ORIGINAL ARTICLE

In Vitro Toxicity Assessment of *Swietenia macrophylla* King Extracts Using a Cell-Based Assay

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ABSTRACT

Introduction: *Swietenia macrophylla* King (family Meliaceae) is a timber species with medicinal value. The seed is used as traditional medicine, however there are reports of liver injury, suspected to be associated with *S. macrophylla* seed consumption. The aim of this study is to assess the toxicity of *S. macrophylla* seed prepared in different solvent of extraction using a cell-based assay. **Methods:** Two methods were employed in the preparation of the seed extracts. In the first method, the dried seed was extracted with ethanol, methanol, or 50% ethanol. In the second method, the dried seed was extracted sequentially with solvents following ascending polarity. HepG2 cells were used as an in vitro liver model. The cells were treated with various concentrations of seed extracts and the cell viability was assessed by the 3, (4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. **Results:** *Swietenia macrophylla* seed was cytotoxic towards HepG2 cells with the chloroform extract being the most cytotoxic, followed by the ethyl acetate and hexane extracts. The ethanol, methanol, and 50% ethanol extracts were also cytotoxic but to a lesser extent than the three non-polar extracts. The water extract does not negatively affect cell viability. **Conclusion:** The findings reveal the cytotoxic effect of *S. macrophylla* seed is dependent on solvent extraction.

Keywords: *Swietenia macrophylla* King; Cytotoxicity; *in vitro* toxicity, MTT assay

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INTRODUCTION

Swietenia macrophylla King is a forest timber species from the family Meliaceae. It is commonly known as big-leaf mahogany. Its other vernacular names include sky fruit (English), tunjuk langit (Malay), xiang tian guo (Chinese), and tettankotai (Tamil) (1). *Swietenia macrophylla* is well known for its wood quality. The fruits are valued for its medicinal properties although almost all parts of the plant are used in traditional medicine (2).

A number of scientific studies have been carried out to investigate the medicinal effects of *S. macrophylla*, most notably the seed (3–18). The aqueous seed extract has been shown to reduce fasting blood glucose levels of diabetic rats (3). Petroleum ether extract of the seed also reduced blood glucose levels of normal rats after glucose loading in intraperitoneal glucose tolerance test (IPGTT) and promoted glucose utilisation by the muscle tissue (4). Compounds isolated from the seed, namely 6-O-acetylswietenolide, diacetyl swietenolide, and swietenine exhibited peroxisome proliferator-actived receptor gamma (PPARγ) ligand activity, stimulated adipocyte differentiation, increased adipogenic markers (adiponectin, adipsin, PPARγ, GLUT4) expression, and induced cellular glucose uptake by muscle cells via translocation of GLUT4 glucose tranporter to the plasma membrane, which are all beneficial for the treatment of diabetes (5). Anti-hypertensive effect of *S. macrophylla* seed (ethanol extract) was demonstrated by vasorelaxation activity in isolated rat aortas and in vivo. The vasorelaxation mechanism was mostly attributed to channel-linked receptor pathways (blockage of voltage-operated calcium channels, potassium channels opener, and inositol triphosphate (IP3) receptor inhibitor), with smaller contributions from the endothelium-

independent β2-adrenergic and the nitric oxide/soluble guanylyl cyclase 3′,5′-guanosine monophosphate (NO/sGC/cGMP) signaling pathways (6–7). The fruit ethanolic extract demonstrated pain relief (8) while a formulated oilment containing 10% w/w of ethanolic seed extract showed wound healing potential (9). Antidiarrhoeal activity of the seed extract was also demonstrated in vivo (10). Other medicinal effects of *S. macrophylla* that have been investigated include anti-inflammation (11–12), antioxidant (3), antibacterial and antifungal (13), antimalarial (14), antiviral (15), anti-cancer (16–17), and neuroprotective (18).

