistance, as proven by the insulin tolerance test (tabulated in Table 3). All rats in EES and metformin groups improved insulin resistance as proven by higher ITT values (3.01 ± 0.91 for EES 100 mg/kg BW; 3.01 ± 1.22 for EES 200 mg/kg BW; 5.86 ± 3.13 for EES 400 mg/kg BW; and 6.44 ± 2.58 for metformin) compared with the fructose-induced group without treatment (ITT = 2.62 ± 1.38). Interestingly, rats treated with an EES dose of 400 mg/kg BW exhibited the highest ITT values of 5.86 ± 3.13, which is almost comparable to that of the metformin group.

Effects of EES on the TNF-α, Adiponectin, and FFA Levels of Fructose-Induced Wistar Rats

All doses of EES significantly reduced TNF-α levels (Table 4) and increased adiponectin levels (Table 5) compared to those of the rats in the fructose-induced group, with $P < 0.05$. All doses of EES revealed a stronger inhibitory activity toward TNF-α compared to metformin. Lower doses of EES (100 mg/kg BW and 200 mg/kg BW) showed stronger activity in increasing adiponectin levels in fructose-induced rats compared to metformin. An inducement with fructose slightly altered the FFA levels of the rats, although not significantly compared to that in the normal control group, and treating the rats with all doses of EES significantly reduced the FFA levels of fructose-induced Wistar rats. Unexpectedly, metformin treatment slightly elevated the levels of FFA (Table 6).

Table 1 The Effect of EES on the BW of Fructose-Induced Wistar Rats

Group	D0	D15	D30	D45	D60	% Weight Gain (D60 – D0)/D0 x 100%
Normal group	181.50 ± 5.07 (P = 0.65)	190.75 ± 18.46 (P = 1.00)	200.75 ± 18.26 (P = 0.43)	210.50 ± 13.48 (P = 0.49)	223.75 ± 13.09 (P = 0.29)	23
Fructose-induced group	194.50 ± 8.58	208.25 ± 18.45	238.25 ± 21.01	249.25 ± 23.37	268.25 ± 26.20	38
Metformin 45 mg/kg BW	190.00 ± 15.77 (P = 1.00)	207.00 ± 31.44 (P = 1.00)	202.50 ± 22.72 (P = 0.53)	207.25 ± 24.66 (P = 0.33)	206.25 ± 25.32* (P = 0.03)	9
EES 100 mg/kg BW	178.75 ± 1.50 (P = 0.25)	191.50 ± 15.35 (P = 1.00)	203.75 ± 9.74 (P = 0.63)	216.75 ± 4.57 (P = 1.00)	230.25 ± 6.34 (P = 0.62)	29
EES 200 mg/kg BW	187.25±2.75 (P = 1.00)	194.75 ± 26.00 (P = 1.00)	213.75 ± 24.10 (P = 1.00)	220.25 ± 26.21 (P = 1.00)	212.75 ± 22.02 (P = 0.07)	14
EES 400 mg/kg BW	182.75±8.38 (P = 0.97)	187.00 ± 24.43 (P = 1.00)	197.50 ± 31.72 (P = 0.28)	201.00 ± 36.29 (P = 0.15)	203.50 ± 38.31* (P = 0.02)	11

Notes: Data are presented as mean (in g) ± SD; an asterisk (*) shows a significant difference compared to the fructose-induced group, with P < 0.05; P value is presented in brackets; EES (extract *E. subumbrans*); n = 5 rats/group.

Table 2 The Effect of EES on the Blood Glucose Level of Fructose-Induced Wistar Rats

Group	The Blood Glucose Level at D0	The Blood Glucose Level at D15	The Blood Glucose Level at D30	The Blood Glucose Level at D45	The Blood Glucose Level at D60
Normal group	87 ± 16.52	94 ± 26.08	74 ± 8.66	72 ± 13.62	73 ± 14.66
Fructose-induced group	90 ± 7.89	84 ± 10.25	73 ± 13.33	78 ± 11.18	73 ± 19.26
Metformin 45 mg/kg BW	82 ± 17.25	76 ± 22.05	78 ± 11.27	78 ± 11.18	73 ± 19.26
EES 100 mg/kg BW	75 ± 1.91	69 ± 16.01	78 ± 25.47	68 ± 16.15	60 ± 21.33
EES 200 mg/kg BW	84 ± 11.09	79 ± 9.50	93 ± 7.35	73 ± 16.52	68 ± 21.24
EES 400 mg/kg BW	90 ± 8.54	69 ± 15.59	76 ± 17.56	88 ± 12.07	82 ± 22.91

Notes: Data are presented as mean (in mg/dL) ± SD; EES (extract *E. subumbrans*); n = 5 rats/group.

