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Research Article

Evaluation of herbal extracts for alpha glucosidase inhibition and cytochrome P450 3A4 modulation

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ABSTRACT

Diabetes mellitus (DM) is one of the chronic non-communicable diseases and has been rapidly increasing globally. Alpha glucosidase is an important enzyme that hydrolyzes polysaccharides into monosaccharides before being absorbed into the bloodstream. The alpha glucosidase inhibitors, such as acarbose are expensive and have several adverse effects. The use of herbal products to prevent or treatment of disease is becoming popular. There are many herbal products that can reduce blood sugar levels. However, taking herbal products with anti-DM medicines may cause herb-drug interactions that affect either the pharmacokinetics or pharmacodynamics of the drugs, leading to adverse effects or ineffective treatment. The aim of this study is to examine the effect of herbal extracts on alpha-glucosidase activity and the level of cytochrome P450 3A4 (CYP3A4) by using HepaRG as a model. Three herbs that reported blood glucose-lowering activity were selected including *Moringa oleifera*, *Momordica charantia*, and *Morus alba*. All herbs were extracted with 95% ethanol and tested for their *in vitro* alpha glucosidase inhibitory activity. The results showed that *M. alba* extract exhibits the highest activity (IC50= 83.75 µg/mL). Then, the extracts were evaluated for their cytotoxicity on the HepaRG cell line. All extracts showed no cytotoxicity at concentrations up to 250 µg/mL. The sub-cytotoxic concentration of each extract was selected to test for the effect on the CYP3A4 enzyme. The results showed that only *M. alba* extract elevated the level of CYP3A4, while the others slightly reduce the level of CYP3A4. However, this is a preliminary study that the major compounds of each extract must be isolated and evaluated in the future.

Keywords:
Herbal extracts, Alpha glucosidase inhibitor, Cytochrome P450 3A4

1. INTRODUCTION

Diabetes mellitus (DM) is one of the chronic non-communicable diseases (NCDs) and has been rapidly increasing around the world. Recently, this disease has been immediately rising in both developed and developing countries[1]. The International Diabetes Federation (IDF) has approximated about 537 million people living with DM, and 643 million adults are predicted to have diabetes by 2030[2]. In Thailand, the number of diabetes patients is 4.2 million people and is expected to be 5.2 million people by 2045[3]. Alpha glucosidase is one of the most important enzymes that can digest oligosaccharides, trisaccharides, and disaccharides into monosaccharides or glucose before being absorbed in the small intestine. This enzyme hydrolyzes alpha-1,4-glycosidic linkages at nonreducing carbohydrate terminals and releases alpha-glucose[4]. Inhibition of this enzyme slows down the absorption of carbohydrates from the small intestine, which helps to control blood sugar levels. There are 2 available anti-alpha glucosidase agents, acarbose and miglitol, that are approved for the treatment of DM. However, these drugs are expensive and have several side effects including constipation, stomach pain, diarrhea, easy bruising, unusual bleeding, and liver problems. Therefore, drugs with fewer side effects and cheap are still essential.

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Currently, the use of herbal products for the prevention or treatment of diseases is highly popular, resulting in a large number of herbal products available in the market. Several reports show that the use of herbs in primary care treatment is up to 80% in some developing countries[5-6]. Additionally, in some countries, herbs have been included in the National Essential Drug List, such as China, Japan, and India[7-8]. In Thailand, more than 70 herbal medicines have been included in the list since 2012[9]. The popularity of herbal medicine is due to its low cost, safety, high efficacy, and minimal side effects compared to modern medicines. However, for those who have been taking medication for NCDs such as DM for a long time, taking herbs with modern medicines may cause interactions that can affect the pharmacokinetics (absorption, distribution, metabolism, and excretion) and pharmacodynamics (efficacy and safety) of the drugs, leading to adverse effects or ineffective treatment. The interaction between drugs and herbs may occur because the herbs or their components can induce or inhibit enzymes involved in drug metabolism or interact with other substances in the body.

