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MOLECULAR DOCKING STUDIES AND ANTI-HYPERGLYCAEMIC PROPERTIES OF *MELICOPE PTELEFOLIA* METHANOLIC LEAVES EXTRACT IN RATS

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ABSTRACT

Type 2 diabetes is one of the most common diseases worldwide which is progressing at an incremental rate every year. A number of research works are being carried out in finding a new compound by targeting its enzymes. The anti-hyperglycaemic properties of *Melicope ptelefolia* and its compounds were analysed by molecular docking with various enzymes and also in *in vivo* anti-hyperglycaemic study in rats. The molecular docking study showed that four compounds (5,5-dimethoxy alloagerasanin, melifolin, melicobisquinolinone A and β-sitosterol) were found to be potential against PPAR-γ and α-amylase whereas the *in vivo* anti-hyperglycaemic study showed a reduction in blood glucose level. The possible mechanism of action of anti-hyperglycaemic property of *Melicope ptelefolia* might be due to the presence of these compounds which bind to the active binding site of the enzymes. Finally, molecular docking and *in vivo* anti-hyperglycaemic results revealed that a group of 4 *Melicope ptelefolia* compounds exhibit to be potential natural products for the treatment of diabetes mellitus.

Keywords: Molecular docking, anti-hyperglycaemic, antidiabetic, *Melicope ptelefolia*

1. INTRODUCTION

Melicope ptelefolia (M. ptelefolia) is a shrub locally known as tenggek burung. It belongs to the *Rutaceae* family which is a large family of trees, shrubs and woody climbers. Their twigs and branches are sometimes armed with spines or thorns. This family consists of about 160 genera and 1650 species [1]. *M. ptelefolia* is mostly distributed in the tropical and subtropical part of the world. The natural habitats of the native species are at the lowland, hills, mountains, and offshore islands. Many species are found below 1300 m

elevation and a few ascend are at higher altitudes.

Hyperglycaemia is defined as an excess of glucose in the blood stream often associated with diabetes mellitus. Diabetes mellitus remains a burdensome and public health problem with increasing global incidence [2]. Although several therapies are in use for treatment, there are certain limitations due to high cost and side effects such as development of hypoglycaemia, weight gain, gastrointestinal disturbances and liver toxicity [3]. Though there are various approaches to reduce the ill effects of diabetes and its secondary complications, herbal formulations are preferred due to lesser side effects and low cost [4]. The World Health Organization Expert Committee on diabetes has recommended that traditional medicinal herbs be further investigated.

Nowadays herbal drugs are frequently used to treat many diseases and are considered to be less toxic and free from side effects. As mentioned by Sulaiman *et al.* (2010), *M. ptelefolia* extracts from leaves, twigs, roots, bark, fruits, and flowers have been noted to have been used traditionally for various ailments. In Malay community, the *M. ptelefolia* leaf is taken raw as salad. They believed that this plant possesses anti-hyperglycaemic effect, however limited research been done to prove its effect [5].

In this study, we conducted virtual screening and *in-vivo* anti-hyperglycaemic study to determine the blood glucose lowering effect of *M. ptelefolia*. Virtual screening is one of the available molecular modelling methods that enables the discovery of lead compounds in a faster, more cost-effective and less resource-intensive manner compared to the experimental methods, such as high-throughput screening. The structure-based approach of virtual screening is commonly used and this includes molecular docking. Anti-hyperglycaemic *in-vivo* study is one of the methods to assess the reduction of blood glucose level in animal settings. Oral glucose tolerance test (OGTT) is the frequently used method to be conducted where the animal will be fasted 12-16 hours prior to treatment.

2. MATERIALS AND METHODS

2.1 Molecular docking

2.1.1 Material

The molecular docking simulations were carried out using the program AutoDock version 4.2.3 [6]. All computational experiments were conducted on a desktop computer, running under the Fedora 17 operating system. AutoDock Tools [7], Discovery Studio Visualiser [8], were used for visualization, and analysis of molecular systems, creation and display of graphical representations, and related data.

