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LC-MS ANALYSIS AND EFFECTS OF MALAYSIAN PROPOLIS ON INSULIN, GLUCAGON, PANCREAS AND OXIDATIVE STRESS STATUS IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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ABSTRACT

This study evaluated the phytochemical constituents using liquid chromatograph-mass spectrometry (LC-MS) analysis and in vivo effects of Malaysian propolis on plasma insulin, glucagon, oxidative stress status and histology of pancreas in diabetic rats. Analysis was carried out on water (WEP) and ethanol (EEP) extracts of Malaysian propolis. Forty rats were assigned into five groups (n = 8 rats per group) i.e. non-DM, DM, DM + 300EEP (300 mg/kg), DM + 600EEP (600mg/kg) and DM + metformin (100 mg/kg). DM was induced and treatments were given daily by oral gavage for 4 weeks. LC-MS profile revealed 10 and 29 compounds in WEP and EEP respectively, which were mainly phenolic derivatives. Food intake, fasting blood glucose (FBG), glucagon, malonaldehyde and protein carbonyl were significantly higher while final body weight, insulin and total antioxidant capacity were significantly lower in DM group compared with non-DM group. There were significantly higher body weight, insulin level and total antioxidant capacity with significantly lower food intake, FBG, glucagon, malonaldehyde and protein carbonyl levels in DM + 300EEP and DM + 600EEP groups compared with DM group. Pancreas histology revealed islet cells regeneration in DM + 300EEP and DM + 600EEP groups. This study showed that Malaysian propolis EEP had higher phytochemical compounds which were mainly phenolic derivatives and in vivo study suggests its potential antidiabetic and antioxidant properties which could be due to the synergistic effect of some of these constituents.

INTRODUCTION

Propolis is a resinous natural product collected by honeybees from various plant sources to establish beehives[1]. Some of its compositions include phenolic acids and esters, flavonoids (flavones, flavanones, flavonols and others), β-steroids, aromatic aldehydes and naphthalene[2-3]. Liquid chromatography-mass spectrometry (LCMS) analysis shows that Portuguese propolis possesses 62 phytochemical compounds which are mainly phenolic derivatives[4]. Brazilian propolis possesses compounds such as phenolics, artepillin C and kaempferide derivative[5]. Some of bioactive compounds in propolis are reported to have medicinal values which include rutin with antihypertensive action, quercetin with antidiabetic property and galangin with antioxidant activity[6]. It also has been demonstrated to have hepatic and pancreatic protective effects in diabetic animal model[7], anti-bacterial[8], potential anti-ulcer[9] and promote wound healing process in diabetic rats[10].

Diabetes mellitus (DM) has long been described as a metabolic disorder associated with abnormal glucose metabolism[11]. There is an increase in DM

KEYWORDS: diabetes, glucagon, LC-MS, propolis, oxidative stress, insulin

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prevalence which is projected to increase up to 642 million by 2040. Its effect on the patient socio-economy, physical and medical state has become a major concern globally[12]. The healthy life style and exercise remain a major effective strategic intervention[13]. Under normal physiological conditions, insulin secretion from pancreatic beta cells is involved to decrease blood glucose level. Insulin secretion is regulated by glucose, hormones and autonomic nervous system activities[14]. Pancreatic beta cells are sensitive to reactive oxygen species (ROS) because of its low antioxidant enzyme contents[15]. Hyperglycaemia increases ROS production which in turn alters mitochondrial function leading to tissue damage[16-17]. In the process of cellular respiration, part of the oxygen taken into living cells is changed to several harmful ROS and free radicals[18]. Once formed, free radicals can start a chain reaction leading to formation of more ROS[19-20]. Superoxide anion radical (O^-_2) is one of the strongest ROS among the radicals that are generated after oxygen is taken into living cells. The O^-_2 changes to other harmful ROS, which are implicated in the aetiology of many diseases including DM[21-22].