The enzymes that play an important role in drug metabolism are the cytochrome P450 (CYP450) enzymes, particularly the CYP450 group[10]. These enzymes are primarily involved in the phase I metabolism of drugs, including various herbs. There are many subgroups of CYP450, most of them are found in liver cells. The subgroups that are important in the metabolism of drugs are CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5, which metabolize approximately 90% of drugs[11]. The metabolism of drugs through the CYP3A enzyme is the most common. The activity of these enzymes can be altered by many factors, such as diseases, genetics, or environmental conditions[12]. Inducing the activity of these enzymes will cause drugs to be metabolized faster, resulting in a decrease in drug levels in the bloodstream faster than it should be, which may affect treatment outcomes. Inhibiting the activity of these enzymes will slow down the metabolism of drugs, resulting in high levels of drugs in the bloodstream for a longer period, which may lead to unwanted effects.

The compound in herbal products could affect CYP3A4 enzymes at multi-levels, including the expression of mRNA or protein, and enzyme activity[13-17]. There are many reports of changes in enzyme activity caused by herbal plants, such as *Vaccinium macrocarpon* or cranberries, which are used to treat urinary tract infections by inhibiting the activity of CYP3A in metabolizing the drug nifedipine, resulting in increased levels of nifedipine in the blood of experimental mice[18]. Similarly, *Echinacea purpurea* can also modulate the activity of CYP3A enzymes[18]. With the increasing popularity of herbal products, there is a chance that patients who are taking long-term medication may also take herbal supplements, which can lead to herb-drug interactions. Reports of herb-drug interactions in NCD patients who take herbal supplements alongside medication have been found, and some types of herbal supplements can significantly alter the levels of drugs in the bloodstream, which is clinically significant[19-21]. The aim of this study is to evaluate the effect of herbal extracts on the alpha glucosidase activity and level of CYP3A4 enzymes in the HepaRG cell line after treated with the extracts. The human hepatocyte-like cell line HepaRG was utilized as a validated *in vitro* model to study the effect of compounds on metabolic enzymes. This cell line is regarded as an alternative to primary *ex vivo* cultured human hepatocytes, particularly in research related to detoxification metabolism, including the activities of CYP3A4 enzymes for anticipating drug-drug interactions[22-24].

2. MATERIALS AND METHODS

2.1. Cell line, enzyme, and reagents

HepaRG cell line was cultured in Dulbecco's Modified Eagle's Medium mixture nutrient F-12 (DMEM/F12) with 10% fetal bovine serum (FBS) containing 100 IU/mL penicillin and 100 µg/mL streptomycin (Invitrogen, USA). The cells were maintained at 37°C with 5% CO_2 and subculture every 3-4 days. The alpha-glucosidase from *Saccharomyces cerevisiae* G5003, acarbose, and p-nitro-phenyl-α-D-glucopyranoside (p-NPG) were obtained from Sigma (St. Louis; USA)

2.2. Preparation of herb extracts

Three herbs, which reported glucose lowering activity, were purchased from V.P. Pharmacy Co., Ltd. All herbs were identified by Assistant Professor Bhanubong Bongcheewin, Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University, Thailand. The voucher specimens were deposited at the Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University, Thailand. Scientific name and part used of each herb was shown in Table 1. All herbs were ground and macerated with 95% ethanol at room temperature for 3 days with agitation 5 times per day. The extract was filter through Whatman filter paper No.1 and subjected to evaporate with rotary evaporator to remove the solvent. The ethanol extracts were dissolved in dimethyl sulfoxide and stored at -40°C until used.

2.3. Determination of total phenolic content

The total phenolic content was determined by the Folin Ciocalteu method described by Singleton and Rossi, et al. with some modifications[28]. All extracts were prepared at a concentration of 1 mg/ml in methanol. The extracts were incubated with 10% Folin-Ciocalteu reagent and 7.5% Na_2CO_3 solutions in the dark for 1 h at room

Table 1. List of herbs, part used, percentage yield of 95% ethanol extracts, and total phenolic content.