2.1.2 Ligand and protein preparation

Eighty-two molecular structures were built on a PC utilizing the HyperChem software package [9]. They were then optimized to obtain lowest energy using the semi-empirical

(AM1 Hamiltonian) gas phase energy minimization. Single point calculations were conducted on the minimized geometries to calculate different electronic properties of the molecules including charges, highest occupied molecular orbital (HOMO), and lowest unoccupied molecular orbital (LUMO) energies and dipole moments by molecular orbital package MOPAC [10]. The ligand was made fully flexible with torsions, free translation, and rotation to improve the interaction. We obtained the X-ray structure of antidiabetic targets in complex with known inhibitor (PDB entry3BAJ and 2Q5S) from the Protein Data Bank (PDB) [11, 12]. Then the energy–grid files were generated after the addition of hydrogen atoms, Kollman charges, and solvation parameters using AutoDock tools.

2.1.3 Docking studies

The docking studies were done separately for all the prepared proteins. The docking energies were calculated from a set of energy grids of the enzyme. A docking box with a grid consisting of 90x90x90 and 0.375 Å spacing was used. The protein coordinates were fixed during calculations, while the ligand was flexible and moves on the grid. Grid searching was performed by Lamarckian genetic algorithm to locate the ligand in the best binding orientation and conformation based on the binding energy. Eighty-two docking experiments were performed and consequently a population of fifty docked conformations were produced for each inhibitor.

2.1.4 Validation of the docking protocol

The validation of the software was carried out by re-docking already bound ligands with the proteins. The root mean square deviation (RMSD) was checked for the reliability of docking method in reproducing the experimentally observed binding mode of the proteins.

2.2 Anti-hyperglycemic studies

2.2.1 Materials

Matured leaves of *M. ptelefolia*, electrical weighing balance AX200 (Shimadzu, Japan), freeze-dryer (Alpha 1-2 LD Plus, Germany), rotary evaporator (Buchi R210, Switzerland), incubator shaker (WiseCube® Incubating Shaker), refrigerator (Mediref Pharmaceutical Refrigerator, Malaysia), Buchner funnel, vacuum pump (Buchi V700, Switzerland), electrical weighing balance AX200 (Shimadzu, Japan), electric grinder (Major Kenwood, Panasonic), oven, beaker, measuring cylinder, beaker, spatula, stirrer and glucometer (Accu-check Active) were used in this study. Glucose (Glucolin), methanol (R&M chemicals), normal saline (0.9% NaCl), stainless steel oral gavage (Singapore), towel, glucose strips (Accu-check Active), blades, syringe 3mL/cc, petri dish, paraffin film and Whatman No. 1 filter paper were also used in this study.

2.2.2 Plant materials

Fresh mature leaves of *M. ptelefolia* were collected from Institute of Bioscience, Universiti Putra Malaysia (UPM). Authentication of plant was carried out by a qualified botanist from Institute of Bioscience, UPM where vouchers SK-15302 have been deposited in the herbarium.

2.2.3 Extraction method

Extraction was carried out using methanol as proposed by Sulaiman *et al.* (2010) with slight modifications [5]. Firstly, the freshly collected leaves of *M. ptelefolia* were air-dried under shade for 48 hours. The dried leaves were grounded using an electrical grinder to powder form. Then, 50 g powdered *M. ptelefolia* was macerated with 250 ml of methanol where the ratio of sample to the solvent is 1:5. The mixture was allowed to stand for 24 hours to ensure that all solvent and sample were completely homogenized. The macerated mixture was filtered using vacuum pump and the filtrate was concentrated and evaporated using rotary evaporator under controlled temperature (55°C) and reduced pressure. Further, the wet residue was freeze-dried using freeze dryer for 24 hours until light green powdered form was produced. The extract was then stored in a refrigerator at 4°C until further use.

2.4.4 Animals

A total of forty-two healthy Sprague-Dawley male rats weighing 150-180 g were used in this study. The animals were left for seven days at laboratory condition for acclimatization. They were maintained on standard pellet diet and water *ad libitum* throughout the experiment. The animals were fasted for 12-16 hours and were allowed for free access of water *ad libitum*.