Previous studies reported Malaysian propolis to have higher number of volatile compounds with ethanol extract (25) compared to distilled water extract (12) using gas chromatography-mass spectrometry (GC-MS) analysis[23] while, in vivo study revealed it to possess antihyperglycaemic property[24]. To date, no study has been reported on non-volatile compounds of Malaysian propolis and its possible ameliorative effect against pancreatic dysfunction and diabetic induced-oxidative stress. Hence, the objective of the study was to determine the non-volatile constituents of Malaysian propolis and its effects on the levels of plasma insulin, glucagon, histology of pancreas and oxidative stress status in streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Collection and preparation of samples

Raw propolis of stingless bee Heterotrigona itama was purchased from the local beekeeper in Kelantan, Malaysia collected from May to August 2015 and stored at - 40 ^{0}C.

Extract preparation

Water (WEP) and 70% ethanol (EEP) propolis extracts were prepared using the method described earlier[25], with a modification. Briefly, raw Malaysian propolis (30 g) was washed with distilled water, frozen at – 80 ^{0}C and ground into powder. Then, it was mixed vigorously with 100 ml of distilled water or 70% (v/v) ethanol at room temperature on a shaker for 6 h daily for 7 days. It was then filtered, concentrated, freeze-dried or lyophilized to remove water and ethanol to obtain WEP and EEP.

LC-MS analysis of propolis extracts

LC-MS analysis was performed on EEP and WEP using Finnegan Surveyor plus HPLC instrument equipped with a DAD and coupled to a MS. The chromatographic system consists of quaternary pump, auto-sampler, degasser, photodiode-array detector and automatic thermostatic column compartment. Ten mg of WEP or EEP was filtered through a 0.2 µm nylon membrane at a rate of 1 ml/min, split out 200 µl/min to mass spectrometer. The experiment was done using helium as collision gas, at energy of 25-40 eV. The peaks mass spectrum in mass per charge ions (m/z) were acquired for the possible compound name in the range of 200-600 nm and identification of compounds was done using ReSPect

(RIKEN MSn spectral database for phytochemicals). The extract with higher number of compounds was used for the in vivo animal study.

Animals

Forty female Sprague Dawley rats of age 8 to 10 weeks (190 – 220 g) were used in this study. The animals were obtained from the Laboratory Research Unit, Health Campus, Universiti Sains Malaysia. They were exposed to 12 h light, 12 h dark cycle at 22 - 24 ^{0}C, and given water and food ad libitum. The study was performed in accordance with the guidelines by the Animals Ethical Committee, Universiti Sains Malaysia (2013/(90)503), which was in accordance with the internationally accepted principles for laboratory animal use and care.

Induction and assessment of DM

Animals were fasted overnight for 16 h and diabetes was induced using single intraperitoneal injection of streptozotocin (STZ; Sigma-Aldrich Co., St Louis, USA) at 60 mg/kg body weight. Diabetes was confirmed following 48 h of streptozotocin injection[26]. Animals with fasting blood glucose (FBG) level of more than 200 mg/dl (Digital Glucometer, Lifescan Inc Milpitas, USA) taken from the tail vein were considered diabetic and used for this study. EEP was used for this animal study since the LC-MS analysis showed that EEP had higher number of compounds compared to WEP.

Experimental design

The animals were randomly assigned into 5 groups (n = 8 rats per group) as follows: (i) non-DM group: Healthy rats received distilled water (1 ml/day) as negative control group, (ii) DM group: Diabetic rats received distilled water (1 ml/day) as positive control group, (iii) DM + 300EEP group: Diabetic rats on 300 mg/kg body weight EEP, (iv) DM + 600EEP group: Diabetic rats on 600 mg/kg body weight EEP and (v) DM + metformin group: Diabetic rats on 100 mg/kg body weight metformin. Treatments were given by oral gavage daily between 8 to 10 am for 4 weeks. Total food intake, body weight, initial and final FBG levels were recorded. After 4 weeks of treatment, animals were fasted overnight and sacrificed under anaesthesia (90 mg/kg ketamine and 5 mg/kg xylazine). Plasma was analysed for the levels of FBG, insulin, glucagon and oxidative stress markers such as total antioxidant capacity (TAC), malonyldehyde (MDA) and protein carbonyl (PCO) using commercial kits (BioAssay Systems. California, USA). Pancreas was carefully dissected, fixed with 10% formalin, sectioned of 5µm and stained with haematoxylin and eosin for histological study.