Effects of EES on the Number of Adipose Cells and Morphology of Adipose Tissue in Fructose-Induced Wistar Rats

All doses of EES did not alter the number of white adipose tissue (WAT) taken from the retroperitoneal of the rats as shown by metformin; however, the extracts significantly increased the number of WAT of the perirenal, and only a dose of 400 mg/kg BW significantly increased the number of WAT of the epididymal (Table 7). In addition, EES significantly affects the brown adipose tissue (BAT) taken from the three studied organs as tabulated in Table 7. Moreover, EES and metformin did not significantly alter the diameter of BAT and only slightly changed the diameter of WAT (Table 8). The histopathology of the adipose tissue reveals that metformin and EES contribute to the formation of BAT (Figures 1–3).

The Chemical Properties of EES

Phenolic compounds and flavonoids are present in the ethanol extract of *E. subumbrans* leaves (EES). The total phenols in EES calculated using a gallic acid standard curve (linear regression equation $y = 0.0511x + 0.1496$; $R^2 = 0.9992$) is

Table 3 The Effect of EES on the Insulin Tolerance Test (ITT) of Fructose-Induced Wistar Rats

Group	ITT
Normal group	8.64 ± 2.00* (P = 0.00)
Fructose-induced group	2.62 ± 1.38# (P = 0.00)
Metformin 45 mg/kg BW	6.44 ± 2.58* (P = 0.02)
EES 100 mg/kg BW	3.01 ± 0.91# (P = 0.00)
EES 200 mg/kg BW	3.01 ± 1.22# (P = 0.00)
EES 400 mg/kg BW	5.86 ± 3.13* (P = 0.07)

Notes: Data are presented as mean ± SD; EES (extract *E. subumbrans*); n = 5 rats/group; an asterisk (*) shows a significant difference compared to the fructose-induced group, with P < 0.05; a # shows a significant difference compared to the normal group; P value is presented in brackets.

Table 4 The Effect of EES on the Serum TNF-α Level of Fructose-Induced Wistar Rats

Group	Serum TNF-α Level (ng/L)
Normal group	0.589 ± 0.195*
	(P = 0.000)
Fructose-induced group	1.550 ± 0.110
Metformin 45 mg/kg BW	0.892 ± 0.269*
	(P = 0.010)
EES 100 mg/kg BW	0.538 ± 0.026*
	(P = 0.000)
EES 200 mg/kg BW	0.434 ± 0.038*
	(P = 0.000)
EES 400 mg/kg BW	0.434 ± 0.017*
	(P = 0.000)

Notes: Data are presented as mean (ng/L) ± SD; an asterisk (*) shows a significant difference compared to the fructose-induced group, with P < 0.05; P value is presented in brackets; EES (extract *E. subumbrans*); n = 5 rats/grou.

Table 5 The Effect of EES on the Serum Adiponectin Level of Fructose-Induced Wistar Rats

Group	Serum Adiponectin Level (mg/L)
Normal group	0.231 ± 0.041*
	(P = 0.002)
Fructose-induced group	0.112 ± 0.006
Metformin 45 mg/kg BW	0.227 ± 0.000*
	(P = 0.001)
EES 100 mg/kg BW	0.243 ± 0.019*
	(P = 0.001)
EES 200 mg/kg BW	0.272 ± 0.019*
	(P = 0.001)
EES 400 mg/kg BW	0.217 ± 0.018*
	(P = 0.004)

Notes: Data are presented as mean (mg/L) ± SD; an asterisk (*) shows a significant difference compared to the fructose-induced group, with P < 0.05; P value is presented in brackets; EES (extract *E. subumbrans*); n = 5 rats/group.

Table 6 The Effect of EES on the Serum FFA Level of Fructose-Induced Wistar Rats

Group	Serum FFA Level (µg/L)
Normal group	0.480 ± 0.031
	(P = 0.704)
Fructose-induced group	0.568 ± 0.035
Metformin 45 mg/kg BW	0.614 ± 0.055
	(P = 0.100)
EES 100 mg/kg BW	0.340 ± 0.018*
	(P = 0.001)
EES 200 mg/kg BW	0.402 ± 0.036*
	(P = 0.002)
EES 400 mg/kg BW	0.458 ± 0.054
	(P = 0.261)

Notes: Data are presented as mean (µg/L) ± SD; an asterisk (*) shows a significant difference compared to the fructose-induced group, with P < 0.05; P value is presented in brackets; EES (extract *E. subumbrans*); n = 5 rats/group.

2.7638 ± 0.0430 mg GAE/g extract, and the total flavonoids in EES calculated using a quercetin standard curve (linear regression equation $y = 0.1417x - 0.0127$; $R^2 = 0.9886$) is 1.9626 ± 0.0152 mg QE/g extract.