2.4.5 Blood glucose measurement

Blood samples were collected by slitting the end of the rat's tail using razor blade and massaging the tail in a downward direction in order to facilitate the blood flow out from the small cut. Blood glucose measurement was carried out by using electronic glucometer.

2.4.6 Experimental design

All forty-two rats were randomly divided into seven groups comprising of six rats in each group as shown in **Table 1**. Each rat was fasted (approximately 12-16 hours) before the baseline blood glucose level was recorded and denoted as time 0 reading. Rats in normal (group 1) and negative control (group 2) were pre-treated with 0.9% normal saline whereas rats in positive control (group 3) were pre-treated with a dose of 0.25 mg/kg of glibenclamide. For groups 4, 5, 6 and 7, the rats were treated with *M. ptelefolia* extracts (100 mg/kg & 300 mg/kg) respectively. Thirty minutes after the pre-treatment phase, all rats were given 2 g/kg glucose (group 2, 3, 6 &7) and 0.9% normal saline (group 1, 4 &5) respectively. All treatment were administered through oral force-feeding using oral gavage. Blood samples were collected at 30, 60, 90, 120 and 180 minutes after all treatment [13].

Table 1: Illustration of each group and its respective treatment

Groups	Pre-treatment	Treatment after 30 minutes
Group 1 (Normal control)	**0.9% normal saline**	**0.9% normal saline**
Group 2 (Negative control)	**0.9% normal saline**	**2 g/kg of glucose**
Group 3 (Positive control)	**Glibenclamide (0.25 mg/kg)**	**2 g/kg of glucose**
Group 4 (100 mg/kg of *M. ptelefolia* + Normal rats)	**100 mg/kg of *M. ptelefolia***	**0.9% normal saline**
Group 5 (300 mg/kg of *M. ptelefolia* + Normal rats)	**300 mg/kg of *M. ptelefolia***	**0.9% normal saline**
Group 6 (100 mg/kg of *M. ptelefolia* + Glucose-fed rats)	**100 mg/kg of *M. ptelefolia***	**2 g/kg of glucose**
Group 7 (300 mg/kg of *M. ptelefolia* + Glucose-fed rats)	**300 mg/kg of *M. ptelefolia***	**2 g/kg of glucose**

2.4.7 Statistical Analysis

The data obtained was statistically analysed using SPSS version 20. The result was expressed as mean ± SEM. The comparisons between groups and within groups were analysed using one-way analysis of variance (ANOVA). Tukey's and Duncan's test were carried out to determine statistical significance between the means. All data were expressed as the mean ± S.E.M. of 6 animals per group. The values were considered significant when P value is less than 0.05 ($P < 0.05$).

3 RESULTS

3.1 Molecular docking of *M. ptelefolia*

The four best compounds were selected according to the lowest free energy of binding and inhibition constant which is summarized as in **Table 2**. The docking poses of the selected natural compound and the known inhibitor of each target were compared and from the result it was revealed that 5,5-dimethoxy alloagerasanin, melifolin, melicobisquinolinone-A, and β-sitosterol possess similar amino acids for the selected target proteins as shown in **Figure 1**. This confirmed that these compounds bound in the active site of the proteins.

Hydrogen bonds interaction analysis showed that 5,5-dimethoxy alloagerasanin had two hydrogen bond interactions with PPAR-γ (Leu217 and Met233), melifolin had six hydrogen bond interactions (Ser342, Ser342, Arg288) with the protein PPAR-γ, whereas melicobisquinoline-A and β-sitosterol had one hydrogen bond interaction each with α-amylase within their binding pocket as tabulated in **Table 3**. Both known inhibitor, Rosiglitazone and acarbose have two hydrogen bond interaction with the enzymes respectively.