Statistical Analysis

All numerical data are expressed as mean and standard deviation (SD). Statistical analysis was performed using Instat. Exe version 3.1. (Charleswork Publishing Services Ltd. Deighton, Huddersfield, UK). One way analysis of variance (ANOVA) followed by Turkey-Kramer test was used to assess the differences among means and $P < 0.05$ was considered as significant.

RESULTS

LC- MS analysis

There were 10 and 29 phytochemical compounds found in WEP and EEP of Malaysian propolis samples, respectively. The glucuronic acid derivative (551.23 m/z), ellagic acid (506.39 m/z) and gallic acid derivative (487.22 m/z) showed high mass spectrum while carboxylic acid (172.10 m/z) acid showed lowest mass spectrum (Table 1).

Food intake and body weight

In DM group, total food intake was significantly higher while final body weight was significantly lower compared with non-DM group. The total food intake was significantly lower and final body weight was higher in DM + 300EEP and DM + 600EEP groups compared with DM group. In DM + metformin group, the final body weight was significantly lower than non-DM group but significantly higher than DM group (Table 2).

FBG, insulin and glucagon levels

FBG level in non-DM group was invariable throughout the study period. On the contrary, the final FBG level in DM group was significantly higher compared with non-DM group. The FBG was significantly lower in DM + 300EEP, DM + 600EEP and DM + metformin groups compared with DM group. However, no significant difference was found between the EEP-treated groups compared with DM + metformin group (Table 3). Plasma insulin was significantly lower while glucagon level was significantly higher in DM group compared with non-DM group. However, plasma insulin level was significantly higher while glucagon level was significantly lower in DM + 300EEP and DM + 600EEP groups compared with DM group. No significant difference was found between DM + metformin group and DM group (Table 4).

Histology of pancreas

The histological section of pancreas in non-DM group revealed normal morphology of pancreas with normal size of islet of Langerhans. DM group showed shrunken islet of Langerhans. However, improved morphological features of islet of Langerhans were observed in DM + 300EEP, DM + 600EEP and metformin groups (Fig.1).

Antioxidant and oxidative stress markers

Plasma TAC was significantly lower while MDA and PCO levels were significantly higher in DM group compared with non-DM group. In DM + 300EEP and DM + 600EEP, plasma TAC was significantly higher while MDA and PCO levels were significantly lower than DM group. However, there were no significant differences in TAC, MDA and PCO levels between all the treatment groups (Fig. 2).

Table 1: Liquid chromatography-mass spectrometry analysis of Malaysian propolis

No	Compound name	Retention time (min)	Mass spectrum (m/z)	WEP	EEP
1.	Glucuronic acid derivative	43.60	551.23	-	+
2.	Ellagic acid	48.00	506.39	-	+
3.	Gallic acid derivative	27.10	487.22	+	+
4.	Di-prenylated compound	42.50	469.19	-	+
5.	Pinobanksin-5-methylether acetate	23.40	467.22	-	+
6.	Pentose derivative	11.70	465.16	-	+
7.	Coumaric acid derivative	23.40	438.24	+	+
8.	Prenylated compound	41.40	427.18	+	+
9.	Malonic acid derivative	34.40	404.21	+	+
10.	Syringic acid derivative	31.70	403.18	-	+
11.	Di-caffeic acid	28.90	389.16	-	+
12.	Vanillic acid derivative	32.20	373.17	+	+
13.	Delta-tocotrienol	26.40	327.16	+	+
14.	Cinnamic anhydride	45.50	325.37	+	+
15.	Kaempferol-dimethyl ether	37.53	319.25	-	+
16.	Pinoresinol	31.90	314.27	-	+
17.	Diprenylated compound	13.00	304.18	-	+
18.	Resveratrol compound	38.90	277.18	-	+
19.	Ferulic acid derivative	10.60	275.17	-	+
20.	L-(-)-phenylalanine	6.30	275.11	+	+
21.	Isoferulic acid	12.50	270.20	-	+
22.	Methylxanthine derivative	27.30	262.17	-	+
23.	Dimethyluric acid derivative	9.20	256.18	-	+
24.	Serotonin derivative	25.50	254.14	-	+
25.	Malonylhexose derivative	25.80	219.10	-	+
26.	4-aminohippurate	21.00	211.14	-	+
27.	Caffeic acid derivative	20.90	206.07	+	+
28.	1,3-Dimethylurare	15.70	206.07	-	+
29.	Carboxylic acid	14.40	172.10	+	+

