Extraction of *Swietenia macrophylla* Seed Oil using Supercritical Carbon Dioxide Technique and Its Antioxidant, Antidiabetic and Toxicity Properties

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Swietenia macrophylla has been used in treatments for diabetes, asthma, premenstrual syndrome and migraine traditionally. The seeds of *S. macrophylla* have been proven to possess antioxidant, antimicrobial, antifungal and hyperglycemic activity. Supercritical carbon dioxide (SC-CO$_2$) extraction is a recent extraction process technology that has been developed to provide better extraction yield, safer and easier process. This study aimed to extract *S. macrophylla* seed oil by using SC-CO$_2$ and investigate the antioxidant, antidiabetic activity and toxicity content of the oil extracted. Antioxidant analysis was performed by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and *in vitro* antidiabetic analysis was done by using the inhibitory of α-amylase and α-glucosidase to validate antidiabetic activity of *S. macrophylla* seed oil. Toxicity test of *S. macrophylla* seed extract was performed using MTT assay on human skin fibroblast cell (HSF 1184). The result showed that *S. macrophylla* seed extracts at studied concentrations of 10 to 0.0001 mg/mL are non-toxic with cell viability above 80 %. α-amylase and α -glucosidase inhibition (%) of the *S. macrophylla* seed extracts was 100.0 ± 0.3 % and 4.1 ± 2.0 %. *S. macrophylla* seed extract was proven to have promising antidiabetic activity to act as an alternative to the commercial antidiabetic drugs.

1. Introduction

Swietenia macrophylla comes from Meliaceae family is a large, deciduous, and economically important timber tree native to the West Indies which is commonly known as mahogani. Especially seed, it has potential of antioxidant, antimicrobial, antifungal and antidiabetic properties (Bhurat et al., 2011). Diabetes is a chronic condition when glucose remains circulating in the blood stream due to the lacking and ineffectiveness of the insulin in the body. The function of insulin is to transport glucose from blood stream into cell to be used as energy (McLaughlin et al., 2015). Oral medicine was acknowledged as the major treatment for diabetes. Commercial antidiabetic drugs such as sulfonylureas and rosiglitazone have been reported to have side effects such as increased risks of cardiovascular disease (CVD) and stroke (Castilla-guerra et al., 2018). Therefore, the search in natural sources for antidiabetic drugs should be taken seriously to provide an alternative to the patients. A novel extraction needs to be studied to provide the best quality of *S. macrophylla* oil. Soxhlet extraction (SE) has been used for centuries as primary extraction method. The drawback of this method has led to environmental effect by using large volume of solvent that leads to wasting and high cost treatment to dispose (de Castro and Priego-Capote, 2010). Meanwhile, the physical properties of carbon dioxide at supercritical state include low critical temperature and critical pressure made SC-CO$_2$ environmentally friendly and green solvent (Sapkale et al., 2010). The selection of extraction method is crucial

to ensure the extract in high purity, rich with components and non-toxic (Lang and Wai, 2001). Thus, the objective of this study was to investigate the oil yield of *S. macrophylla* seed oil extracted using SC-CO$_2$ and its antioxidant, antidiabetic activity and toxicity content.

2. Materials and methods

2.1 Extraction of *S. macrophylla* by using Supercritical Carbon Dioxide (SC-CO$_2$)

Extraction of *S. macrophylla* seed oil was conducted according to method from Hartati et al. (2018) with modification. Approximately 3 g of *S. macrophylla* seed were placed into 10 mL extraction vessel. During the process, the vessel was installed in the oven to maintain the temperature at 60 °C. A flow rate of CO$_2$ was 2 mL/min, particle size of 0.75 mm and pressure of 30 MPa was set prior extraction process. As the desired temperature achieved, carbon dioxide was pumped into the vessel. The extraction yield was collected by a vial and the solvent was evaporated using an oven. The extracted yield was weighed and the concentration of yield was calculated based on cumulative mass of extract. After the extraction has completed, the oil extract was stored at 4 °C refrigerator for further analysis. Soxhlet extraction (SE) (60 mL/g solvent to solid ratio for 6 h) with ethanol 95 % as the solvent was used as conventional method to be compared with SC-CO$_2$ extraction for all the analysis results.