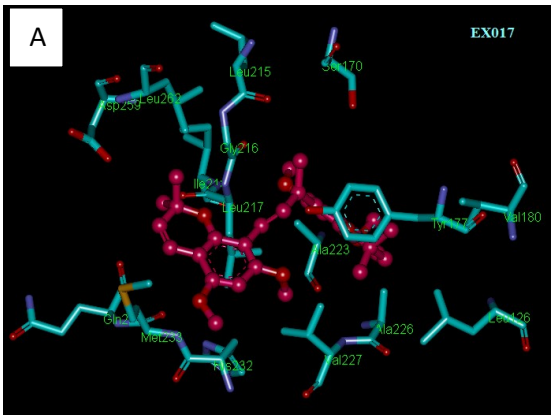
3.2 Anti-hyperglycaemic activity

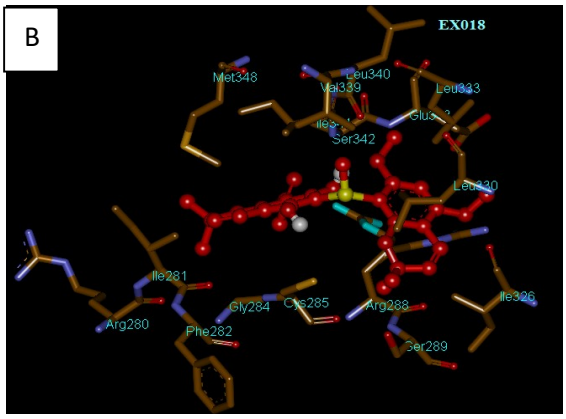
The effect of *M. ptelefolia* extract on glucose tolerance was assessed in normal and glucose-fed rats at various time intervals (0, 30, 60, 90, 120 and 180 minutes). The result of the study was tabulated in **Table 4**. There was a significant increase in blood glucose level in all groups after 30 minutes' administration of 2 mg/kg glucose. After 60 minutes of glucose loading, blood glucose began to decrease and almost approach its baseline value at 180 minutes of glucose administration in each group respectively. There was a reduction of blood glucose level for group 3, 6 and 7 when compared to the blood glucose level at 30 minutes within group and it was statistically significant at 180 minutes. The percentage reduction of blood glucose level for group 3, 6 and 7 was 50.9 %, 68.9 % and 78.5 % respectively.

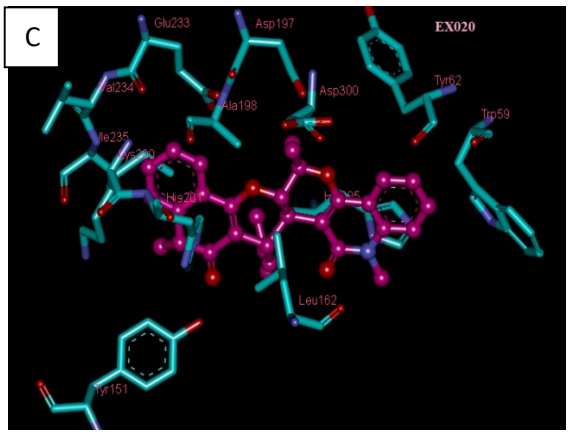
Table 2: The lead compounds for each selected anti-hyperglycemic targets.

Target proteins	Lead compounds and known inhibitors	ΔG	K_i
PPAR-γ	5,5-dimethoxy alloagerasanin	-12.29	0.97
	Melifolin	-11.22	5.94
	Rosiglitazone	- 9.09	6.30
α-Amylase	Melicobisquinolinone A	-9.24	168.73
	β-sitosterol	-9.21	178.69
	Acarbose	-8.84	323.30

ΔG = free energy of binding in Kcal mol^{-1} ; K_i = inhibition constant in nanoMolar (nM) concentration