WEP; water extract propolis, EEP; ethanol extract propolis. Positive sign (+) indicates the presence of compound and negative sign indicates the absence of compound (-).

Table 2 Food intake and body weight of all the experimental groups

Groups	Total food intake (g)	Initial body weight (g)	Final body weight (g)
non-DM	516.24 (40.03)	207.49 (6.63)	248.04 (6.11)
DM	945.56 (192.20)[a]	210.65 (8.74)	174.99 (10.86)[a]
DM+300EEP	717.46 (144.26)[a b]	207.74 (7.38)	232.8 (8.86)[a b]
DM+600EEP	658.78 (118.50)[b]	209.43 (7.90)	239.29 (14.22)[b]
DM+metformin	799.56 (122.42)[a]	203.16 (4.96)	230.80 (8.12)[a b]

Values are mean (SD), n = 8/group. [a] $P < 0.05$ compared with non-DM group, [b] $P < 0.05$ compared with DM group (one way ANOVA followed by Tukey-kramer post hoc test).

Table 3 Initial and final fasting blood glucose (FBG) levels of all the groups

Groups	Initial FBG levels (mg/dl)	Final FBG levels (mg/dl)
non-DM	90.88 (2.03)	90.50 (1.07)
DM	437.13 (67.37)[a]	541.88 (62.45)[a]
DM+300EEP	447.50 (27.70)[a]	307.50 (33.63)[a, b]
DM+600EEP	417.38 (64.79)[a]	270.88 (86.25)[a, b]
DM+metformin	494.63 (50.42)[a]	284.25 (74.01)[a, b]

Values are mean (SD), n = 8/group. [a] $P < 0.05$ compared with non-DM group, [b] $P < 0.05$ compared with DM group (one way ANOVA followed by Tukey-kramer post-hoc test).

Table 4 Plasma insulin and glucagon levels of all the groups

Groups	Insulin levels (µ IU/ml)	Glucagon levels (ng/ml)
non-DM	27.16 (0.56)	35.89 (13.30)
DM	8.24 (2.00)[a]	100.85 (18.72)[a]
DM+300EEP	11.71 (0.55)[a, b]	55.19 (17.90)[b]
DM+600EEP	14.52 (0.99)[a, b]	43.69 (6.61)[b]
DM+metformin	9.05 (2.23)[a, d]	92.98 (21.71)[a, c, d]

Values are mean (SD), n = 8/group. [a] $P < 0.05$ compared with non-DM group, [b] $P < 0.05$ compared with DM group, [c] $P < 0.05$ compared with DM+300EEP group, [d] $P < 0.05$ compared with DM+300EEP group (one way ANOVA followed by Tukey-kramer post-hoc test).

