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Phytochemical composition and activity against hyperglycaemia of Malaysian propolis in diabetic rats.

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Abstract

Diabetes mellitus (DM) is a disease associated with hyperglycaemia and loss of body weight. Brazilian propolis is shown to have hypoglycaemic effect in diabetic rats. However, the role of Malaysian propolis on food intake, body weight gain and fasting blood glucose in diabetes has yet been reported. We aimed to determine the phytochemical compounds in ethanol extract of Malaysian propolis (EEP) and its hypoglycaemic effect on diabetic rats. Thirty female rats were randomly assigned into five groups (n= 6/group): Non-DM (non-diabetes on distilled water 1 ml/day), DM (diabetes on distilled water 1 ml/day), DM+300EEP (diabetes on 300 mg/kg/day EEP), DM+600EEP (diabetes on 600 mg/kg/day EEP) and DM+metformin (diabetes on metformin 100 mg/kg/day) groups. DM was induced by a single dose of streptozotocin (60 mg/kg) intraperitoneally and treatments were given by oral gavage for four weeks. There were 36 volatile phytochemicals compounds identified in EEP using gas chromatography-mass spectrometry (GC-MS) analysis. Total food intake and fasting blood glucose were significantly higher while body weight gain was significantly lower in DM group compared to Non-DM group. DM+600EEP group had significantly lower total food intake compared to DM group. Significantly higher body weight gain and lower fasting blood glucose were found in DM+300EEP, DM+600EEP and DM+metformin groups compared to DM group. In conclusion, the GC-MS analysis of EEP revealed 36 volatile phytochemical compounds. EEP significantly reduced fasting blood glucose level and total food intake, and increased body weight gain in streptozotocin-induced diabetic female rats.

Keywords: Phytochemistry, Hypoglycaemia, Propolis, Diabetes

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Introduction

Diabetes mellitus (DM) is a complex metabolic disease characterized with hyperglycaemia, increased thirst, polyuria, polyphagia and weight loss. Gradually it alters and virtually affects all the systems in the body leading to an increase in acute and chronic metabolic problems as well as micro and macro vascular complications [1]. There were 382 million people in the world with diabetes in 2013 and it is projected to increase up to 592 million by 2035. Its effect on the patient socio-economy, physical and medical state has become a major concern globally [2]. The healthy life styles, pre-diabetic check-up, adequate evaluation of family history of diabetes and exercise have been a major effective strategic intervention in preventing the disease progression, cardiovascular involvement and further complications [3]. Several medicine and natural products are used to treat DM whereby, some of these materials may negatively influence the diabetic symptoms. Thus, the investigation for new compounds has become necessary to come up with treatment with little or no side effects with scientific proof [4].

Bee glue known as propolis is a natural product derived from plant resins, collected by honeybees for shelter development as beehives [5]. It has a long history of medicinal use by humans [6] and has captured researchers attention as a result of its properties such as, antiviral and antifungal [7], antibacterial [8,9] anti- inflammatory [10] and antioxidant properties [11], has hepatic and pancreatic protection against oxidative stress in diabetic male rats [12] as well as improved sperm parameters [13] and protect reproductive system against aluminium chloride toxicity in male rats [14]. However, gas chromatography-mass spectrometry (GC-MS) report shows propolis to contain at least 200 compounds with more than 100 being commonly present in all the samples in respect of any factor [15]. These include phenolic acids and esters, flavonoids (flavones, flavanones, flavonols and others), terpenes, β-steroids, aromatic aldehydes and alcohols derivatives of

sesquiterpenes, naphthalene and stibenes [16,17]. Some of these flavonoids are reported to have medicinal values which include rutin with antihypertensive action, quercetin with antidiabetic property and galangin with antioxidant activity [18].