The liquid chromatography analysis of standard quercetin revealed that this flavonol is eluted at t_R of 1.7 minutes (depicted in Figure 4a) and the mass spectrometry analysis confirmed a peak at *m/z* 301.1000 (Figure 4b). Eventually, the

 https://doi.org/10.2147/DDDT.S472660

Table 7 The Effect of EES on the Number of Adipocytes of Fructose-Induced Wistar Rats

Group	The Number of Adipocytes (Mean ± SD)					
	Retroperitoneal		Perirenal		Epididymis	
	WAT	BAT	WAT	BAT	WAT	BAT
Normal group	34.0 ± 4.0*	22.9 ± 0.7	18.7 ± 2.9*	22.8 ± 0.4	42.8 ± 3.0	9.8 ±3 0.6
	(P = 0.006)	(P = 0.299)	(P = 0.021)	(P = 0.060)	(P = 0.455)	(P = 0.273)
Fructose-induced group	39.8 ± 4.0	22.4 ± 1.8	17.8 ± 6.2	22.2 ± 4.6	29.6 ± 2.8	10.5 ± 6.7
Metformin 45 mg/kg BW	45.8 ± 1.6	28.6 ± 9.6*	26.8 ± 4.0*	11.4 ± 3.6*	37.2 ± 1.4	6.1 ± 0.9
	(P > 0.05)	(P = 0.001)	(P = 0.000)	(P = 0.021)	(P = 0.422)	(P = 0.336)
EES 100 mg/kg BW	43.5 ± 1.1	13.2 ± 3.4	38.1 ± 13.9*	25.4 ± 14.0*	29.9 ± 0.5	17.0 ± 2.4*
	(P > 0.05)	(P = 0.965)	(P = 0.000)	(P = 0.007)	(P = 0.144)	(P = 0.000)
EES 200 mg/kg BW	43.6 ± 0.6	31.1 ± 6.7*	25.2 ± 5.4*	28.2 ± 14.2*	29.2 ± 2.4	16.4 ± 2.8*
	(P > 0.05)	(P = 0.001)	(P = 0.040)	(P = 0.040)	(P = 0.063)	(P = 0.000)
EES 400 mg/kg BW	43.1 ± 4.3	25.1 ± 4.1*	27.5 ± 4.7*	22.2 ± 5.6*	49.2 ± 2.6*	11.4 ± 2.6*
	(P > 0.05)	(P = 0.023)	(P = 0.037)	(P = 0.001)	(P = 0.034)	(P = 0.000)

Note: Data are presented as mean ± SD; an asterisk (*) shows a significant difference compared to the fructose-induced group, with P < 0.05; P value is presented in brackets; EES (extract *E. subumbrans*); n = 5 rats/group.
Abbreviations: WAT, white adipose tissue; BAT, brown adipose tissue.

Table 8 The Effect of EES on the Diameter of Adipocytes of Fructose-Induced Wistar Rats

Group	The Diameter of Adipocytes (Mean ± SD)					
	Retroperitoneal		Perirenal		Epididymis	
	WAT	BAT	WAT	BAT	WAT	BAT
Normal group	71.0 ± 9.6	67.6 ± 0.7	61.8 ± 12.6*	48.5 ± 3.5	61.3 ± 0.2*	56.5 ± 7.3
	(P = 0.72)	(P = 0.28)	(P = 0.01)	(P = 0.28)	(P = 0.00)	(P = 0.141)
Fructose-induced group	60.6 ± 4.6	64.1 ± 9.7	73.8 ± 6.1	70.0 ± 6.8	76.5 ± 3.2	56.7 ± 3.5
Metformin mg/kg BW	58.9 ± 3.8	50.6 ± 13.4*	77.7 ± 0.6	69.2 ± 5.2	79.7 ± 2.5	55.4 ± 2.8
	(P = 0.48)	(P = 0.04)	(P > 0.05)	(P > 0.05)	(P = 0.06)	(P = 0.207)
EES 100 mg/kg BW	66.8 ± 5.7	48.4 ± 4.1	62.8 ± 6.1*	52.5 ± 8.8	67.9 ± 3.3*	62.0 ± 0.1
	(P = 0.65)	(P = 0.60)	(P = 0.02)	(P > 0.05)	(P = 0.01)	(P = 0.133)
EES 200 mg/kg BW	65.7 ± 18.2*	59.7 ± 6.5	70.9 ± 0.9	64.2 ± 7.6	66.5 ± 3.1*	55.2 ± 3.7
	(P = 0.04)	(P = 0.43)	(P = 0.07)	(P > 0.05)	(P = 0.01)	(P = 0.417)
EES 400 mg/kg BW	57.5 ± 7.7*	55.9 ± 4.8	64.7 ± 1.4	48.7 ± 4.7	68.6 ± 3.4*	54.3 ± 4.3
	(P = 0.04)	(P = 0.73)	(P = 0.09)	(P = 0.223)	(P = 0.02)	(P = 0.165)

Notes: Data are presented as mean (μm) ± SD; an asterisk (*) shows a significant difference compared to the fructose-induced group, with P < 0.05; Significant differences between groups were analyzed using one-way ANOVA continued with the post-hoc LSD, n = 4x observations; P value is presented in brackets; EES (extract *E. subumbrans*).
Abbreviations: WAT, white adipose tissue; BAT, brown adipose tissue.