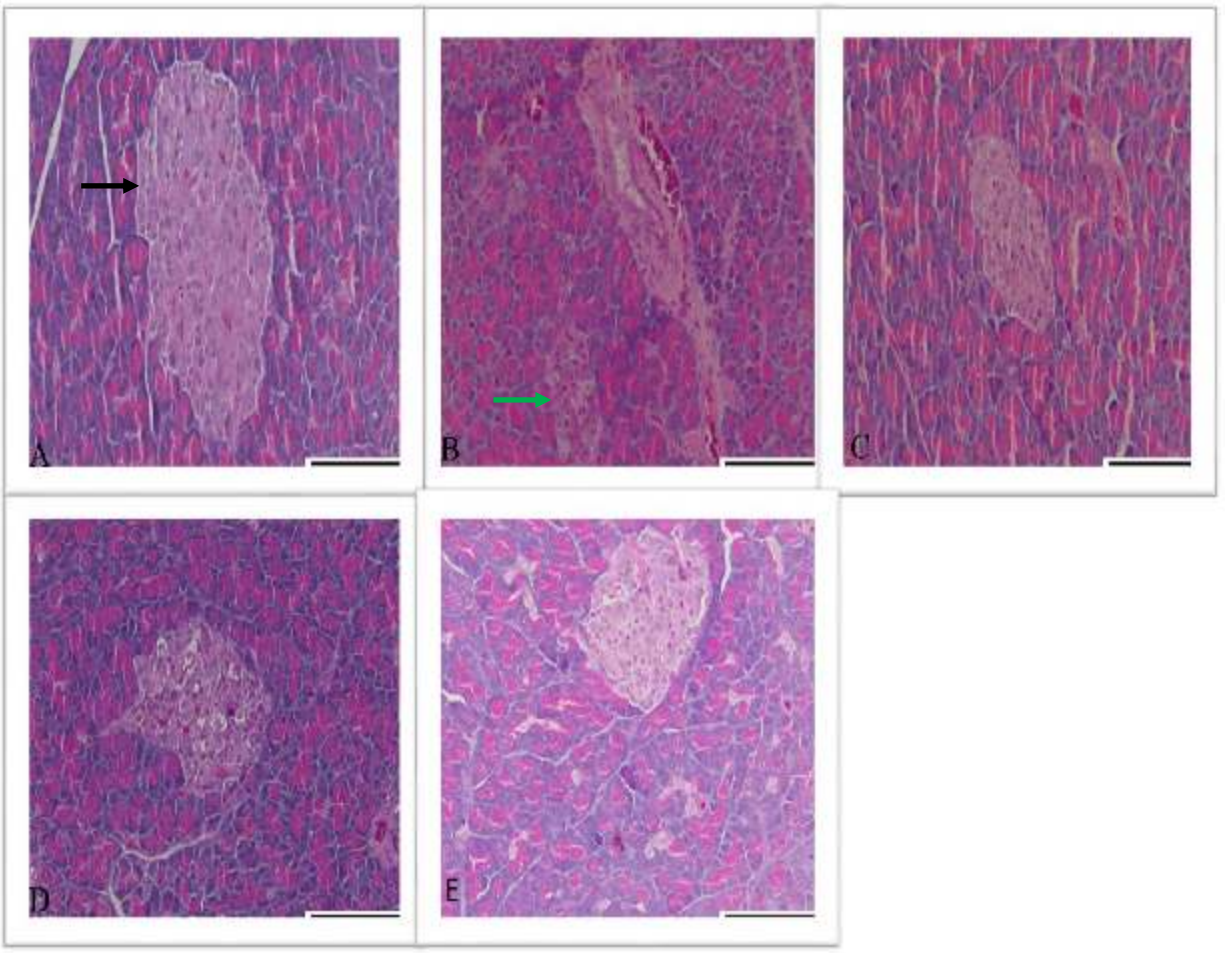


Fig. 1: Representative photomicrograph of pancreas. (A) non-DM group showing normal islet of Langerhans with normal size (dark arrow). (B) DM group showing shrunken islet of Langerhans with reduced size (green arrow). (C) DM+300EEP, (D) DM+600EEP and (E) DM+metformin groups showing improvement for the size of islet of Langerhans (magnification 100x; haematoxylin and eosin staining; scale bar: 100 μm).