2.2 Antioxidant analysis by using 2,2-Diphenyl-1-picrylhydrazyl assay

Antioxidant activity of *S. macrophylla* seed oil extract was determined by using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging activity as referred to Jusoh et al. (2018) with modification. 40 µL of *S. macrophylla* solution (5 mg/mL) were mixed with 220 µL DPPH solution. The reaction mixture was incubated under dark at room temperature for 1 h. The absorbance was recorded at 517 nm against the ethanol as blank using EPOCH Microplate Spectrophotometer (BioTek Instrument Inc., USA). The percentage of inhibition or scavenging of free radicals was calculated by using Eq(1).

$$\text{DPPH Scavenging Activity (\%)} = \frac{\text{absorbance of control-absorbance of sample}}{\text{absorbance of control}} \times 100 \tag{1}$$

2.3 *In vitro* models of anti-diabetic effects

2.3.1 α-glucosidase enzyme inhibition assay

α-glucosidase inhibition activity was determined according to methods by Jemain et al. (2011) with modification. Sample (10 µL) with various concentrations (0-100 µg/mL), 1-Deoxynojirimycin (positive control) and DMSO (negative control) were transferred to 96-well plate (Greiner Bio-one) by using micropipettes (Eppendorf, ResearchPlus). About 20 µL of α-glucosidase, 40 µL of phosphate buffer saline (PBS) and 20 µL of distilled water were pipetted into each well and incubated at 37 °C for 10 min (pre-incubation) by using thermos-shaker incubator (Allsheng, MSC-100). 10 µL of pNPG was added into all wells in the dark and the first absorbance at 405 nm ($A^{0 \text{ min}}$) was immediately measured by using spectrophotometer (BMG, FLUOstar Omega). The plate was incubated at 37 °C for 30 min and the absorbance at 405 nm ($A^{30 \text{ min}}$) was measured. Inhibition of α-glucosidase (%) was determined by using Eq(2).

$$\text{Inhibition (\%)} = \frac{\left(A^{30 \text{ min}} - A^{0 \text{ min}}\right)_{\text{Control}} - \left(A^{30 \text{ min}} - A^{0 \text{ min}}\right)_{\text{sample}}}{\left(A^{30 \text{ min}} - A^{0 \text{ min}}\right)_{\text{control}}} \times 100 \tag{2}$$

2.3.2 α-amylase enzyme inhibition assay

α-amylase inhibition activity was determined by methods from Jemain et al. (2011) with modification. Sample (10 µL) with 1 µg/mL, acarbose (positive control) and DMSO (negative control) were transferred to test and blank well in 96-well plate (Greiner Bio-one) by using micropipettes (Eppendorf, ResearchPlus). α-amylase solution (50 µL) and distilled water (40 µL) were added into each well (90 µL of distilled water was added in blank wells) and the plate was incubated at 25 °C for 5 min (pre-incubation) by using thermos-shaker incubator (Allsheng, MSC-100). Then, 100 µL of starch solution was added into every well and incubated at 25 °C for 7 min. 100 µL of DNS color solution was added into all wells in the dark and was incubated at 85 °C for 30 min. The plate was let cool to the room temperature before measuring the absorbance at 540 nm. Inhibition of α-amylase (%) was calculated by using Eq(3).

$$\text{Inhibition (\%)} = \frac{(A_{540}\text{Control-Blank}) - (A_{540}\text{Sample-Blank})}{(A_{540}\text{Control-Blank})} \times 100 \tag{3}$$

2.4 *In vitro* toxicology test on Human Skin Fibroblast 1184

This section described the methodology used for *in vitro* toxicology test which include cell recovery, cell subculture, cell cryopreservation, cell viability and toxicology test using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay following previous method (Freshney, 2005).

2.4.1 Cell recovery

Cryogenic vial containing human skin fibroblast 1184 (HSF 1184) cells was removed from the liquid nitrogen storage. The cells and media which was modified eagle medium (MEM) was thawed bath (Edelstahl) at 37 °C for 1 to 5 min or until its completely thawed. The thawed cells were cultured in MEM using t-flask. The cells were maintained at 37 °C in a humidifier atmosphere of 5 % CO_2 (Branstead, USA) until it achieved 90 to 95 % confluent monolayer.