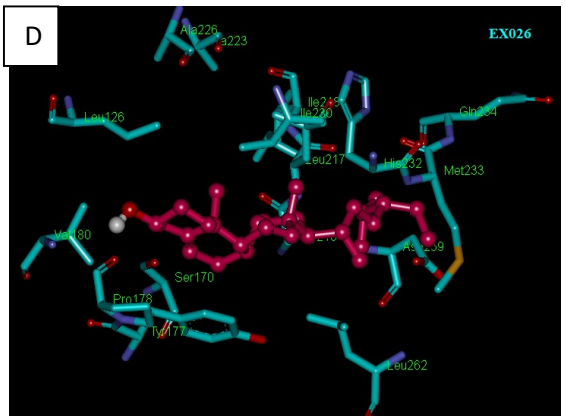


Figure 1: Amino acid residues in the binding pocket of A) 5, 5-dimethoxy alloagerasanin, B) melifolin, C) melicobisquinolinone A and D) β-sitosterol

Table 3: Hydrogen bonds interaction analysis of 5,5-dimethoxy alloagerasanin, melifolin, melicobisquinoline-A and β-sitosterol

Ligand	Target	Amino acid	No of H bonds
5,5-dimethoxy alloagerasanin	PPAR-γ	Arg288 Arg288	2
Melifolin	PPAR-γ	Ser342 Ser342 Arg288 Arg288 Arg288 Arg288	6
Rosiglitazone	PPAR-γ	Ser289 His449	2
Melicobisquinoline-A	α-amylase	Tyr151	1
β-sitosterol	α-amylase	Lys200	1
Acarbose	α-amylase	Gly106 Ser105	2

Table 4: Blood glucose level from time 0 to 180 minutes for all rats

Groups	Time (minutes)					
	0	30	60	90	120	180
Group 1 (Normal control)	4.82 ± 0.25	5.15 ± 0.22[b]	4.68 ± 0.26[b]	4.59 ± 0.25[b]	4.56 ± 0.24	4.49 ± 0.27
Group 2 (Negative control)	5.45 ± 0.2	8.73 ± 0.21[a]	7.48 ± 0.22[a]	6.50 ± 0.4[a]	6.37 ± 0.13	6.95 ± 0.09
Group 3 (Positive control)	5.50 ± 0.18	7.00 ± 0.29[a]	5.92 ± 0.23	5.40 ± 0.18	4.85 ± 0.15	4.20 ± 0.16*
Group 4 (100 mg/kg of *M. ptelefolia* + Normal rats)	5.58 ± 0.25	5.40 ± 0.31[b]	4.80 ± 0.26[b]	4.60 ± 0.25[b]	4.60 ± 0.25	4.24 ± 0.53
Group 5 (300 mg/kg of *M. ptelefolia* + Normal rats)	5.75 ± 0.16	5.72 ± 0.19[b]	5.35 ± 0.22[b]	5.32 ± 0.2	4.87 ± 0.18	4.87 ± 0.23
Group 6 (100 mg/kg of *M. ptelefolia* + Glucose-fed rats)	5.48 ± 0.42	9.51 ± 1.21[a, *]	8.16 ± 0.97[a]	6.04± 0.24[b]	5.89± 0.28 *	5.73 ± 0.35*
Group 7 (300 mg/kg of *M. ptelefolia* + Glucose-fed rats)	5.08 ± 0.29	9.11 ± 0.97[a, *]	7.98 ± 0.47[a]	5.69 ± 0.29	5.91 ± 0.49 [a]*	5.12 ± 0.27*

Each column represents the mean ± SEM in mm/L of 6 animals in each group (n=6); a = P < 0.05 when compared to group 1 between group; b = P < 0.05 when compared to group 2 between group;* = P < 0.05 when compared at 30 minutes within group