With such a vast number of scientific findings, it is not surprising that *S. macrophylla* is a much sought after medicinal plant (19). However, in 2018, the Health Sciences Authority of Singapore (HSA) reported several cases of liver injury suspected to be associated with the consumption of *S. macrophylla* seed and advises consumers to exercise caution when considering using *S. macrophylla* (20). From 2015 till October 2018, 7 cases of liver injury suspected with the use of *S. macrophylla* were recorded in Singapore. The severity of the liver injuries varied, from transaminitis to jaundice and liver failure, while the pattern of liver toxicity was either hepatocellular or hepatocellular and cholestatic. The onset of liver injury was between 30–45 days after consumption of *S. macrophylla*, though in one patient it was 6 months. Patients either consumed *S. macrophylla* seed in its raw form (10 seeds a month to 18 seeds daily) or as formulation in capsules. The age of patients was between 40–70 years old. Most of the patients had other underlying medical conditions such as diabetes, hypertension, hyperlipidaemia, fatty liver and they were consuming the seed to control diabetes, hypertension, and/or for general well-being. Most patients took other medications concomitantly. Other than liver injury, some patients were also present with acute kidney injury, polythralgia, and mesenteric panniculitis. No fatalities were reported and the patients recovered after cessation of the use of *S. macrophylla* (20–22). After the first report in Singapore, more cases of liver injury related to *S. macrophylla* were published in China (23–26, 28), Hong Kong (27), and India (29).

Only limited toxicity studies of *S. macrophylla* were found in the literature. Sprague Dawley rats fed with a single dose of ground seed suspended in olive oil (2 g/kg of body weight (b.w.)) did not show any sign of toxicity during the 14-day study period based on body weight, food and water intake, vital organs (lung, liver, spleen, heart, kidney, testis, stomach) weight and histology, haematological and biochemical parameters, and changes in general behavioural. The study concluded that *S. macrophylla* up to 2 g/kg b.w. is not toxic, which is equivalent to human dose of 325 mg/kg b.w (30). In another study, a single dose administration of ethyl acetate fraction of the seed extract up to 2 g/kg b.w. on Balb/c mice did not produce any lethal signs of morbidity and mortality when monitored up to 14 days based on the haematological, biochemical, and histopathological analyses (18). A single oral administration of ethanolic and aqueous extracts of *S. macrophylla* fruit up to 2 g/kg b.w. in albino mice, with 14 days observation also did not produce any mortality (8). There is no record of chronic toxicity study found in the literature.

Phytochemical investigations have reported that the seed contains alkaloids, flavonoids, saponins, phenols, steroids, glycosides, resins, tannins, oils, and limonoids, from the class of triterpenoids (15, 31–37). Compounds that have been isolated from the seed/fruit of *S. macrophylla* include swietenine, swietenolide, swietenine acetate, 8,30 epoxy swietenine acetate, 6-deoxyswietenine, 2-hydroxyswietenine, swietenolide diacetate, swietenolide tiglate, 6-O-acetylswietenolide, 3,6-di-O,O-acetylswietenolide, 3-O-tigloylswietenolide, swietemahonins E–G, 6-O-acetylswietamahonin G, swietemahonolide, swielimonoids A–F, swieteliacate A–E, seco-mahoganin, augustineolide, febrigugina, andirobin, proceranolide, khayasin T, humilin B, 3β,14-dihydroxymexicanolide, 3β,6-dihydroxydihydrocarapin, 7-deacetoxy-7-oxogedunin, 7-deacetoxy-7α-hydroxygedunin, methyl angolensate, methyl 3β-tigloloxy-2,6-dihydroxy-1-oxo-meliac-8(30)-enate, methyl 3β-tigloloxy-2-dihydroxy-1-oxo-meliac-8(30)-enate, methyl 3β-tigloloxy-2-dihydroxy-8α-30α-epoxy-1-oxo-meliacate, methyl 3β-acetoxy-2,6-dihydroxy-8α-30α-epoxy-1-oxo-meliacate, and methyl 3β-isobutyryloxy-2,6-dihydroxy-8α-30α-epoxy-1-oxo-meliacate (15, 32–37). There are diverse constituents present in the seed. The current study aimed to investigate the possible toxicity of *S. macrophylla* seed. Different polarity of extraction solvents were used to extract all the non-polar and polar constituents from *S. macrophylla* seed for toxicity evaluation on HepG2 cells.

MATERIALS AND METHODS

Plant material and extraction

Swietenia macrophylla ripe fruits were collected from Jalan Kaki Bukit Wang Kelian, Perlis (N06.65111°, E100.21152°). The plant material was identified and voucher specimen (TL(SM)-PLS-2019/08) deposited at the Natural Products Division, Forest Research Institute Malaysia (FRIM) Herbarium.