2.4.2 Cell subculture

The subculture procedures were done immediately after the cells achieved 90 to 95 % confluent monolayer. The media were aspirated and the cells in the t-flask were washed with 5 mL phosphate buffer saline (PBS) to remove any cellular debris or serum which could retard the action of trypsin. The PBS was removed and 3 mL of trypsin-EDTA was added. After that, the flask was incubated for 5 min at 37 °C. MEM was added and the cell mixture was placed into centrifuge tube and then centrifuged (Hettich, Rotina 420R) for 10 min at 3300 rpm. The supernatants were discarded and the pellets were diluted with MEM to the volume required. The cells were incubated at 37 °C in 5 % CO_2 air atmosphere.

2.4.3 Cell cryopreservation

Cells at log phase were used for freezing. Cells were trysinized and centrifuged. The freezing medium containing 90 % fetal bovine serum (FBS) and 10 % DMSO were slowly added. The cells were aliquots into 1.2 mL pre-labelled cryogenic vials and were placed into the NalgeNunc freezing container (for adaptation process) with temperature of -70 °C for 24 h. The vials were transferred to liquid nitrogen storage.

2.4.4 Cell viability

The viable cells in cell suspension were counted using dye exclusion test (Freshney, 2005) using Trypan blue exclusion test and Neubauer improved bright line haemocytometer (Fortuna®, Germany). The number of unstained (live cells) and stained (dead cells) were counted under a light microscope (Zeiss, axiovert 40CFL, Germany). The concentration of viable cells was calculated using the Eq(4).

$$C = N_A \text{ x } D \text{ x } 10^4 \qquad (4)$$

Where C is concentration of cells (cells/mL), N_A is average number of cells counted and D is dilution factor.

2.4.5 MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay

The effect of *S. macrophylla* seed extracts on cell viability was assessed by using the MTT assay according to (Bakorova et al., 2011). A monolayer of fibroblast was trypsinized and neutralized. After that, the cell suspension was centrifuged for 10 min at 3300 rpm. The supernatant of the cells was discarded and the pellet was collected and resuspended with MEM and the cells were counted. The test samples were prepared by dissolving the extracts in MEM to yield the final concentrations of the crude extracts at 10, 1, 0.1, 0.01, 0.001, 0.0001 mg/mL. The absorbance was measured at 540 nm and reference wavelength at 630 nm using ELISA plate reader (BIOTEK, ELx-808). The percentage of cell viability was calculated by using the following Eq(5).

$$\text{Cell viability (\%)} = \frac{\text{Mean absorbance of experimental cells}}{\text{Mean absorbance of control cells}} \text{ x } 100 \qquad (5)$$

3. Results and discussion

3.1 Seed oil yield extract

SC-CO_2 and SE produced oil yield of 28.88 ± 0.21 % and 28.52 ± 0.16 %. From this result, considering the extraction time, SC-CO_2 extraction was able to extract higher oil yield in shorter duration of 2 h while SE required 6 h of extraction time. SC-CO_2 extraction used lower volume of solvent (which was carbon dioxide) compared to Soxhlet extraction that used 80 percent ethanol concentration. Only small amount in volume of carbon dioxide (240 mL) was used compared to ethanol (480 mL) in SE. Previous study by Hamid et al. (2018) has reported the optimum yield of alpha mangosteen from *Garcinia Mangostana* extracted using SC-CO_2 method. This study supported that SC-CO_2 extraction is safe, non-toxic, non-flammable, inexpensive and no remaining solvent in extracted oil.

3.2 Antioxidant analysis of *S. macrophylla* seed oil

The result of DPPH radical scavenging activity (%) on *S. macrophylla* seed oil showed that the oil extract produced from SC-CO_2 has higher antioxidant level by inhibiting 45.95 ± 0.3 % the radical scavenging activity compared to oil extract from SE with 34.68 ± 0.2 %. Previous research on *S. mahagoni* by Sahgal et al. (2009) has reported the oil extracted by using methanol extract showed a DPPH-scavenging activity of 23.29 % at 1 mg/mL concentration, this value was less than that of ascorbic acid. Another study by Alqahtani et al. (2018) has reported that antioxidant activity of *Lepidium sativum* seed oil was 22.15 ± 0.2 % inhibition at the same seed oil concentration, 5 mg/mL. Meanwhile, the seed oil extract from *Vaccinium myrtillus L.* inhibited 50 % of radical activity (IC_{50}) at concentration of 5.5 mg/mL (Gustinelli et al., 2018). These results showed that antioxidant activity produced by *S. macrophylla* seed oil extract from SC-CO_2 was higher.

