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Labisia pumila extracts standardization and cytotoxicity analysis against uterine fibroid cells

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Labisia pumila or locally known as Kacip Fatimah is widely used as traditional medicine in Malaysia. The present study aimed to evaluate physicochemical parameters, analyze metabolites content and determine cytotoxic activity on uterine leiomyoma cells (SK-UT-1) for the standardization of *L. pumila* for potential anti-uterine fibroid botanical drugs. Physicochemical analysis was evaluated according to Malaysia Herbal Monograph. Metabolites content was analyzed using published protocols. Cytotoxic activity was determined by (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) MTT assay. Data on physicochemical will served as standard for both extracts. Total flavonoids (8.489 ±0.15) and total phenolics (5.305 ±0.21) were highest in 50% ethanol extract compared to water extract. Total proteins were highest in water extracts (1.100 ±0.16) compared to 50% ethanol extract. Cytotoxic activity showed that 50% ethanol extract exhibited more activity than water extract with IC_{50} of 22 µg/mL and 28 µg/mL respectively. Results showed a possible anti-uterine fibroid effect, which may have interesting applications in the treatment of uterine fibroid and improve better health.

Keywords: Standardization, Labisia pumila, SK-UT-1, cytotoxic, uterine fibroid

INTRODUCTION

Serious consideration of herbal medicine as an alternative or complementary form of medical treatment requires rigorous examination, which involves fundamental issues as source materials, standardization and evidences of efficacy. For Source material, each batch of herbs must be properly identified, first by morphology, and then by microscopic examination. The source material also must adhere to the limit of contamination such as foreign material, microbial, total ash content, extractive values, heavy metals and other extraneous matter (Cappaso et al. 2000). Standardized herbal product must have a known content of active or marker compounds. Due to the complexity of chemical component in herbal, fingerprint analysis is an acceptable strategy for the assessment of herbal medicine. Chemical fingerprint obtained must be simple, rapid and environmental friendly, which can be an attractive option for regulatory laboratories, testing laboratories and laboratories supporting manufacturing activities. Evidence of efficacy is vital. However, because the composition of the product varies between manufacturers, evidence of efficacy should be considered to be extract specific.

L. pumila or popularly known as Kacip Fatimah is a well-known herb among Malaysian which has received much attention since more than a decade ago. There are three known varieties of *L. pumila,* which are *L. pumila* var *pumila*, *L. pumila* var *alata* and *L. pumila* var

lanceolata (Stone, 1988). Till now, it is consume by the locals to maintain healthy female reproductive system, treating menstrual irregularities, as well as enhancing sexual function. Plants from the Myrsinaceae family are known to exhibit a number of interesting pharmacological properties such as anti-inflammatory, antiviral, anti-tumor (Kobayashi and De Meji'a, 2005), cytotoxic (Chang et al., 2009), antibacterial (Karimi et al., 2011), anti-inflammatory (Ibrahim et al., 1996; Rasadah et al. 2001) and antioxidant (Mohamad et al., 2009) properties. Uterine fibroids, or leiomyomas, are the most common type of solid tumor presenting in women of reproductive age, with an incidence of 20-25% (Buttram and Reiter, 1981). Although they may be asymptomatic, fibroids often cause a variety of health problems for women, including menorrhagia, chronic pelvic pain, pressure symptom on adjacent pelvic organ, recurrent miscarriage, obstructed labour, postpartum haemorrhage and sepsis (Stewart, 2001). As a result of this problem, uterine fibroid is the most common indication for hysterectomy in Australia and USA (Farquhar and Steiner, 2002). As a result, women are increasingly searching for alternative treatments rather than hysterectomy for fibroid disease.

The reports on anti-cancer activity of *L. pumila* are scarce especially on leiomyomas. Therefore, these study aims to assess the potential cytotoxic activity of various extracts of *L. pumila* against uterine fibroid leiomyomas and also determine its pharmacognostic parameter as well as their chemical content.

MATERIALS AND METHODS

Preparation of Standardized Plant Extracts

L. pumila var. *alata* whole plants (leaf, stem and root were purchased from HERBAGUS Sdn Bhd. The plant was pulverized into fine powder using electric grinder (Retsch, Germany). Two hundred grams of powdered plant was extracted with 50% ethanol (LPEW) by soxhlet extraction and 100% water (LPW) by maceration technique.

Physicochemical analysis of *L. pumila*

Physico-chemical parameters such as moisture content, total ash content, acid insoluble and water soluble ash content and ethanol extractable matter were determined according to the methods described in guidelines of Malaysian Herbal Monograph Vol 1 (Ismail et al., 1999)

Analysis of metabolites

Total Proteins Content

Total proteins content was determined using method as described by (Bradford, 1976). Twenty µL of extracts were added with 1 mL Bradford dye reagent, 1X and vortex for 30 seconds. The solution was incubated for 5 min at room temperature and the absorbance was read at 595 nm. Bovine serum albumin was used as standard in the range of 12.5-250 µg/mL. The total proteins content was expressed in mg/g of protein. Analysis was performed in triplicate and data was expressed as average percentage ± SD.

Total Flavonoids Content

Total flavonoids content was determined by aluminum chloride colorimetric assay as described by Kale et al. (2010). One hundred µL of extract were mixed with 300 µL of methanol and then 20 µL of 10% aluminium chloride were added, followed by addition of 20 µL of 1M potassium acetate and 560 µL of distilled water. Mixture was incubated for 30 minutes at room temperature. Absorbance was read at 415 nm using spectrophotometer. The standard calibration curve was plotted using different concentration of quercetin solutions at range of 3.125-100 µg/mL. Total flavonoids content was expressed in mg/g of quercetin equivalent. All the determination was perform in triplicate and data was expressed as average percentage ± SD.