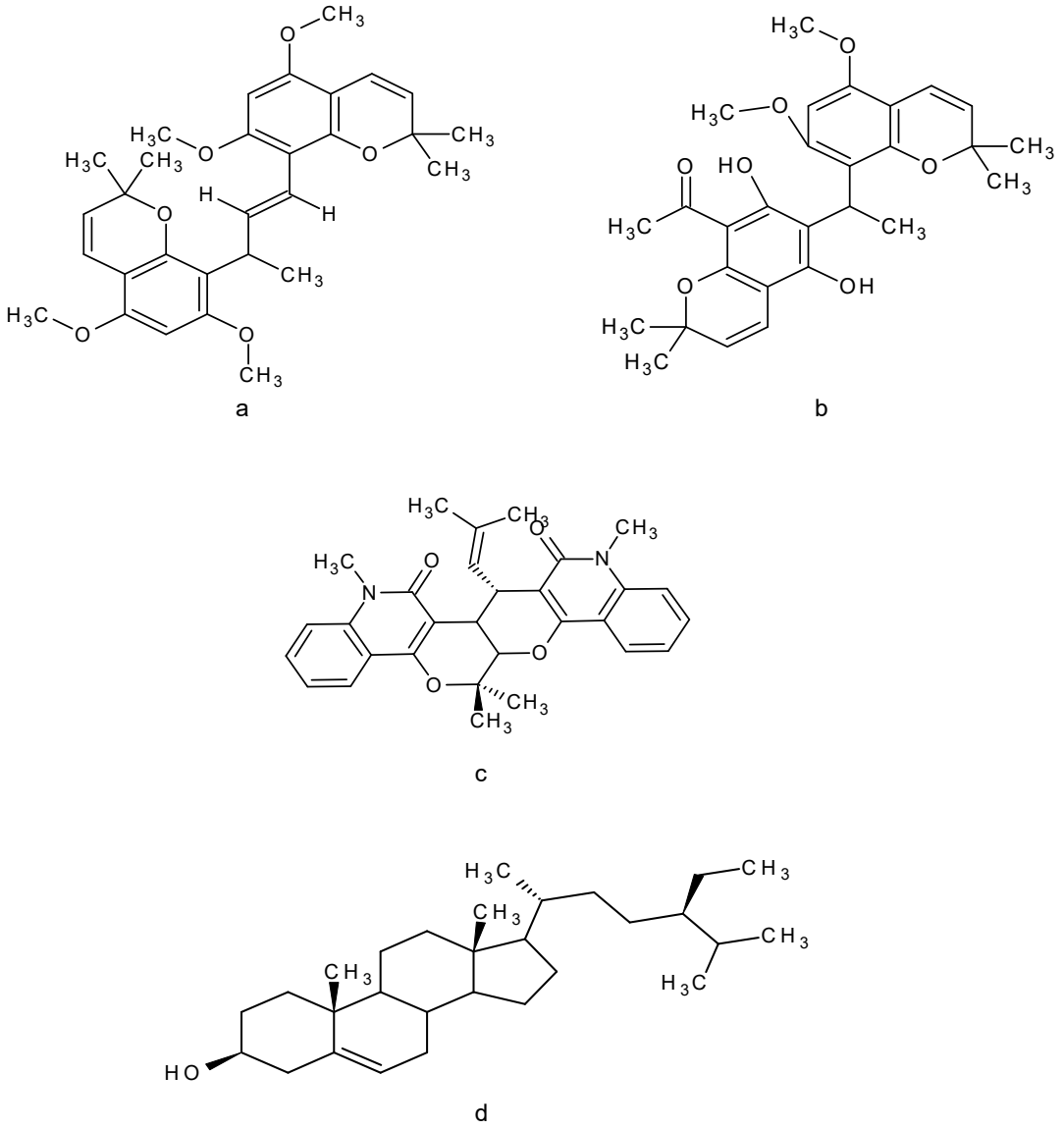


Figure 2: Structure of the lead compounds a) 5, 5-dimethoxy alloagerasanin, b) melifolin, c) melicobisquinolinone A and d) β-sitosterol

DISCUSSION

According to the docking results, there are four compounds that were selected with best autodock score and low bound energy which are 5,5-dimethoxy alloagerasanin, melifolin, melicobisquinoline-A and β-sitosterol. They were found to be potential against PPAR-γ and α-amylase. The docking interaction between the selected natural compounds and the known inhibitors (acarbose and rosiglitazone) of each target were compared. It was revealed that these compounds poseses better score than acarbose (-8.84 kCal/mol) and rosiglitazone (-9.09 kCal/mol) previously bound one. The bioactive compounds also interacted the proteins with more numbers hydrogen bonds in their binding pocket as

the previous study by Lewis et al., 2009 and Brayer, et al., 1995 for PPAR γ and α-amylase respectively. Any agonist and inhibitor of these enzymes will contribute in a substantial decrease in blood as the breakdown of complex starch will be inhibited and delayed glucose absorption into the blood. This is in line with the desired outcome in treating diabetic patients [14, 15].