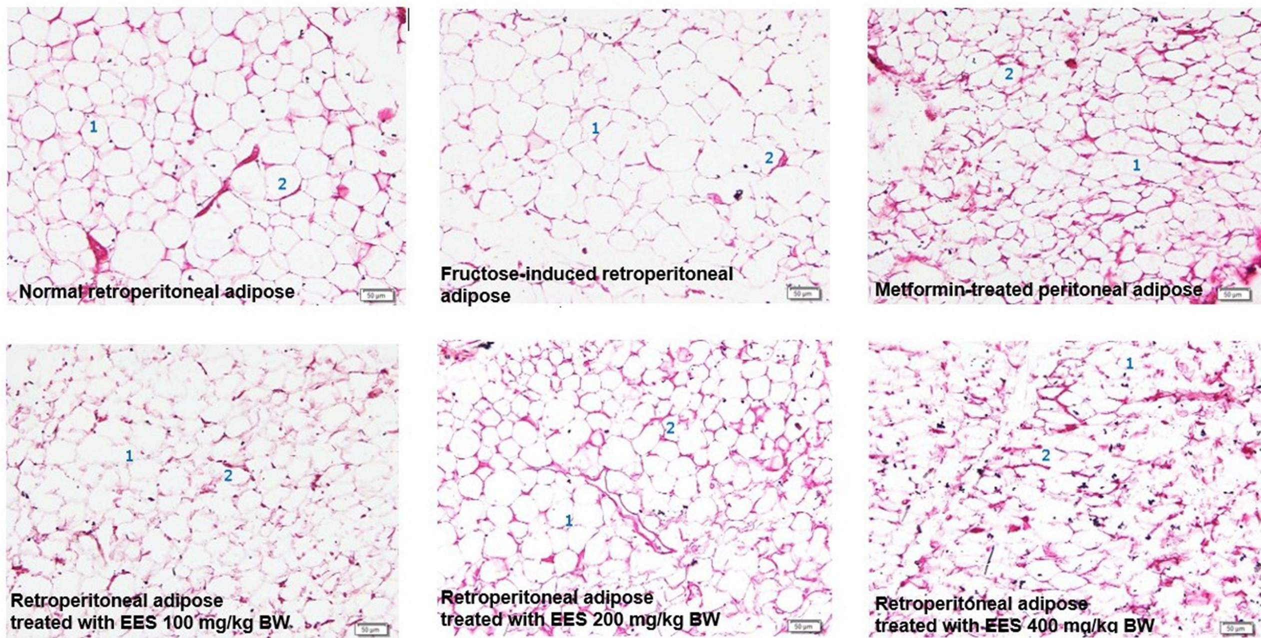

Figure 1 Microscopic morphology of the retroperitoneal adipose of Wistar rats using hematoxylin-eosin color reagent in 200x magnification. WAT comprises the major component in the normal group; A small number of BAT (scored +1) is observed in the fructose-induced group; Extensive formation of BAT (scored +4) is observed in the metformin-treated group; A small number of BAT (scored +1) is observed in the group treated with EES 100 mg/kg BW and 200 mg/BW; Extensive formation of BAT (scored +4) is observed in the group treated with EES 400 mg/kg BW. Number 1 represents white adipose tissue (WAT); number 2 represents brown adipose tissue (BAT).