Scientific name	Part used	% Yield (W/W)	Total phenolic content (mg GAE/g dry extract)	References
Moringa oleifera lamk.	Leaves	15.51	28.31 ± 0.80	25
Morus alba L.	Leaves	4.36	35.76 ± 1.85	26
Momordica charantia L.	Fruit	5.76	16.17 ± 0.49	27

temperature. After that, the absorbance was measured at 765 nm. Gallic acid was diluted in methanol and used to generate a standard curve. The total phenolic content of the extracts was calculated and expressed as gallic acid equivalents (GAE) per gram of dry extract.

2.4. Alpha glucosidase inhibitory assay

Alpha glucosidase inhibitory assay was performed as previously described by Yamaki and Mori with some minor modifications[29]. The assay was performed in 96 well plates with a total volume of 200 µL. Herb extracts and acarbose at various concentrations were incubated with alpha glucosidase (0.1 U/mL) in 100 mM phosphate buffer pH 6.8. at 37°C for 30 minutes. The reaction was initiated by adding p-NPG and incubated at 37°C for 20 minutes before being terminated with 50 µL of 0.1 M Na_2CO_3. The release of p-nitrophenol from pNPG was measured by SPECTROstar Nano (BMG LABTECH; Germany) at 405 nm. The results were calculated and expressed as a percentage of inhibition by the following equation.

$$\% \text{ Inhibition} = [(\text{Acon-Asam})/\text{Acon})] \times 100$$

While: Acon is the absorbance of negative control (without test substances).

Asam is the absorbance in the presence of test substances

2.5. Cytotoxic assay

HepaRG cells were seeded in a 96-well plate at 1×10^4 cells/well and incubated at 37°C with 5% CO_2 for 24 h. After incubation, the medium was replaced with a two-fold serial dilution of plant extracts in a culture medium containing 3% FBS and further incubated for 72 h. The untreated culture was used as a negative control. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) solution was added to each well and further incubated for 3 h at 37°C with 5%CO_2. Cell viability was measured by spectrophotometer (Tecan, Switzerland) at 490 nm.

2.6. Effect of herbs extracts on cytochrome P450 3A4

HepaRG cells were seeded in a 6-well plate at 5×10^6 cells/well and incubated at 37°C with 5% CO_2 for 24 h. The treated cells were washed with PBS twice and culture media with several concentrations of herb extracts were added and further incubated for 24 h. After incubation, cells were washed with ice-cold PBS and harvested by cell scraper, and lysed by homogenizer. The cell lysates were kept at -40°C until used. The level of CYP3A4 was examined by Human Cytochrome P450 3As ELISA kit (Cat. No. 730400; MyBioSource, USA) according to the manufacturer's protocol. Rifampicin and ketoconazole were used as positive control for enzyme inducer and inhibitor, respectively.

3. RESULTS

3.1. Extraction of herb

After extraction with 95% ethanol, the percentage of yield of each extract is shown in Table 1. The *M. olefera* gave the highest yield with 15.51%, while the *M. alba* gave the lowest yield. *M. alba* exhibited the highest total phenolic content at 35.76 mg GAE/g, while *M. charantia* showed the lowest total phenolic content at 16.17 mg GAE/g (Table 1).

3.2. Alpha glucosidase inhibitory activity

The *in vitro* alpha glucosidase inhibitory effect of all plant extracts was evaluated compared to acarbose, which was used as a positive control (Figure 1). The *M. alba* extract showed the best activity among all extracts, but lower than acarbose. *M. charantia* had little activity at the highest concentration (500 µg/mL), but no activity at the lower concentration. While *M. oleifera* showed very low inhibitory activity at every concentration tested. In this study, acarbose gave the highest percentage of inhibition with the 50% inhibition concentration (IC50) at 35.15 µg/mL. *M. alba* extract also showed the high percentage of inhibition with IC50 at 83.75 µg/mL.

3.3. Cytotoxicity of herb extracts

All herb extracts were examined for their cytotoxicity on the HepaRG cell line. The concentration range between 0.125-1 mg/mL were used. The results showed that at low concentrations (0.125-0.25 mg/mL) two extracts, *M. oleifera*, and *M. alba* showed a percentage of cell viability more than 80% (Figure 2). The extract of *M. charantia* at a concentration of 0.25 mg/mL has 60% cell viability. From this result, the extract at concentrations 0.125 and 0.25 mg/mL were selected for the further experiment.