According to Berger et al., (2002), *M. ptelefolia* extract show antidiabetic properties mediated by the action of peroxisome proliferator-activated receptor gamma (PPAR-γ) [16]. PPAR-γ is a member of the nuclear receptor superfamily that regulates the gene expression of proteins involved in the control of glucose and lipid metabolism. It acts by regulating the insulin sensitivity and developing synthesis PPAR-γ agonists, which could be of therapeutic use in patients affected by Type 2 diabetes mellitus [17].

α-amylase is a salivary and pancreatic enzyme that breaks down starches and complex carbohydrates in the small intestine. It is classified as a hydrolase enzyme. It acts by catalysing the cleavage of internal glycosidic bonds (α-1,4-glucosidic links) in a complex carbohydrate into oligosaccharides. This will allow rapid absorption of glucose in the body and cause an increase of blood glucose in the body for energy utilization [15].

5,5-dimethoxy alloagerasanin and melifolin belongs to flavonoids groups, melicobisquinolinone A is a quinolone alkaloid and β-sitosterol is known as plant sterol.

From the in vivo anti-hyperglycaemic study, there was a significant reduction of blood glucose level for the group treated with 100 mg/kg and 300 mg/kg *M. ptelefolia* after giving 2 mg/kg glucose after 180 minutes. The anti-hyperglycaemic properties of *M. ptelefolia* can be attributed by the presence of polyphenolic compounds like flavonoids (5,5-dimethoxy alloagerasanin and melifolin) present in it. Polyphenolic compounds, especially flavonoids, are among the classes of compounds that have received the most attention with regard to their anti-hyperglycaemic properties [18]. There is also another study that have been done by Iwai *et al.* (2006) and Cabrera *et al.* (2006) about the beneficial effects of polyphenols on management of blood glucose in diabetes. The hypoglycaemic effects of polyphenols are mainly attributed in lowering intestinal absorption of dietary carbohydrate, modulation of the enzymes involved in glucose metabolism, improvement of β-cell function and insulin

action and stimulation of insulin secretion [19, 20].

The anti-hyperglycaemic properties of *Melicope ptelefolia* is also due to melicobiquinolinone A and β-sitosterol which are present in it. Studies have reported that *Melicope ptelefolia* contains alkaloids of the quinolone type [21-24]. It was reported by Semwal *et al.,* 2007 that the hypoglycaemic action of medicinal plant is due to the presence of alkaloids and steroids in the plant extracts [25].

According to Iwai (2008) and Tadera *et al.* (2006), one of the most well-known properties of the polyphenols, especially flavonoids, phenolic acids and tannins, on carbohydrate metabolism is inhibition of α-amylase, the key enzyme responsible for digestion of dietary carbohydrates to glucose [26, 27]. Through in vitro and in vivo studies, Ahmed *et al.* (2010) were able to demonstrate that the flavonoid had the ability to positively influence insulin activity and insulin resistance in type 2 diabetic rats [28]. Their results showed flavonoid decreased glycaemia, lipidaemia, serum insulin concentrations, and liver glycogen content, and hexokinase activities. The presence of polyphenolics and alkaloids was also effective in increasing the expression of the receptor PPAR-γ, leading to improved muscle insulin sensitivity and insulin signalling by increasing insulin-stimulated GLUT 4 receptor activity [9].

In conclusion *M. ptelefolia* extract has anti-hyperglycaemic properties due to the presence of lead compounds (melicobisquinolinone A, melifolin. 5,5-dimethoxy alloagerasanin, β-sitosterol) which acts by inhibiting the PPAR-γ and α-amylase.

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