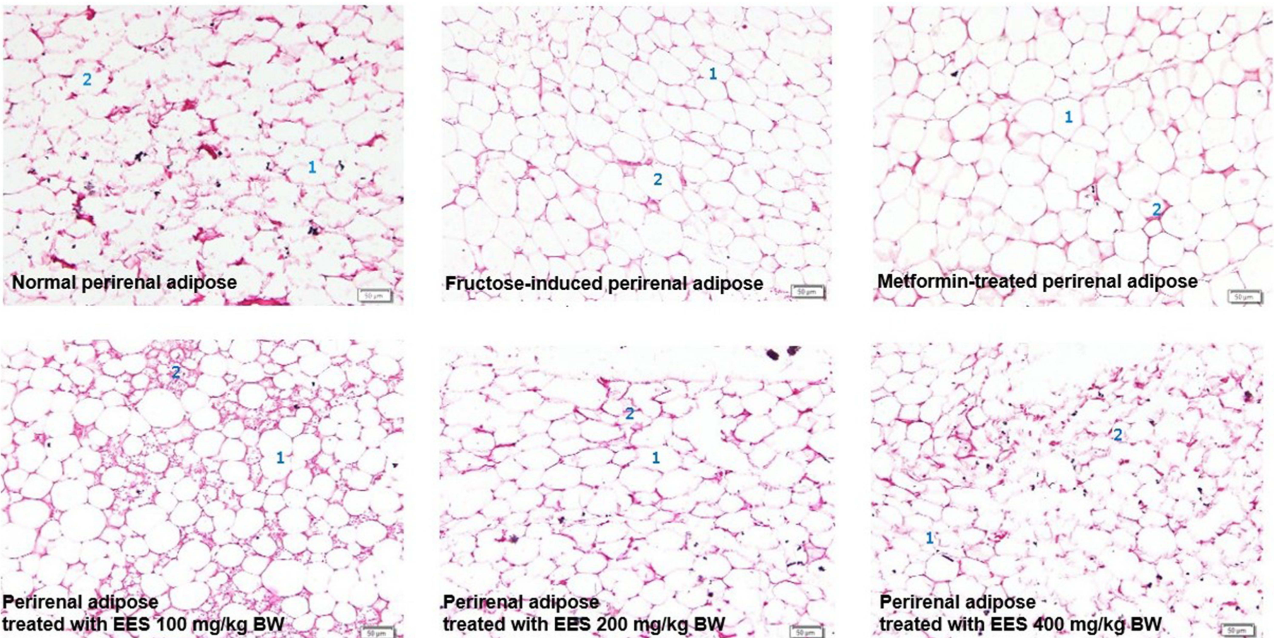

Figure 2 Microscopic morphology of perirenal adipose of Wistar rats using hematoxylin-eosin color reagent in 200x magnification. Extensive BAT comprises the major component in the normal group; A small number of BAT (scored +1) is observed in the fructose-induced group and the metformin-treated group; A small number of BAT (scored +2) is observed in the group treated with EES 100 mg/kg BW, 200 mg/BW, and 400 mg/kg BW. Number 1 represents white adipose tissue (WAT); number 2 represents brown adipose tissue (BAT).

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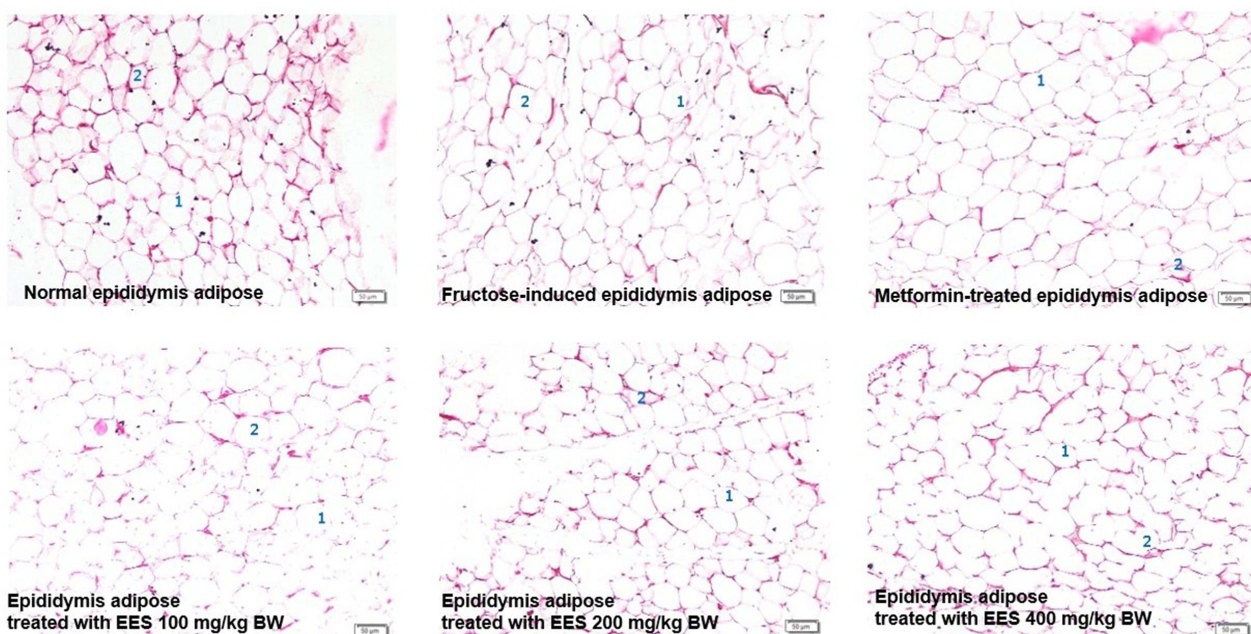

Figure 3 Microscopic morphology of epididymal adipose of Wistar rats using hematoxylin-eosin color reagent in 200x magnification. WAT comprises the major component in the normal group with only a small number of BAT (scored +1); A small number of BAT (scored +1) is observed in the fructose-induced group and the metformin-treated group; A small number of BAT is observed in the group treated with EES 100 mg/kg BW and 200 mg/BW; Extensive formation of BAT (scored +4) is observed in the group treated with EES 400 mg/kg BW. Number 1 represents white adipose tissue (WAT); number 2 represents brown adipose tissue (BAT).

liquid chromatography analysis of EES indicates that quercetin is detected at t_R of 1.8 minutes and present in the extract in the amount of 0.246 µg/mL (Figure 4c). Additionally, the mass spectrometry analysis reveals that quercetin is confirmed as a small peak, fragmented at *m/z* 301.4744 (Figure 4d) in EES. Quercetin is predicted to contribute to the pharmacology activity of EES.