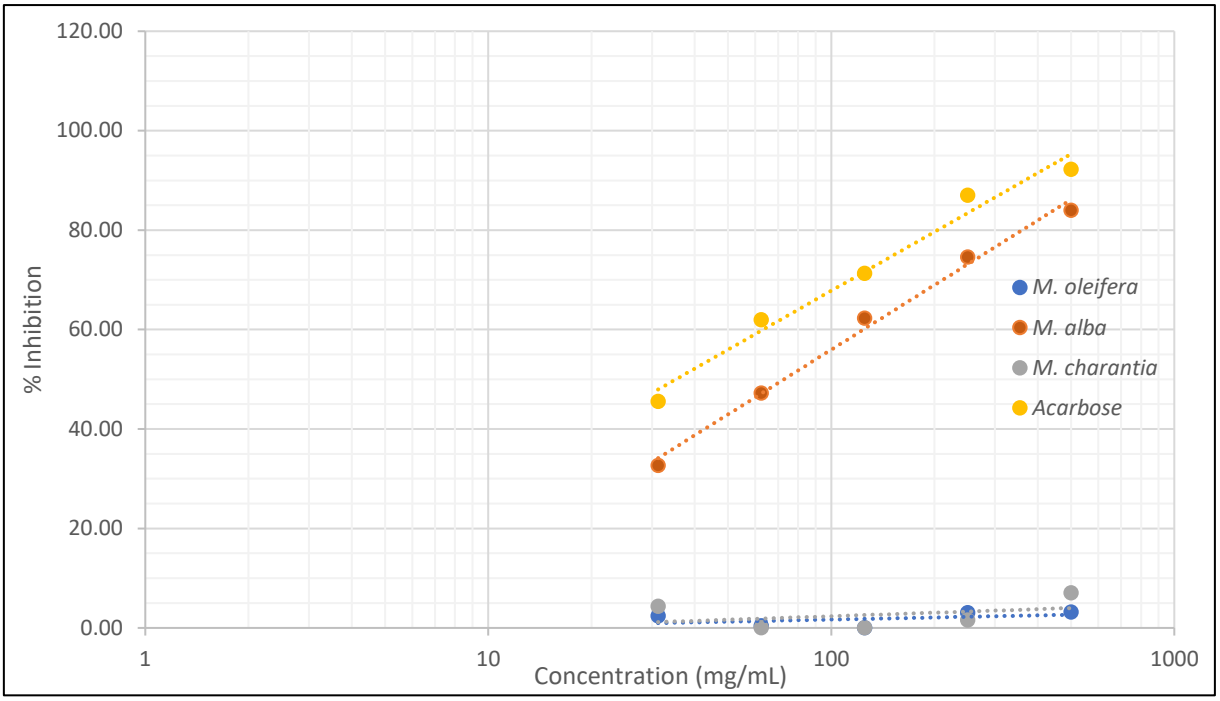

Figure 1. Alpha glucosidase inhibitory activity of plant extracts. All plant extracts at concentration 31.25-500 µg/mL were evaluated for their activity compared to acarbose. Results are expressed as relative percentage of inhibition to the negative control (without inhibitor).