The seeds were peeled and dried in an oven (~ 45–50°C) till the moisture content was below 10%. The dried seeds were then ground into a fine powder and extracted with either ethanol, methanol, or 50% ethanol at the ratio of 1:10. Each of the extract was filtered, and concentrated under reduced pressure (38). For sequential extraction, the powdered seed

was sequentially extracted with solvents following the ascending polarity; hexane, chloroform, ethyl acetate, methanol, and water. The organic solvents were removed by rotary evaporator while the water extract was freeze-dried. All extracts were stored at -20°C.

Cell culture and cell-based experimentation

HepG2 cells were used as model hepatocytes. The cells (JCRB 1054) were purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank. They were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, 1% penicillin/streptomycin, and maintained at 37°C and 5% CO_2/95% air.

Exponentially growing cells were seeded in a 96-well plate at the density of 1 x 10^4 cells/well, 100 µL/well of complete culture medium (unless otherwise stated). After an overnight incubation, the culture medium was removed, cells washed with phosphate-buffered saline (PBS), and treated with the extracts at various concentrations (0–1000 µg/mL) in serum-free medium for 24 h. After the treatment period, medium containing the extracts was removed, cells washed with PBS and cell viability was assessed by modified MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay (39).

Data analysis

Percentage of cell viability was calculated using the following formula (40):

$$\text{Cell viability (\%)} = \frac{(\text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{blank}})}{(\text{Absorbance}_{\text{non-treated control}} - \text{Absorbance}_{\text{blank}})} \times 100\%$$

Where:
Sample: Cells treated with the extract.
Non-treated control: Cells treated with the vehicle solvent used to dissolve the extract.
Blank: Without cell but contained the extract or vehicle solvent.

The percentage of cell viability was plotted against concentration of the extract in log to show the dose-response curve. The 50% inhibitory concentration (IC_{50}) was determined from the dose-response curve by non-linear regression. All data are expressed as mean ± standard error of mean (S.E.M.) in at least 3 independent experiments (unless otherwise stated). Comparison of means was performed by either one-way or two-way analysis of variance (ANOVA), followed by a Bonferroni post-hoc test (GraphPad Prism 8.4.3, GraphPad Software). Statistical significance was accepted at $p < 0.05$ level.

RESULTS

Fig. 1 shows the fruits and seeds of *S. macrophylla*. Extraction of the seed with ethanol produced an extract that was liquid-like while the methanol extract was gluey and the 50% ethanol extract was dry. The yield of the ethanol extract (expressed as dry weight of the extract over dry weight of raw material) was 27% (w/w), 55% (w/w) for the methanol extract, and 18% (w/w) for the 50% ethanol extract. Sequential extraction of the seed produced the following yield, hexane extract: 47% (w/w), chloroform: 14% (w/w), ethyl acetate: 2% (w/w), methanol: 13% (w/w), and water: 3% (w/w). The physical texture of the extracts changed from oily/liquid-like to solid as the extraction solvent increased in polarity.

Fig. 1 : *Swietenia macrophylla* fruit and seeds. (A) Ripe fruit with the seeds inside, (B) the unpeeled seeds, and (C) the peeled seed.

Toxicity assessment of the ethanol, methanol, and 50% ethanol extracts are shown in Fig. 2. The extracts were dissolved in dimethyl sulphoxide (DMSO) before further diluting in serum-free culture medium. The final concentration of DMSO in each cell culture well was maintained at 0.5% (v/v). Treatment with the ethanol, methanol, or 50% ethanol extracts for 24 h at 1000 µg/mL decreased viability approximately 47%, 61%, and 45%, respectively (Fig. 2A). Based on the IC50 values (Table I), the methanol extracts (IC_{50}: 484 ± 160) was more toxic than the ethanol and 50% ethanol extracts (IC_{50}: > 1000). Increasing the treatment time to 48 and 72 h did not further decrease cell viability, the viability was still above 50% (Fig. 2B & 2C). However, based on the IC50 values, it seems that the methanol extract is less toxic after 48- and 72-h of treatment (IC_{50}: > 1000) compared to 24-h (IC50: 484 ± 91). This is because 2% serum was added to the 48- and 72-h culture medium to prevent cell death due to serum starvation.