3.3 The inhibitory of α-amylase and α-glucosidase enzyme activity

The inhibitory of α-amylase and α-glucosidase enzyme activity was examined to determine the ability of *S. macrophylla* to serve as an alternative for antidiabetic agent. Table 1 shows the α-amylase and α-glucosidase inhibitory activity of *S. macrophylla* with acarbose and 1-Deoxynojirimycin as positive control for α-amylase and α-glucosidase enzyme respectively. The percentage of α-amylase inhibition showed that *S. macrophylla* has higher inhibition at 100.0 ± 0.3 % and 98.7 ± 3.3 for SC-CO_2 and SE respectively, which is comparable to the positive control at 100.0 ± 0.8 %. It showed low percentage of inhibition of α-glucosidase for *S. macrophylla* seed oil extracted by using SC-CO_2 with the value of 4.1 ± 2.0 % while 82.4 ± 2.0 for SE. This is due to the different solvent used in these methods. The ethanol used in SE is a polar solvent which can extract better quality of phenolic compound than CO_2 which is non-polar solvent used in SC-CO_2. Ty´skiewicz et al. (2018) has reported that extraction using hexane (non-polar solvent) produced low phenolic compound due to the low solubility of phenolic compound in non-polar solvent. In this case, phenolic content is important to inhibit high α-glucosidase activity. Previous research by Subhadip et al. (2013) has reported that the extraction of *S. macrophylla* by using petroleum ether maceration produced 64.84 ± 0.52 % α-amylase inhibition while 4.37 ± 0.19 % α-glucosidase inhibition was reported by using *S. macrophylla* extract from aqueous maceration extraction (Wresdiyati et al., 2015). Other extraction method was investigated on α-glucosidase inhibition including ethanol maceration, aqueous reflux and ethanol reflux which produced inhibition of 18.65 ± 3.86 %, 5.31 ± 0.00 % and 14.31 ± 3.52 %. Based on these results, *S. macrophylla* oil extract by using SC-CO_2 was more promising against α-amylase compared to α-glucosidase. The simultaneous inhibition of both enzymes would cause unusual bacterial fermentation in the colon due to the undigested sugars which also leads to abdominal distention, flatulence and diarrhea (Figueiredo-González et al., 2016).

Table 1: Inhibitory activity of α-amylase and α-glucosidase from S. macrophylla seeds extract

Sample	Concentration	α-Amylase Inhibition (%)	α-Glucosidase Inhibition (%)
S. macrophylla (SE)	100 µg/ml	98.7 ± 3.3	82.4 ± 2.0
S. macrophylla (SC-CO_2)	100 µg/ml	100.0 ± 0.3	4.1 ± 2.0
Acarbose (control)	100 µg/ml	100.0 ± 0.8	-
1-Deoxynojirimycin (control)	1.0 mg/ml	-	91.4 ± 1.2

3.4 *In vitro* toxicity analysis of *S. macrophylla* seed oil

The *in vitro* study was performed to investigate the toxicology level of *S. macrophylla* seed oil on human skin fibroblast (HSF 1184). The toxicology test result in Figure 1 shows that the cell viability (%) of *S. macrophylla* seed oil extract was above the control with the highest reading of 146.1 % followed by 141.1 %, 122.5 %, and 109.6 % for concentration of 1 mg/mL, 0.0001 mg/mL, 0.1 mg/mL and 0.01 mg/mL, respectively. There are only two concentrations of *S. macrophylla* seed oil extract that produce cell viability (%) below the control which were 84.1 % and 95.4 % for concentration of 10 mg/mL and 0.001 mg/mL.