Brazilian and Nigerian propolis has been shown to have hypoglycaemic effect in diabetic male rats [12,19]. However, the chemical compositions of propolis that modulate the pharmacological activities are seen to be influenced by geographical location and seasonal variation [20]. Previous study has reported the presence of 12 and 25 volatile compounds in water and ethanol extracts of Malaysian propolis respectively [21]. Another study using GC-MS shows propolis samples from different region of Turkey i.e., Kazan and Marmaris to have 24 compounds (Kazan) and 18 compounds (Marmaris), respectively. The samples were obtained and analysed in the same time of period [22]. Brazilian red propolis reveals 20 phytochemical compounds using ethanol as solvent [23]. The chemical composition of propolis depends on many factors including the geographical location [24]. However, investigating the propolis chemical composition will guide in the understanding and promoting it biological properties [22].

To date, no study has been done on the role of Malaysian propolis in diabetic animal model or to study if Malaysian propolis may benefit diabetic patients. Hence, the aim of this study was to investigate the phytochemical composition and *in vivo* anti- diabetic effect of ethanol extract of Malaysian propolis (EEP) in streptozotocin-induced diabetic female rats.

Material and Methods

Collection and Extraction of Propolis

Propolis was purchased from a local beekeeper in Kota Bharu, Kelantan, Malaysia during the period of Jan to April, 2015 (dry season) and stored at -80°C. EEP was prepared by the method described earlier [25] with a modification. Briefly, raw propolis (30 g) was washed with distilled water, frozen at -80°C and ground into powder by a mill. Then, it was mixed vigorously with 100 ml 70% (v/v) ethanol at room temperature, 6 hours daily for 7 days. The extract was filtered, concentrated and lyophilized to obtain EEP.

Chemicals

All the chemicals and reagents used for the study were of analytical grade. Streptozotocin was purchased from Sigma Aldrich Company Ltd, Gillingham Dorset, United Kingdom. Ketamine and xylazine were purchased from Troy laboratories PTY Ltd, Glendenning, Australia.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

EEP was dissolved in methanol (0.01 g/ml) and vortexed for 1 min. The GC-MS analysis was carried out using Hewlett-Packard gas chromatograph 5890 series II Plus linked to a Hewlett-Packard 5973 mass spectrometer system. The temperature was set from 30 to 60°C and helium was used as a carrier gas at a flow rate of 0.8 ml/min. The split ratio was 1:10 and the injector temperature was 280°C at 70 eV ionization voltage. The compound identification was performed based on the database of the National Institute Standard and Technology (NIST) and Wiley Registry of Mass Spectral data's, New York (WILEY) libraries. Information of the component of the test materials such as retention time and percentage peak area was also obtained using this analysis. The compounds with percentage peak area more than 0.07% were considered significant components and recorded.

Animals

A total of thirty 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°C, and provided with food and water *ad libitum*. The study was performed in accordance with the guidelines by the Animals Ethical Committee, Universiti Sains Malaysia (USM/Animal Ethics Approval/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 (60 mg/kg body weight) in a sterile normal saline. Six control rats were injected intraperitoneally with normal saline alone at a single dose of 1 ml/kg. They were allowed free access to food and water *ad libitum* and hyperglycaemia was confirmed after a minimum of 48 h following streptozotocin injection [26,27]. Animals were considered diabetic and selected for this study on the basis of fasting blood glucose levels ≥ 200 mg/dl (Digital Glucometer, Lifescan lnc., Milpitas, USA) taken from the tail vein.

Study Design

The animals were randomly assigned into 5 groups (n = 6 rats/group) as follows:

1. Non-DM: Non diabetic rats received distilled water (1ml) as normal control group
2. DM: Diabetic rats received distilled water (1 ml) daily as diabetic control group
3. DM+300EEP: Diabetic rats received 300 mg/kg EEP as low dose treatment group
4. DM+600EEP: Diabetic rats received 600 mg/kg body weight EEP as high dose treatment group
5. DM+metformin: Diabetic rats received 100 mg/kg body weight metformin

All treatment was given by oral gavage daily between

9 to 10 am for 28 days. Food intake was measured and recorded daily while fasting blood glucose and body weight were measured weekly. At the end of the study period of 4 weeks, animals were fasted overnight and sacrificed under anaesthesia using 90 mg/kg ketamine and 5 mg/kg xylazine.