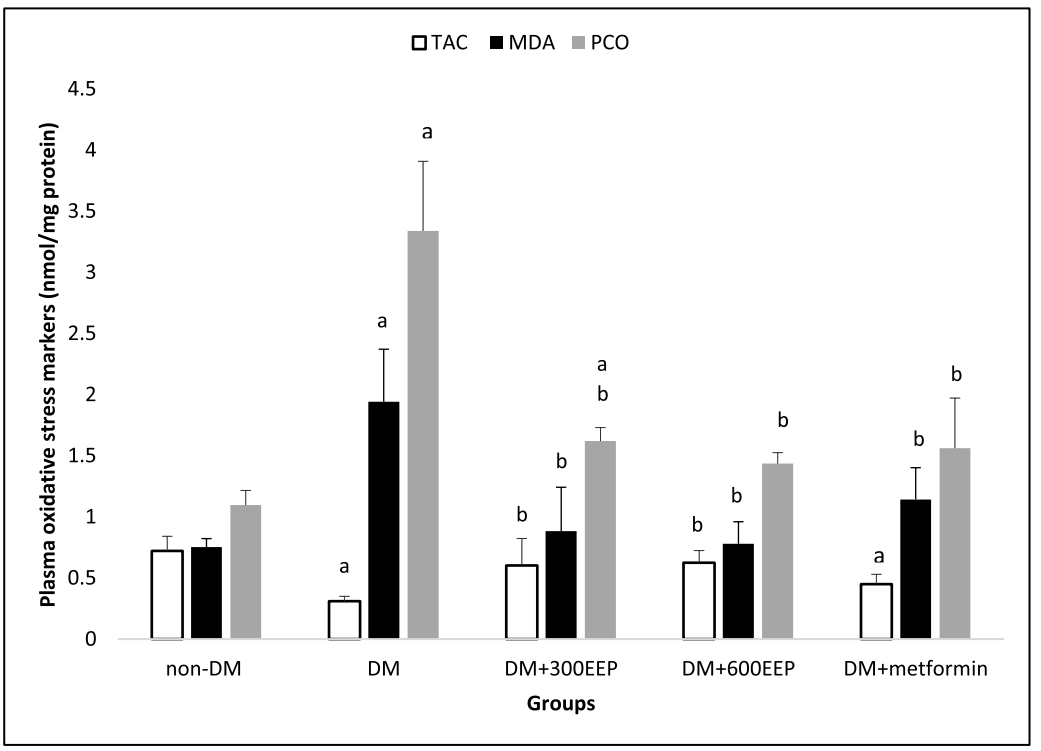
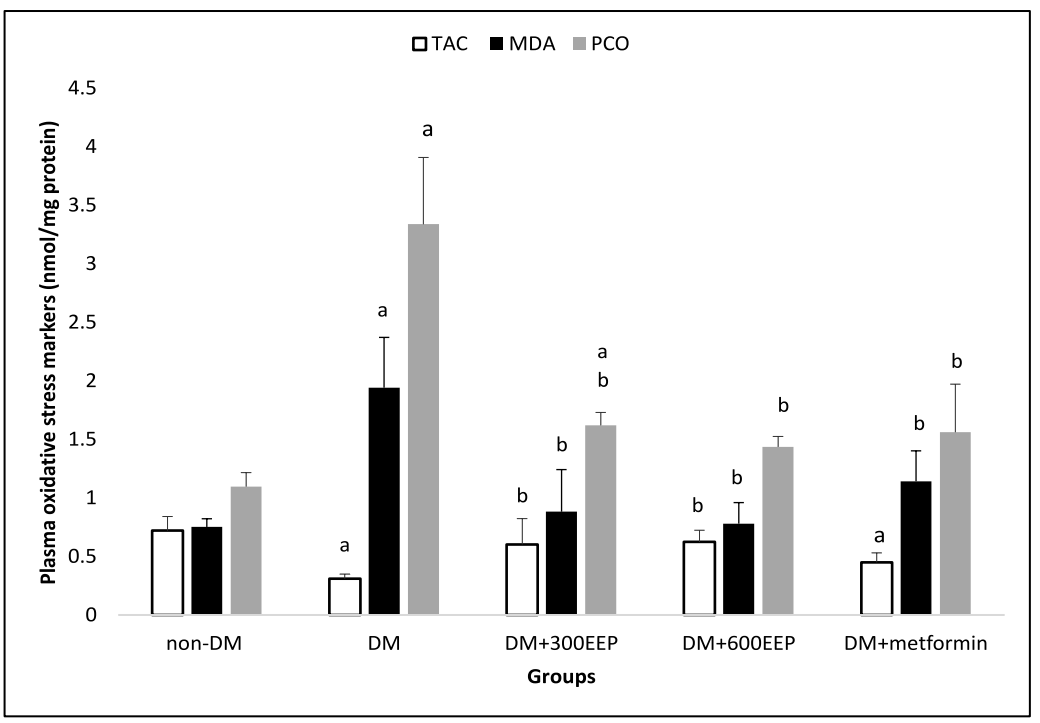