Discussion

Erythrina subumbrans (Hassk). Merr. possesses a lot of potential to be utilized as a medicinal plant. The main findings of our study are that (1) the ethanol extract of *E. subumbrans* (EES) could significantly lower % weight gain, reduce TNF-α levels, and increase adiponectin levels in fructose-induced Wistar rats; (2) EES significantly affects the formation of brown adipose tissue (BAT) similar to that of metformin; (3) EES contains polyphenol compounds (2.7638 ± 0.0430 mg GAE/g extract), flavonoids (1.9626 ± 0.0152 mg QE/g extract), and quercetin 0.246 µg/mL at *m/z* 301.4744.

In our study, the 60-day feed with fructose 60% w/v did not induce the occurrence of hyperglycemia in the rats. However, the 60-day feed with fructose 60% w/v has induced the occurrence of insulin resistance, as proven by the insulin tolerance test (tabulated in Table 3). We compared this result to a former study by Lê et al (2006) who reported that 4 weeks of feeding a high-fructose meal in seven healthy participants resulted in significant increases in plasma triglycerides, VLDL-triglycerides, lactate, glucose, and leptin, but no changes in body weight, insulin sensitivity, intrahepatocellular lipid, and intramyocellular lipid were found.[24] Another study on the effects of oral fructose compared with glucose in fourteen healthy participants showed that only the glucose significantly elevated glucose and insulin levels, but not fructose. Both glucose and fructose could raise triglyceride levels, however, fructose increased VLDL-triglycerides.[25] It is suggested that a fructose diet should be restricted to avert metabolic syndrome.[26] A previous investigation reported the effects of dietary fructose on risk factors associated with metabolic disorders. The evidence described that fructose may contribute a predisposing cause in the development of insulin resistance in correlation with the induction of hypertriglyceridemia. Studies in high fructose-induced animals have proven this association, albeit the conflicting results due to the heterogeneity of the studies.[27]