Statistical Analysis

Data are represented as mean ± standard deviation (SD). Statistical analysis was carried out using Instat. Exe version 3.1 (Charlesworth Group Ltd, Huddersfield, United Kingdom). One way analysis of variance (ANOVA) followed by Turkey Kramer multiple comparison test were used to assess the differences in fasting blood glucose, body weight gain and total food intake between groups. $P < 0.05$ was considered as significant.

Results

The GC-MS analysis of EEP is presented in Table 1 while the data for fasting blood glucose, body weight gain and total food intake in Table 2. Thirty six volatile phytochemical compounds were identified in EEP. The three major compounds with the highest total percentage peak area were phenol, 3-pentadecyl (1.01%), cycloartenol or 9,19- cylolanost- 24- en-3-en-3-ol-9, 3-beta (4. 06%) and 1, 3-benzenediol 5-pentadecyl (4.18%).

Before treatment, the levels of fasting blood glucose were significantly higher in diabetic rats (DM+300EEP, DM+600EEP and DM+metformin groups) than non-diabetic rats (Non-DM group). After 4 weeks, fasting blood glucose level was significantly higher in DM group compared to Non-DM group. Significantly lower fasting blood glucose levels were found in DM+300EEP, DM+600EEP and DM+metformin groups compared to Non-DM group. However, no significant differences were found for fasting blood glucose levels among Non-DM, DM+300EEP, DM+600EEP and DM+metformin groups.

Table 1*: GC-MS analysis of Malaysian ethanol extract propolis (EEP)*

Compounds name	Retention time	Percentage peak area (%)
2- Furanmethanol	3.515	0. 070
2, 5- Furandione- 3- methyl	5.777	0. 170
2, 4- dihydroxy- 2, 5- dimethyl-3(2h) furan- 3- one	6.365	0. 200
2, 5- furandione or succinic anhydride	6.946	0. 190
Penylacetaldehyde	7.177	0. 150
Bezeneethanol	7.870	0. 190
Benzoic acid or Retardex	8.368	0. 390
2- Coumaranone	8.774	0. 130
Benzeneacetic acid	8.928	0. 520
Decanioc acid or Capric acid	9.544	0. 110
Isocaryophyllene or gamma caryophyllene	9.920	0. 200
Bela selinene	10.100	0. 150
Alpha Copaene	10.200	0. 270
Alloaromadendrebe	10.270	0. 130
Alpha Selinene	10.320	0. 180
Gamma Cadinene	10.410	0. 130
Delta Cadinene	10.440	0. 150
Lauric acid	10.560	0. 220
Spathulenol	10.740	0. 160
Caryophyllene oxide	10.780	0. 130
Hexadecanoic acid, methyl ester	12.160	0. 190
Hexadecanoic acid ethyl ester	12.420	0. 280
5- Octadecene	12.770	0. 280
8- Octadecenoic acid, methyl ester	12.840	0. 450
9- Octadecenoic acid (z) ethyl ester	13.890	0. 870
9- Octadecen- 1- 0 9z	13.420	0. 800
Phenol, 3- pentadecyl	14.200	1. 010
Hydroxymethyloethyl ester	14.240	0. 170
1, 2- Benzenedicarboxylic acid, bis (2- ethylhexyl) ester	14.370	0. 220
5- Heptylresorcinol	14.470	0. 340
1, 3- Benzenediol, 5- pentadecyl	15.830	4. 180
Lanosta- 8, 24- dien- 3- ol (3 beta)	17.220	0. 920
Cycloartenol or 9, 19- Cylolanost- 24- en- 3- ol- 9, 3 beta	18.100	4. 060
9, 19- Cyclolanostan- 3- ol, 24- methylene (3 beta)	18.490	0. 550
9, 19- Cyclo- 9 beta- lanostane- 3 beta 25 diol	18.630	0. 260
9, 19- Cyclolanostan- 3- ol, 24- methylene (3 beta)	20.560	0. 320