Fig. 2: Plasma oxidative stress markers. Total antioxidant capacity (TAC); malonaldehyde (MDA) and protein carbonyl (PCO). Data are presented as mean (SD) (n = 8 rats per group). [a] $P < 0.05$ compared with non-DM group, [b] $P < 0.05$ compared with DM group (one way ANOVA followed by Tukey-Kramer post-hoc test).



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DISCUSSION

In this study, LCMS analysis was done to determine and compare the number of phytochemical compounds obtained from water and ethanol extracts of Malaysian propolis. Twenty nine phytochemical compounds were identified from EEP while WEP revealed only ten compounds and many of them were phenolic derivatives. Interestingly, despite the difference in the number of compounds between the two samples, all the 10 compounds (gallic acid derivative, coumaric acid, prenylated compound, malonic acid derivative, vanillic acid derivative, delta-tocotrienol, cinnamic anhydride, L-phenylalanine, caffeic acid derivative and carboxylic acid) in WEP were also obtained in EEP of the same sample. A higher number of volatile compounds is also found in Malaysian EEP (25) compared to WEP (12) using GC-MS analysis[23]. A similar study using LC-MS shows ethanol extract of Portuguese propolis has 62 different phytochemical compounds and many of them are phenolic derivatives[4]. The difference in the number and type of phytochemical composition in WEP and EEP could be associated with polarity or solubility of compounds[27]. Another similar study on Brazilian propolis identifies 40 compounds that include phenylpronoids, prenylated and pherylpropanoids using GC-MS analysis. However, this difference can be explained by the fact that, some factors like bees type, vegetation, geographical location, season of harvest may also affect the amount and type of compounds as reported with Brazillian propolis[28], in which many of these compounds such as phenolic, flavones, flavonols, flavanones and dihydroflavonols possesses antioxidant potential[29]. This knowledge of propolis chemical composition will promote regional research and provide a guide to establish scientific basis for understanding and promoting its biological properties and functions[30]. Therefore, EEP was further used in the in vivo study.

In DM group, total food intake was significantly higher while final body weight was significantly lower compared with non-DM group. The significantly lower food intake, higher final body weight and lower FBG in DM+300EEP and DM+600EEP groups compared with DM group suggest the antidiabetic potential of EEP. The finding on body weight is consistent with our previous pilot study on Malaysian propolis extract[24] and other study using African propolis[7]. The significant improvement in body weight might be as a result of propolis antihyperglycaemic effect as chronic hyperglycaemia may cause cellular starvation, proteolysis and lipolysis[31]. The significantly higher plasma insulin and significantly lower FBG and glucagon levels in the extract groups compared with DM group, may explain the EEP's antihyperglycaemic effect as seen in this study. Previous report shows phenolic compound with antioxidant property may reduce oxidative damage which in turn may increase insulin release from isolated islets cells[32]. These findings are in accordance with other study on propolis from Egypt that shows significantly lower FBG level and increase in plasma insulin level in streptozotocin-induced diabetic rats[33] and propolis from Brazil that significantly improves FBG level and plasma insulin level in human subject of type 2 diabetes mellitus[34]. Moreover, the improved FBG level and, plasma insulin and reduced glucagon levels may suggest the improved function of beta cells in secreting more insulin which in turn may reduce the glucagon secretion.