For the toxicity assessment of the extracts produced from sequential extraction, the extracts were initially dissolved in the respective extraction solvents before further diluting in serum-free culture medium. The

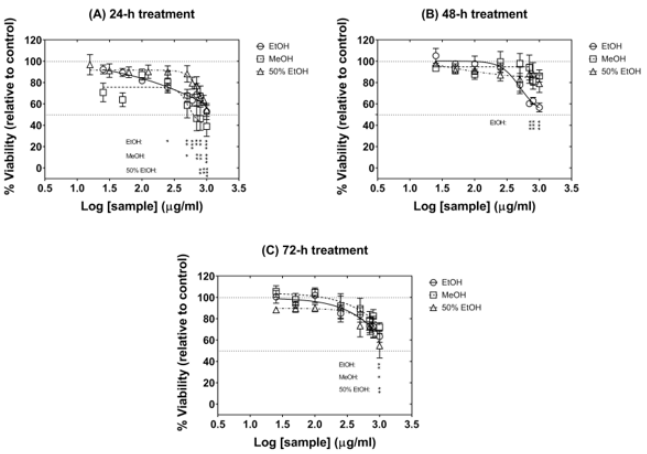

Fig. 2 : Dose-response curves of HepG2 cells treated with ethanol (circle), methanol (square), or 50% ethanol (triangle) extracts for (A) 24-, (B) 48-, and (C) 72-h. Cells were seeded in a 6-well plate (6 x 10^5 cells/well, 2 mL/well of complete culture medium). Treatment ranged from 0–1000 µg/mL. Extracts were dissolved in DMSO before further diluting in serum-free medium (except for the 48- and 72-h treatments where 2% of serum was added to prevent serum starvation). The final concentration of DMSO was maintained at 0.5%. Data are expressed as mean ± S.E.M (n ≥ 3 independent experiments). Each dose-response curve was statistically analysed using one-way ANOVA followed by Bonferroni post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001 compared to untreated).

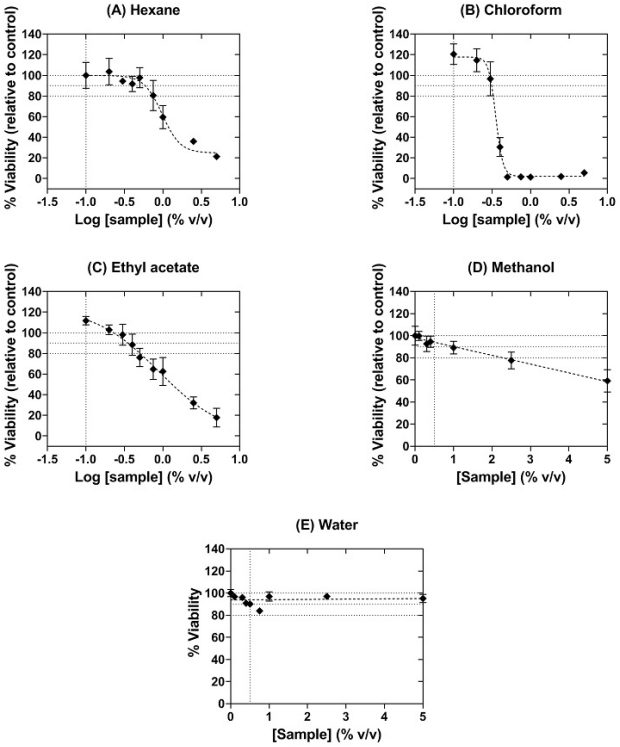

Fig. 3 : Determination of solvents non-toxic concentrations to be used to dissolve the extracts. (A) Hexane, (B) chloroform, (C) ethyl acetate, (D) methanol, and (E) water. Cells were seeded in a 96-well plate (2 x 10^4 cells/well, 100 µL/well of complete culture medium). After an overnight incubation, cells were treated with the solvents for 24 h. Cell viability was assessed using the MTT assay. The dose-response curve for each solvent was plotted and non-linear regression was used to generate the best fit curves for hexane, chloroform, and ethyl acetate while linear regression was used for methanol and water. Data are expressed as mean ± S.E.M (n ≥ 3 independent experiments, except for D and E which are n = 3 technical replicates).