Table 2: *Fasting blood glucose, body weight gain and total food intake in all experimental groups*

Groups	Fasting blood glucose (mg/dl)		Body weight gain (g/rat)	Total food intake (g/rat)
	Before treatment	After treatment		
Non-DM	92.17 (3.19)	91.17 (2.56)	37.52 (6.04)	507.88 (38.20)
DM	435.50 (90.52)[a]	533.17 (70.37)[a]	-34.98 (4.66)[a]	947.87 (217.88)[a]
DM+300EEP	446.17 (31.67)[a]	308.67 (39.38)[a, b]	21.80 (3.83)[b]	730.77 (162.09)
DM+600EEP	425.00 (81.48)[a]	243.00 (82.00)[a, b]	31.75 (19.94)[b]	672.30 (127.93)[b]
DM+metformin	519.00 (58.87)[a]	252.50 (63.82)[a, b]	25.90 (12.34)[b]	815.62 (121.93)

Results are expressed as mean ± SD and n= 6 for each group.
[a]$p<0.05$ compared to Non-DM group; (ANOVA followed by Turkey Kramer multiple comparison test)
[b]$p<0.05$ compared to DM group (ANOVA followed by Turkey Kramer multiple comparison test)

Body weight gain after 4 weeks of study was significantly lower in DM group compared to Non-DM group. Meanwhile, DM+300EEP, DM+600EEP and DM+metformin groups had significantly higher body weight gain compared to DM group. No significant differences were found for body weight gain among Non-DM, DM+300EEP, DM+600EEP and DM+metformin groups.

Total food intake in DM group was significantly higher compared to Non-DM group. Interestingly, total food intake was significantly lower in DM+600EEP group compared to DM group but not significantly different with Non-DM group. No significant differences were observed for total food intake among DM+300EEP, DM+600EEP and DM+metformin groups.

Discussion

This is the first study showing the *in vivo* hypoglycemic action of Malaysian propolis extract together with its phytochemical compounds using GC-MS analysis. Malaysian propolis harvested during the period of dry season (Jan to April) revealed 36 compounds by GC-MS analysis when extracted with ethanol. However, previous study conducted in comparison of water and ethanol extracts using propolis from the same region but harvested during the period of raining season (September to December), showed 12 compounds with distilled water extraction and 25 compounds with ethanol extraction [21]. This is in accordance with the fact that, other than type of bees in the area and geographical location, season of harvest can also affect the amount and type of compounds as seen in Brazilian propolis [20]. Interestingly, despite the difference in the number of compounds and seasonal variation, three phytochemicals namely phenol 3-pentadecyl, cycloartenol (9, 19-cylolanost-24-en-3-ol-9,3 beta) and 1,3-benzenediol 5-pentadecyl were the main chemicals found in Malaysian EEP collected both during dry and raining seasons [21]. This present study showed that Malaysian EEP had more compounds as compared to propolis samples from different region of Turkey (Kazan and Marmaris regions) in which there are 24 compounds in Kazan propolis and 18 compounds in Marmaris propolis using GC-MS analysis [22]. This present study also showed Malaysian propolis to have more compounds compared to Brazilian red propolis which revealed 20 compounds using GC-MS analysis [23]. These findings are in line with the fact that composition of propolis may also depend on the geographical location [20].