The extract is not toxic as the cell viability reaches above control. This is because, human skin cell manages to keep growth even exposed to the extract. If the calculation on cell viability reaches below control, it indicates that there are dead cells as it exposed to the extract. Therefore, from Figure 1 it shows that the extract is non-toxic at concentration level of 1, 0.1 and 0.01 mg/mL. At concentration 0.001 mg/mL, the cell viability slightly below control meanwhile, at concentration of 0.0001 mg/mL the cell viability reaches above control. Which means, at concentration of 0.0001 mg/mL it is non-toxic. *S. macrophylla* seed oil has enhanced cell growth as the cell viability increase compared to control. According to López-García et al. (2014), it stated that ISO 109\93-5 that the cell viability above 80 % is non-toxic while below 40 % cell viability it is strongly

toxic. Thus, it shows that oil extract at concentration of 10 mg/mL for SE and SC-CO_2 extraction method was non-toxic.

S. macrophylla seed oil was extracted by using ethyl acetate extraction showed no lethal indications of morbidity and mortality in intense oral toxicity test. The seed oil extract was also tested by using MTT assay to study the cell viability %. The seed oil was reported to have a significant neuroprotective effect because it increased the cell viability and exhibited protection to the neuronal cells against tert-Butyl hydroperoxide (TBHP) induced oxidative stress (Sayyad et al., 2017). A study from Soderberg et al. (1996) of toxicology test on tee tree oil (TTO) indicates that TTO was non-toxic below the concentration of 0.1 mg/mL but showed notable increase of toxicity level for concentration above 0.1 mg/mL. Cytotoxicity studies on human skin fibroblast are important as endpoint to evaluate the irritant potential of substances on human skin. Further studies on biomarker should be considered to show specific irritation effect of the substances on human skin (Welss et al., 2004).
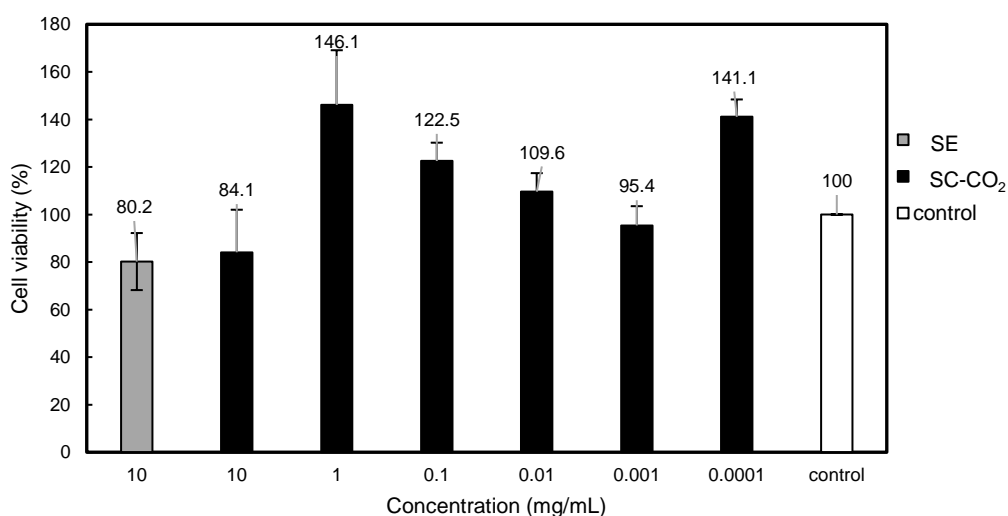

Figure 1. Cell viability on different concentrations of S. macrophylla seed extract

4. Conclusion

From this study, the extract from *S. macrophylla* seed was proven to have promising antidiabetic activity to act as an alternative to the commercial antidiabetic drugs. The percentage of α-amylase inhibition of *S. macrophylla* seed extract was comparable to the positive standard, acarbose, at 100.3 % and 100.8 % respectively. It also has been proven to be non-toxic by analysis on cell viability toxicity MTT assay. With the cell viability above 80 % of extract concentrations (10, 1, 0.1, 0.01, 0.001 and 0.0001 mg/mL), it showed that *S. macrophylla* seed oil extract using SC-CO_2 was non-toxic. MTT assay is the starting procedure for further in vivo toxicity study on acute and sub-acute toxicity at high dosage up to 5000 mg/mL extract. Further study on potential of *S. macrophylla* seed oil extracted using SC-CO_2 can be used on wound healing and antidiabetic applications.

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