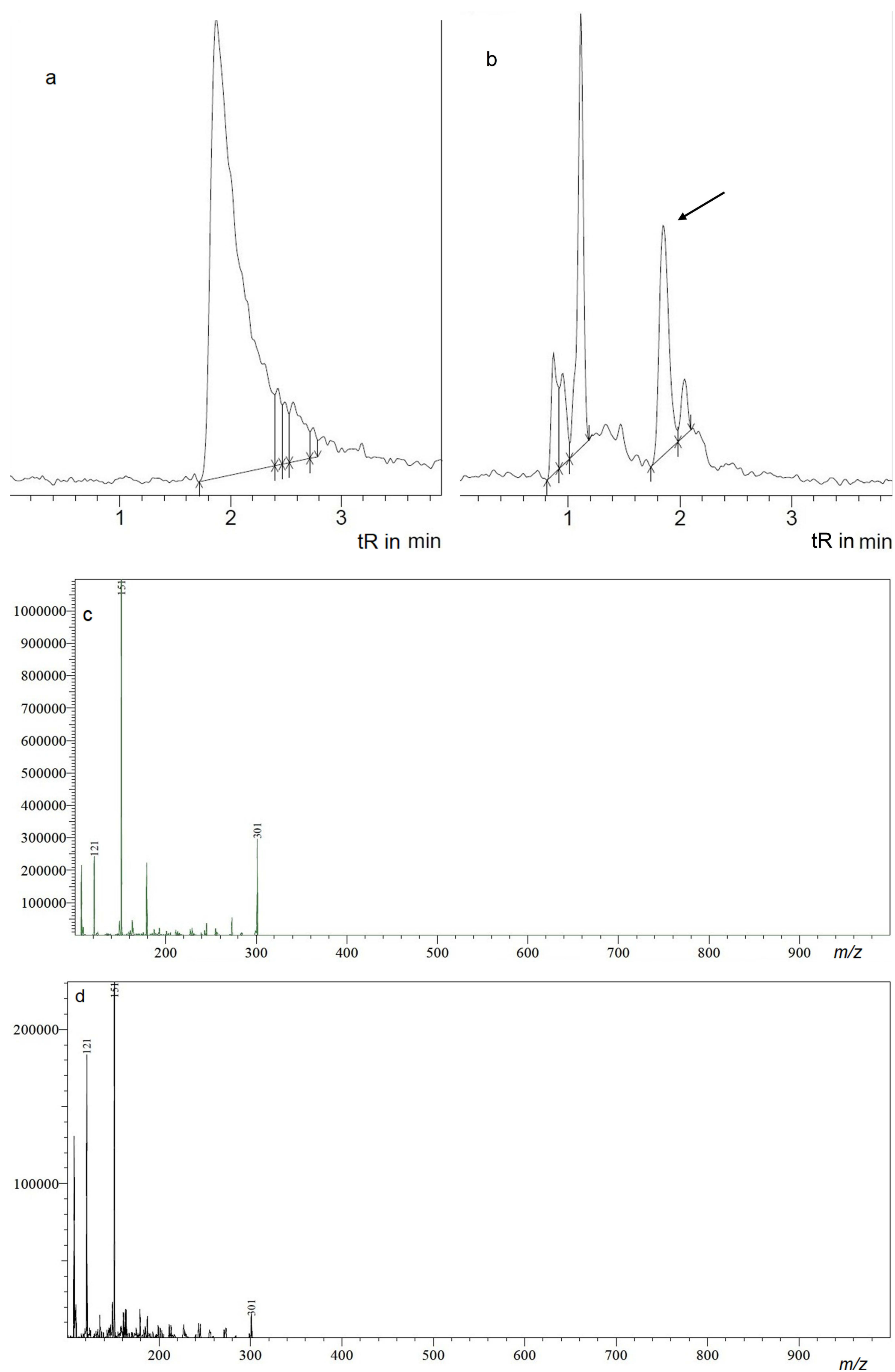

Figure 4 (**a**) LC chromatogram of standard quercetin with a single peak at t_R 1.7 minutes; (**b**) LC chromatogram of quercetin in EES eluted at t_R 1.7 minutes (black arrow); (**c**) MS spectrum of standard quercetin with an *m/z* of 301.1000; and **(d)** MS spectrum of quercetin in EES with an *m/z* of 301.4744 (black arrow).

Furthermore, it is delineated that the pathogenesis of insulin resistance is linked to FFA accumulation, elevated hepatic glucose synthesis, and reduced glucose uptake in insulin-sensitive tissues. An abnormal regulation of fatty acid metabolism may contribute pivotally to the pathogenesis of insulin resistance.[28] Considering this, we measured the levels of FFA, which showed that an inducement with fructose slightly altered the FFA levels of the rats, although not significantly compared to that in the normal control group, and all doses of EES significantly reduced the FFA levels of the rats (Table 6). In our study, metformin treatment in fructose-induced rats has implied a slight increase in the FFA levels although not significant compared to the fructose-induced group (the negative control). This result is not pleasantly expected and is contradictive to the established knowledge, considering that metformin has been known for its activity on adipocytes to suppress lipolysis, decrease plasma FFA levels, and improve insulin sensitivity.[29] A double-blind, placebo-controlled study in 19 insulin-resistant outpatients in the University Medical Centre Utrecht revealed that metformin in insulin resistance did not lead to improved FFA dynamics, despite its trend to improve insulin sensitivity.[30]

Our study confirmed that EES strongly inhibits TNF-α more powerfully compared to metformin. Moreover, EES could increase adiponectin levels in fructose-induced rats similarly to that of metformin. It was described previously that metformin could reduce the synthesis of nitric oxide, prostaglandin E_2, IL-1β, IL-6, and TNF-α, by inhibiting the translocation of NF-kappaB to the nucleus, and parallelly maintaining the levels of anti-inflammatory cytokines, IL-4 and IL-10.[31] Metformin is the first-line therapy for diabetes mellitus, and numerous studies have exhibited its cardioprotective properties not dependent on its hypoglycemic effect. Metformin significantly reduces adipokine secretion and increases adiponectin secretion.[32] A study in postmenopausal obese females with an insulin-resistant (IR) condition confirmed that 6 months of therapy using metformin could increase circulating adiponectin levels and decrease the BMI and IR of the patients.[33] A meta-analysis and systematic review study by Duan et al (2021) elucidated that metformin treatment was correlated with a significant increase in serum adiponectin levels and a decrease in circulating TNF-α and C-reactive protein levels in women with polycystic ovary syndrome.[34]

In this study, the effect of EES on the number of adipocytes in WAT and BAT taken from the fat layer of the retroperitoneal, epididymal, and perirenal of fructose-induced Wistar rats, was also evaluated, which revealed that EES did not alter the number of WAT taken from the retroperitoneal of the rats similar to that of metformin; however, EES significantly increased the number of WAT of the perirenal, and only a dose of 400 mg/kg BW significantly increased the number of WAT of the epididymal. In addition, EES significantly affects the BAT taken from the three studied organs (Table 7). Our work did not evaluate the morphological differences between WAT and BAT, which may be listed as a limitation.