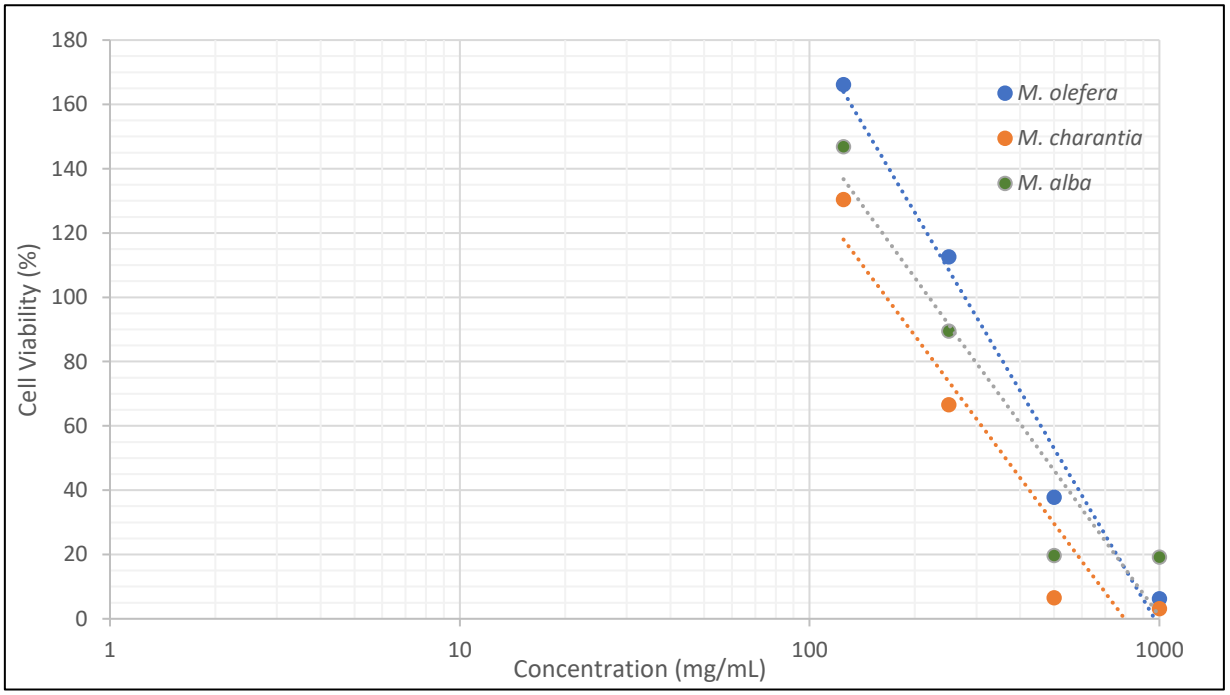

Figure 2. Cytotoxicity of three herb extracts on HepaRG cell line. HepaRG cell line was incubated with various concentration of herb extracts. The cell viability was determined by MTS assay. Data represent mean±SD from three independent experiments.

3.4. Effect of herb extracts on CYP450 3A4

All three extracts were subjected to examine their effects on the level of CYP3A4 enzyme using Human Cytochrome P450 3A4 ELISA kit. The results presented in the fold induction (ratio of CYP3A4 induced by herb extracts and 5%DMSO). It showed that all extracts did not significantly induce the expression of CYP3A4 (Figure 3). However, *M. alba* extracts at the concentration 125 µg/mL induced the expression of CYP3A4 almost 2-fold. But at higher concentration (250 µg/mL) the induction was lower than 125 µg/mL. While, rifampicin at the concentration of 20 µM, which was used as positive control for CYP3A4 inducer, induced the expression of CYP3A4 more than 2-fold. Rifampicin at 5 µM did not induce the expression of CYP3A4. The CYP450 inhibitor, ketoconazole reduced the expression of CYP3A4 at both concentrations tested (5 and 20 µM).