final concentration of hexane, chloroform, and ethyl acetate in each well was maintained at 0.1% (v/v) while the concentration of methanol and water was maintained at 0.5% (v/v). These particular concentrations of solvent were chosen because they did not decrease cell viability, the viability was still above 90% (Fig. 3). The effect of the extracts on HepG2 cells after 24 h treatment is shown in Fig. 4. The hexane extract appears as if it did not affect cell viability (Fig. 4A). The dose-response curves of the chloroform, ethyl acetate, and methanol extracts were nearly similar (Fig. 4B–4D), the highest concentration exhibited about 50% cell viability. The water extract, on the other hand, showed a rise in percentage of cell viability, based on increased metabolic activity, although not statistically significant (Fig. 4E). When the same experiment was repeated by dissolving all the extracts in DMSO, the hexane extract decreased cell viability (Fig. 4A). The chloroform and ethyl acetate extracts dissolved in DMSO also showed more toxic effect (Fig. 4B–4C) and with lower IC_{50} values compared to the initial experiment (Table II). The dose-response curves for the methanol and water extracts were almost similar whether they were dissolved in DMSO or the

Table I : IC_{50} values of *S. macrophylla* seed extracts

Extracts	IC_{50} (µg/mL)		
	24-h treatment	**48-h treatment**	**72-h treatment**
Ethanol	> 1000 [a]	> 1000 [a]	> 1000 [a]
Methanol	484 ± 160 [b]	> 1000 [a]	> 1000 [a]
50% Ethanol	> 1000 [a]	> 1000 [a]	> 1000 [a]

Data are expressed as mean ± S.E.M (n ≥ 3 independent experiments) and statistically analysed using two-way ANOVA followed by Bonferroni post-hoc test. Similar letters within treatment time are not significantly different between type of extracts (*p < 0.05).

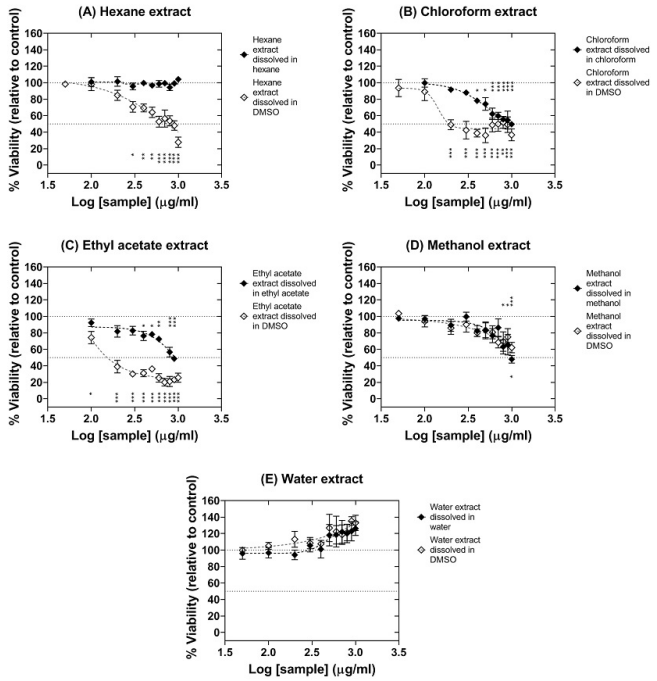

Fig. 4 : Dose-response curves of HepG2 cells treated with (A) hexane, (B) chloroform, (C) ethyl acetate, (D) methanol, and (E) water extracts for 24 h. Cells were seeded in a 96-well plate (2 x 10^4 cells/well, 100 µL/well of complete culture medium). Treatment ranged from 0–1000 µg/mL. The extracts were dissolved in either the extraction solvents (closed symbols) or DMSO (open symbols) before further diluting in serum-free medium. The final solvent concentration for hexane, chloroform, and methanol was maintained at 0.1%, and 0.5% for methanol, water, and DMSO. Data are expressed as mean ± S.E.M (n > 3 independent experiments). Each dose-response curve was statistically analysed using one-way ANOVA followed by Bonferroni post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001 compared to untreated).

extraction solvent (Fig. 4D & 4E). Based on the IC_{50} values, *S. macrophylla* seed extracted with chloroform, ethyl acetate, and hexane were more toxic than the methanol and water (Table II).

DISCUSSION

The incidence of drug-induced liver injury (DILI) is reported to be from 2.7–19 per 100,000 inhabitants a year (41). In the Global North, the most common causes of DILI are due to the use of antibiotics, anticonvulsants, and psychotropic drugs whereas in Asia, DILI is predominantly due to herbal- and dietary-supplements (21, 42).

Our interest in *S. macrophylla* stems from reports of its usage potentially linked to idiosyncratic toxicity (20–29). Diagnosis of idiosyncratic DILI is difficult as it lacks gold standard for confirmation. It relies heavily on the availability of well-documented data. The relatively rare cases of hepatotoxicity due to *S. macrophylla* are a diagnostic challenge. Although *S. macrophylla* is perceived as a traditional medicine, especially in the Southeast Asia, there is no clinical data on the efficacy or adverse reactions associated with it.