WAT deposits energy in the form of triacylglycerol, whereas BAT expends energy as heat to maintain body temperature. WAT and BAT have distinct morphological and metabolic differences. WAT has a unilocular lipid droplet, few mitochondria, and a low oxidative rate.[35,36] WAT was reported to mainly store gene pathways associated with inflammatory processes.[36]

Hematoxylin-eosin (HE) dye was employed to stain the adipocytes in our study. Hematoxylin, is a basic dye that stains acidic proteins a blue color, while eosin is an acidic counterstain dye added after hematoxylin that targets basic structures and produces a pink color. The universal character of HE staining makes it applicable to define intracellular organelles and proteins.[37]

It was detailed that there might be a correlation between obesity and IR because TNF-α is overexpressed in the adipose tissue of obese patients. The activation of proinflammatory pathways after exposure to TNF-α induces a condition of IR (glucose uptake in adipose tissue) that disrupts insulin signaling. In BAT, the mechanism involves the phosphorylation of serine residue (Ser307) mediated by TNF-α activation of mitogen-activated protein kinases.[38] A cross-sectional study of sixty-three healthy participants and sixty-five patients with T2DM at the primary care clinics at King Khalid University Hospital, Riyadh, Saudi Arabia, has confirmed that TNF-*α* is associated with concurrent obesity and T2DM and correlates with HbA1c.[39] Thus, it should be noticed that an imbalance of anti-inflammatory and pro-inflammatory cytokines in obese individuals may provoke the incidence of fatty liver disease.[8]

Albeit the advantage of *Erythrina subumbrans* extracts in lowering weight gain, reducing TNF-α, and increasing adiponectin levels in high fructose-induced rats, we have to admit that there are limitations in our study, such as the use of only male rats instead of both sexes. Male Wistar rats were selected because they demonstrate more prominent pathologic phenotypes than females,[40] and female rodents have more active BAT than males,[41] therefore, investigators usually avoid female rodents because their hormonal cycle may induce variability that leads to the complication of the experimental results.[42]

Flavonoids could modulate numerous phases of inflammatory processes, such as gene transcription and expression, the inhibition of certain enzyme activities, and the scavenging of reactive oxygen radicals.[9] These plant metabolites contribute an important role in regulating carbohydrate hydrolysis, as well as insulin signaling and secretion.[10] Flavonoids are widely distributed polyphenols and comprise a large class of bioresources. Polyphenols contained in human diets are proficient in increasing plasma antioxidants due to the accumulation of these compounds, along with endogenous antioxidants, and protect the cell and cellular components from oxidative stress.[43] In our study, the levels of polyphenol compounds and flavonoids in the leaf extract were also determined. We reported that EES contains a small quantity of polyphenol compounds (2.7638 ± 0.0430 mg GAE/g extract) and flavonoids (1.9626 ± 0.0152 mg QE/g extract). Moreover, the LC-MS analysis revealed the presence of quercetin in a very small amount of 0.246 µg/mL at *m/z* 301.4744. Similarly, a previous study by Muthukrishnan et al (2016) exploring the leaves of *E. variegata* collected from Kolli hills in the Namakkal district, Tamil Nadu, India, also reported a negligible amount of quercetin, rutin, and ferulic acid in the ethanol extract of the leaves.[44] The total phenols in *E. indica* leaves were 24.91 ± 0.00 mg GAE/g aqueous extract and 25.62 ± 0.00 mg GAE/g ethanol extract,[45] which were higher than that of *E. subumbrans* collected from West Java, Indonesia, in our study. The total phenols of *E. abyssinica* and *E. brucei* leaves collected from Southern Ethiopia were 2.04% and 1.06%, respectively[46] which also confirms similar small levels.

The LC-MS analysis authenticated the presence of quercetin in EES as a minor peak at *m/z* 301.4744 along with two medium peaks at *m/z* 121.1901 and 151.2309. These peaks were compared to those recorded in the MassBank (https://massbank.eu/MassBank/, accessed 7 April 2024) and matched to quercetin, with a molecular weight of 302.238, analyzed using LC-in tandem with electrospray ionization-quatre quadrupole MS/MS.

Conclusion

The present study explored the ethanol extract of a plant collected from a small village in Ciamis, West Java, Indonesia, *Erythrina subumbrans* (Hassk) Merr., which belongs to the Erythrina genus plants with numerous pharmacological activities. Our study revealed that the ethanol extract of *Erythrina subumbrans* (Hassk) Merr., (EES) contains a small quantity of polyphenol compounds and flavonoids. We announce that this is the first report on the activity of EES to significantly lower weight gain, improve ITT value, reduce TNF-α and FFA levels, increase adiponectin levels in fructose-induced Wistar rats, and affect the formation of brown adipose tissue (BAT) similarly to that of metformin. These findings provide evidence that EES can be further explored for its activity to improve insulin resistance conditions by inhibiting inflammation in type 2 diabetic patients.

Acknowledgments

The authors thank (1) the Directorate of Higher Education of the Ministry of Education and Culture of the Republic of Indonesia for funding the research via the Doctoral Dissertation Grant, and (2) the Rector of Padjadjaran University via the Directorate of Research and Community Engagement.

Funding

This project is funded by the Directorate of Higher Education of the Ministry of Education and Culture via the Doctoral Dissertation Grant years 2022 to 2024, and the APC is funded by the Rector of Padjadjaran University via the Directorate of Research and Community Engagement.

Disclosure

The authors report no conflicts of interest in this work.

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