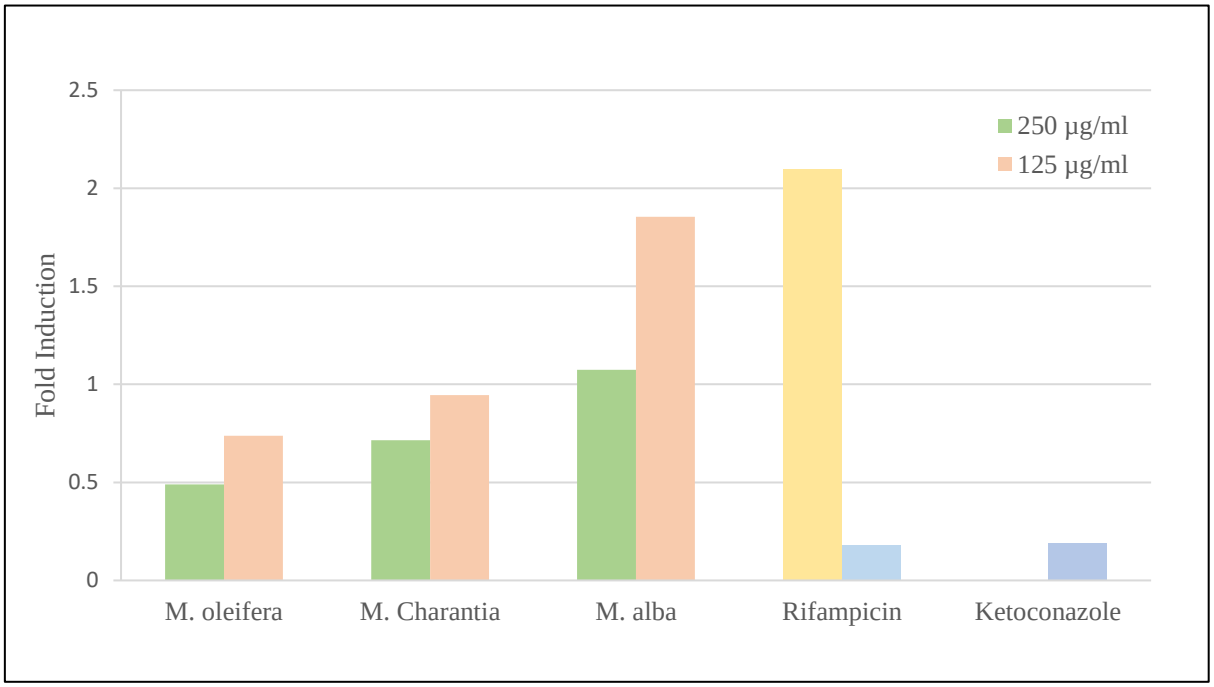

Figure 3. Effect of herb extracts on cytochrome P450 3A4. The extracts were incubated with HepaRG cell line for 24 h. The cell lysates were examined for the level of CYP450 3A4.

4. DISCUSSION

Alpha glucosidase is an important enzyme that converts polysaccharides into glucose. This enzyme is one major target of anti-diabetic agents. However, the available alpha glucosidase inhibitors, acarbose, and miglitol, are expensive and have several side effects. At present, the use of herbal products for the treatment of chronic non-communicable diseases (NCDs) including diabetes mellitus, is highly popular. There are a large number of herbal products being sold in the market, both in the form of individual herbs and herbal formulas. Most of the herbal products are composed of various ingredients from the respective herbal plants that have not yet been clearly tested for their properties but have biological activity. Therefore, the effects of herbal extracts on the alpha glucosidase activity were investigated. Three herb extracts that have reported glucose-lowering activity including *Moringa oleifera, Momordica charantia*, and *Morus alba* were examined for their alpha-glucosidase inhibitory activity. The results showed that at a concentration of 500 µg/mL, *M. alba* extract exhibited the highest percentage of inhibition (37.05%) followed by *M. charantia* (7.07%) and *M. olefera* (3.02%), respectively. Inhibition by *M. alba* extract occurred in a dose-dependent manner (Figure 1). However, the inhibitory activity is lower than acarbose at the same concentration, which has 66.87 %inhibition. This result is different from the previous studies by Hwang et al.[30]. This is probably due to the extraction method. Phenolic compound is largest group of phytochemicals that account for biological activities. Flavonoids are the largest group of naturally occurring phenolic compounds and possess many biological activities[31]. *M. alba* extract, which has the highest total phenolics content gave the best activity against the alpha-glucosidase enzyme. The result indicated that the active compound of *M. alba* is phenolics, corresponding with the other studies[30,32-34].