Non-polar solvents such as hexane, chloroform, and ethyl acetate are generally used to extract non-polar constituents whereas polar solvents such as water, methanol, and ethanol are used to extract the polar constituents (43). Solvents dissolve constituents that have similar or closely related polarity index and hence selection of solvent and its polarity mediates the quantity and quality of an extract. (44). Sequential extraction was applied to extract both the non-polar and polar constituents from the same plant materials. The physical texture and amount of yield of the hexane extract suggests that *S. macrophylla* seeds contained a substantial amount of oil. Hexane is a widely used solvent for oil extraction and the seed of *S. macrophylla* has being studied for its oil production (45–47).

The choice of solvent used to dissolve the extract during experimentation is also important to avoid inaccurate interpretation, especially when it involves non-water soluble extract in a water-based assay system, such as cell culture. For example, when hexane was used to dissolve the hexane extract, results showed that the

Table II : IC_{50} values of *S. macrophylla* seed extracts from sequential extraction after 24-h treatment

Extracts from sequential extractions	Median inhibitory concentration, IC_{50} (µg/ml)	
	Dissolved in extraction solvents	Dissolved in DMSO
Hexane	> 1000 [a]	615 ± 96 [a]
Chloroform	614 ± 171 [b]	146 ± 26 [b]
Ethyl acetate	892 ± 41 [a,b,c]	308 ± 153 [a,b]
Methanol	977 ± 20 [a,c,d]	> 1000 [c]
Water	> 1000 [a,d]	> 1000 [c]

Data are expressed as mean ± S.E.M (n ≥ 3 independent experiments) and analysed using two-way ANOVA followed by Bonferroni post-hoc test. Similar letters within the dissolving solvent are not significantly different between type of extract (*p < 0.05).

extract did not affect cell viability, which was most likely due to the immiscibility of hexane in aqueous solution (48). Chloroform and ethyl acetate also have low aqueous miscibility but both are more polar than hexane (48). In contrast, DMSO, a solvent widely used to dissolve both polar and non-polar compounds, is miscible in water (48). Hence when DMSO was used, the cytotoxic effect of the non-polar extracts on the cells was more prominent, although what was seen may not be the full extent of the cytotoxicity of the non-polar extracts. Employment of micelle preparation for the non-polar extracts could aid their solubility in a water-based assay system.

In this study, we found that *S. macrophylla* seed was cytotoxic towards HepG2 cells with the chloroform extract being the most cytotoxic, followed by the ethyl acetate and hexane extracts. The ethanol, methanol, and 50% ethanol extracts were also cytotoxic but to a lesser extent than the three non-polar extracts. The water extract does not affect viability negatively but may contain certain constituents that increased viability based on MTT assay. These findings imply that cytotoxic constituents of *S. macrophylla* seed are present in the non-polar extracts.

Some of the compounds found in *S. macrophylla* seed, e.g. swietenolide, swietenine acetate, 7-deacetoxy-7α-hydroxygedunin, and methyl anglolensate have been shown to have hepatotoxic effect towards HuH-7 (human liver cancer) cell line, with recorded IC_{50} of 68 ± 1, 83 ± 3, 105 ± 4, and 116 ± 5 μM, respectively (15). Swieteliacate B, another compound found in *S. macrophylla* was cytotoxic against SW480 (human colon cancer) and HL-60 (leukemia) cell lines with IC_{50} of 30.6 and 32.9 μM (37). Meanwhile swietemahonin G was toxic towards Artemia salina (brine shrimp) with a 50% lethal concentration of 220.1 ppm in a 24-h bioassay (32).

CONCLUSION

Swietenia macrophylla seed contains cytotoxic constituent(s) that can be differentiated by solvent extraction, though we have not identified the toxic compound(s). Our next objective is to investigate how *S. macrophylla* seed extracts from sequential extraction affect metabolic pathways such as glycolysis and the tricarboxylic acid (TCA) cycle pathways and impacts intra-cellular ATP supply. We could thereby link genetic polymorphisms in these processes to idiosyncratic toxicity of *S. macrophylla*. We also hope that the toxic compound(s) in *S. macrophylla* seed could be identified in the future.

ACKNOWLEDGMENT

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