Pancreas is the key organ affected in this diabetic model with functional loss due to the destruction of the islet of Langerhans cells by streptozotocin which leads to impaired insulin production and secretion[35]. Histology of pancreas section of diabetic control rat showed severe damage to the islet of Langerhans, shrunken islet cells and reduced size of islet of Langerhans compared with non-DM and DM+300EEP, DM+600EEP and DM+metformin groups. The histology of pancreas showed some improvements as characterized by increment in size of islets of Langerhans in the EEP treated groups compared with that of control DM group. This is in accordance with similar findings using propolis from Africa[7] and Saudi Arabia[36]. Interestingly, the improved histological finding may suggest the improved function of beta cells in producing and secreting insulin as indicated by higher level of insulin in DM+300EEP and DM+600EEP groups compared to DM group. Although there was an improvement in the histology of pancreas in DM+metformin group, the levels of insulin and glucagon were not improved suggesting the higher ameliorating effect against islet of Langerhans cell damage and or regenerating effect of EEP compared to metformin.

Previous study shows that chronic hyperglycaemia leads to increased production of oxidative stress and decreased antioxidant activity or action in DM[22]. As a result of these events, the balance between normal reactive oxygen species or free radical production and antioxidant activity or production to protect against oxidative stress will be altered[37]. This is shown in the present study whereby the levels of oxidative stress markers such as MDA and PCO were significantly higher and the level of TAC was significantly lower suggesting the presence of oxidative stress in DM group compared to non-DM group. The beta-cells are highly prone to oxidative stress and damage due to its low antioxidants enzymes activity[38]. Therefore, it can be suggested that the degenerative and shrunken changes on the islets of Langerhans may be due to the increased oxidative stress in DM group.

However, treatment with EEP at 300 and 600 mg/kg/day for 4 weeks significantly lower MDA and PCO levels and significantly improved TAC levels in DM+300EEP and DM+600EEP groups compared with DM group which may suggest a good antioxidant property of Malaysian propolis. The improved histological findings in rats treated with EEP may be due to reduced cellular oxidative damage by the EEP which in turn could possibly enhance islet cell regeneration and subsequent insulin production and secretion. In addition, treatment with metformin significantly reduced PCO and MDA levels but shows no significant change for TAC levels compared with DM group suggesting a possibility that EEP has higher capacity in ameliorating hyperglycaemia-induced oxidative stress. In addition, the similar findings found in DM+300EEP group was comparable with DM+600EEP group suggesting that these effects were not dose-dependent. These findings are similar with other studies whereby African propolis at 200 mg and 300 mg/kg/day for 4 weeks[7] and Egyptian propolis at 200, 300 and 400 mg/kg/day for 4 weeks[33] significantly reduces MDA levels. In other studies, Iranian propolis at 100 mg and 200 mg/kg/day significantly reduces MDA and total antioxidants capacity after 6 weeks of treatment[31] while Saudi Arabian propolis at 300 mg/kg/day significantly reduces MDA level after 21 days of treatment[36] in type 1 DM animal model. The phytochemical compounds that have antioxidant property such as

gallic acid derivative, cinnamic anhydride, caffeic acid derivative, ellagic acid, coumaric acid, delta-tocotrienol, diprenylated compound and carboxylic acid found in EEP could be responsible for its antidiabetic potential by ameliorating oxidative stress status and subsequently improve islet cells functions. Moreover, the reduced oxidative stress following EEP treatment may possibly enhance cellular response to insulin which further contributes to this improved plasma insulin and hence antidiabetic effects.

CONCLUSION

LC-MS analysis of EEP showed more phytochemical compounds than WEP which were mainly phenolic derivatives and EEP significantly reduced food intake, oxidative stress status and levels of FBG and glucagon as well as significantly improved body weight, insulin level and islet of Langerhans regeneration in streptozotocin-induced diabetic rats. This study suggests the potential antidiabetic and antioxidant properties of Malaysian propolis (EEP) which may be partly due to the synergistic effect of its constituents. However, further studies are needed to evaluate its exact molecular mechanism of action, potential ameliorating effects in other organs and its use as adjuvant supplement among diabetic patients.

Author contributions

UZU conceived the study and drafted the paper. MAM and ABA designed the study. AAM acquired the histology and analysed the data. All authors interpreted the data, revised the paper critically for important intellectual content and approved the final version.

Conflict of interests

The authors report no conflict of interests.

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