Usually, herbal medicine has to be taken continuously for a long time. Therefore, the risk of interactions between herbs and current medications is increased, especially in patients with NCDs that require continuous medication for a long time. The herb-drug interactions can occur similarly to drug-drug interactions, affecting both the pharmacokinetics and pharmacodynamics of the drugs. These interactions may not affect treatment outcomes or may provide synergistic or antagonistic effects. Therefore, the effects of herbal extracts on the cytochrome P450 3A4 (CYP3A4) enzyme, was investigated. The cytotoxicity of all extracts on the HepaRG cell line was evaluated by MTS assay. The result showed that all extracts had no cytotoxic effect at the lower concentration up to 250 µg/mL. Then, the sub-cytotoxic concentrations (125 and 250 µg/mL) of all extracts were selected for their effect on CYP3A4 by using a HepaRG cell line.

Although human primary hepatocytes are recommended for studying the metabolism of drugs or herbs, limitations in finding these types of cells make it impossible to use them in this study. The use of animal testing or primary hepatocytes separated from animals has been reported to provide different results from humans[35]. In this study, HepaRG cell lines were used as a model for studying herb-drug interactions. This cell line is separated from the liver of cancer patients, and hepatocellular carcinoma, which have histological characteristics and can express liver-specific functions[22,36]. These cells also

expressed enzymes involved in drug metabolism processes, such as cytochrome, at a level similar to normal liver cells and can respond to stimulation[37-38]. Therefore, this cell line is suitable for studying the metabolism of drugs and substances that affect the liver[39]. When treated these cell lines with lower concentrations of three herb extracts, only *M. alba* extract can elevate the level of CYP3A4 enzymes. While the other extracts had no effect or slightly reduced the level of CYP3A4 enzymes. The effect of *M. alba* extract on CYP3A4 is controversial. Sheng and colleagues report the aqueous and ethanol leaf extract of *M. alba* stimulated rat hepatic CYP3A4 activity *in vivo*[40]. Udomsak et al. reported the *M. alba* fruit powder had no effect on CYP2C19, CYP2D6, and CYP3A4 mRNA levels in HepG2 cells[41]. On the other hand, Kar et al. reported the CYP3A4 inhibitory activity of hydroalcoholic extract of *M. alba*[42]. Our study demonstrated that the ethanol extract of *M. alba* increased the level of CYP3A4 at the lower concentration (125 µg/mL), while almost no effect at higher concentration (250 µg/mL). These studies have many differences such as the cell line, extraction method, and detection methods.

The effect of *M. charantia* and *M. oleifera* extract on the CYP3A4 is corresponded with previous studies. Fasinu et al., reported the methanol extract of *M. charantia* strongly inhibits CYP3A4 using Fluorescent cytochrome P450 (CYP) assays[43]. The CYP3A4 inhibitory activity of *M. oleifera* aqueous and ethanol extract was reported by Taesotikul[44]. Not only CYP3A4, *M. oleifera* also inhibited CYP1A2, CYP2D6, and CYP2E1, in a dose-dependent manner. Our results showed that both extracts decrease the level of CYP3A4 in a dose-dependent manner.

5. CONCLUSION

The effect of herbal extracts on alpha glucosidase and CYP3A4 was reported in this study. The *M. alba* extract exhibited alpha glucosidase inhibitory activity in a concentration-dependent manner. While, the extract of *M. charantia*, and *M. oleifera* had a slight effect on this enzyme. The extract of *M. charantia*, and *M. oleifera* decreased the level of CYP3A4 in HepaRG treated cells. While the extract of *M. alba* slightly increased the level of CYP3A4. From these results, *M. alba* extract could be used as glucose-lowering agents. However, the CYP3A4 is increased, therefore the herb-drug interaction has to be considered when taking the *M. alba*. This is a preliminary study. The major compounds and standardization of the extract need to be performed in the future.

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Conflict of interest
None to declare.

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Author contribution
KC: Performed experiments; Analyzed and interpreted the data.
NV: Performed experiments; Analyzed and interpreted the data.
KS: Contributed regents and materials.
TK: Perform experiments; Analyzed and interpreted the data; Discussion of the results.
KT: Conceptualization; Conceived and design the experiments; Funding acquisition; Contribute reagents and materials; Analyzed and interpreted the data; Discussion of the results; Manuscript preparation.

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