Streptozotocin is a cytotoxic chemical used to induce DM in experimental animals [27]. DM develops following the pancreatic beta cells destruction [26]. In this study, we observed a significant sustained elevation of fasting blood glucose in DM group compared to Non-DM group showing the establishment of diabetic animal model. Significant reductions in fasting blood glucose levels were found in the extract-treated groups (DM+300EEP and DM+600EEP) and metformin-treated group compared with DM group. However, no significant difference were observed for fasting blood glucose between the extracts and standard drug (metformin) indicating the hypoglycaemic potential of the propolis extracts. There was also no significant different observed between the fasting blood sugar of the two extracts groups (low and higher dose) demonstrating that the extracts may not be dose dependent. The hypoglycaemic effect of EEP in the present study is in line with the previous studies using African propolis [12], and Brazilian propolis [19].

The different phytochemical compounds will be essential for some of the activities of propolis and analysing its chemical composition will guide in understanding, and promoting it biological properties [22]. The reduction in the blood glucose level may be as a results of some bioactive components of propolis that have protective effect on pancreatic beta cells as suggested in previous study [12,28]. It was suggested that propolis could also have acted by (i) stimulating the remaining surviving or functioning beta-cells of the islets of the pancreas to secrete more insulin or (ii) inducing regenerative effect on beta cells through its antioxidant property or (iii) indirectly enhancing the cellular response to the insulin action by inhibition of glucose release from the liver [29]. The hypoglycaemic effect of natural products such as bee product and medicinal plant extracts is believed to be dependent upon the degree of islet beta cell destruction [30]. Previous research shows propolis to contain bioactive constituents such as phenolic acids with hypoglycaemic properties [31] and it is suggested to improve blood glucose level by increasing insulin secretion in streptozotocin-induced diabetic rats [28].

In diabetes, the decrease in body weight is due to proteolysis in skeletal muscle and lipolysis in adipose tissue [33]. The body weight gain was significantly reduced in DM group compared with Non-DM, DM+300EEP, DM+600EEP and DM+metformin groups. The low and high doses of the extracts in EEP treated group showed no significant different suggesting that the effect of extracts on body weight was not dose dependent. This is in accordance with previous studies using Nigerian [12] and Egyptian propolis [33] where by propolis significantly reduced blood glucose level and improved body weight gain in diabetic male rats [12]. The increase in body weight gain may be as a result of propolis hypoglycaemic action, increased glucose utilization or prevention of hyperglycaemia associated bone loss or both [28]. The weight loss in untreated DM may be due to pancreatic beta cells destruction action of streptozotocin leading to insulin deficiency, cellular starvation in the midst of plenty plasma glucose, hence causing mobilization of fats and proteins to produce energy. The beneficial effect of EEP may be partly attributed to the presence of bioactive compounds such as phenolic and flavonoids compounds [35] 3,4,5-tri-caffeoylquinic acid [29] and other useful volatile compounds as obtained in this present study. Some of these bioactive substances found in neutraceuticals and functional food ingredients may help in the management of metabolic disorders [36].

Total food intake was significantly lower in DM+ 600EEP but not in DM+ 300EEP and DM+ metformin group when compared to DM group which is in line with the previous studies using Nigerian and Brazilian propolis [12,28]. The above finding may be resulted from the ability of propolis at higher dose (600 mg/kg/day) to ameliorate hyperglycaemia which in turn suppresses satiety centre in diabetic rats. However, further experiments are needed to investigate the possible mechanisms involved in the hypoglycaemic effect of EEP on diabetic rats as well as to compare the efficacy between Malaysian and other propolis such as Brazilian propolis on DM. Furthermore, as pre-diabetes and family history of diabetes are able to predispose an individual to a future development of diabetes [3] it is also plausible to suggest a study to investigate the possible role of Malaysian propolis in preventing the development of diabetes among those with pre-diabetes condition or family history of diabetes.

Conclusion

GC-MS analysis indicated the presence of 36 volatile phytochemical compounds in Malaysian propolis. Malaysian EEP significantly reduced fasting blood glucose level and food intake as well as increased body weight gain in diabetic female rats. Further study is needed to assess non-volatile phytochemical compounds in Malaysian propolis and investigate its mechanism of action against hyperglycaemia in diabetic animal model.

Conflict of Interest

We declare that there is no conflict of interests.

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