Total Phenolics Content

Colorimetric assay of total phenolics content was determined using method as described by Ghasemzadeh et al., (2010). Ten mg of extract were prepared in 1 mL distilled water and 100 µL of Folin-Ciocalteau reagent were added. Mixture was incubated for 5 min at room temperature followed by the addition of 200 µL of 20% sodium carbonate. The mixtures were incubated further for 1h in the dark. The absorbance was measured by spectrophotometer at 650 nm. The standard calibration curve was plotted using different concentration of gallic acid solutions at range of 0.20-200 µg/mL. Total phenolic content was expressed in mg/g of gallic acid equivalent. Determination of total phenolic content was performed in triplicate and data was expressed as average percentage ± SD.

Total Phospholipid Content

Phospholipid content in extracts was determined in according to Stewart (1980). One

mg/mL extracts were prepared in chloroform, and 0.5 mL of thiocyanate reagent was added, vortex for 1 min and centrifuged at 27000 rpm for 5 min. Black layer was then removed and the absorbance was read at 488 nm using letcithin as a standard. Letcithin was prepared in the range of 0.1-100 µg/mL and calibration curve was constructed. Means of three replicates were calculated and total phospholipids content was expressed as average percentage ± SD.

Total Polysaccharides Content

Total polysaccharides content was measured acording to the method described by Laurentin and Edwards (2003). One mg/mL of extracts was prepared in distilled water. From this, 0.04 mL of solution was taken into test tube, vortex and mixed gently. The mixture was allowed to stand for 15 min at 4°C. Subsequently, anthrone sulphuric acid reagent was prepared by dissolving 0.1 g of anthrone in 50 mL cold sulphuric acid. One hundred µL anthrone sulphuric acid reagent were added, mixed, vortex and heated for 3 min at 92°C then allowed to cold at room temperature for 5 min. Mixture was then heated at 45°C for 15 min. The absorbance was read at 630 nm. Glucose was used as the standards in the range of 10-100 µL. Total polysaccharides content was expressed in mg/g of glucose equivalent. Determination of total polysaccharides was performed in triplicate and data was expressed as average percentage ± SD.

Total glycosaponins content

Gravimetric method as described by Hussain et al. (2008) was used for determination of glycosaponins in the extract. One gram of extract was refluxed with 50 mL methanol three times separately for 30 min and filtered. The filtrate of the 3 cycles was concentrated using rotary evaporator to approximately 10 mL. Fifty mL acetone were added drop wise to the extract and the saponins were precipitated at the bottom of weighed beaker. The precipitate was collected by centrifuge at 4°C at 8000 rpm for 10 min and dried in an oven at 100°C to a constant weight. Means of three experiments were collected and total saponins estimation was expressed in term of percentage average ± SD.

High Performance Thin Layer Chromatography (HPTLC)

Qualitative HPTLC was performed using Camag HPTLC system and WinCATS software (Camag, Switzerland) on HPTLC aluminum plates (10 x 10 cm) silica gel 60 F_{254} (Merck, USA). Both samples of *L. pumila* extracts (20 mg/mL) and the reference markers Gallic acid, Caffeic acid, Chlorogenic acid, rutin, myricitin, and ellagic acid (0.5 mg/ml) were applied to TLC plates as 5 mm bands with 6 mm space from bottom edge, 12 mm from the left margin at a constant rate of 50 nL/second using a nitrogen aspirator. The plates were then developed in a glass chamber using toluence : ethyl acetate : formic acid (6:4:1) (Merk, USA) as a mobile phase. The mobile phase was saturated with the solvent mixture for 10 min before developing to a distance of 8 cm. The developed plate was scanned using Camag TLC Scanner 3 under 330 nm wavelength and visualized under 254 and 366 nm wavelength.

Cytotoxicity effect of *L. pumila* on uterine leomyoma cancer cell SK-UT-1

MTT assay was performed on the uterine leiomyoma cells (SK-UT-1) using method as described by Halim et al., (2017). Cells were seeded in a 96-well plate (1×105 cells/well) using complete growth medium. After 24h of incubation, cells were treated with *L. pumila* extracts at various concentrations (0-100 µg/mL) in triplicate and serially diluted. The cells were then incubated for 72 h and then 20 µL of MTT reagent (5.0 mg/mL) was added into each well and further incubated for four 4 h. Next, media was discarded and 100 µL of 100% dimethyl sulfoxide (DMSO) solution were added and incubated for 15 min. The solubilized formazan product was measured using microplate reader at 570 nm, and 630 nm as the reference (Infinite M200, Tecan, Switzerland). The IC_{50} value was determined from a graph plotted of percentage of cell viability against the concentration of the extract. Doxorubicin was used as the positive control.

RESULTS AND DISCUSSION

Physicochemical analysis

Physicochemical analysis has been performed to evaluate the safety and quality of the raw material. Summary of the physicochemical analysis was presented in Table 1. Pharmacognosical parameters have been used as indicators for the safety and quality of the raw materials which included foreign matter, ash content, acid insoluble ash, water soluble ash, hot method, cold method, 50% ethanol, 100% ethanol and loss on drying. According to Farquhar and Steiner (2002), standardization of herbal drugs is series of protocols, which assure the quality,

efficiency and safety of plant drugs. The values obtained in the study will serve as a reference for quality standard measures for standardization of *L. pumila* whole plant in the dried form. To the best of our knowledge, the results from this study can be used to set up the maximum limit for *L. pumila* whole plant powder since the standardization data for the whole plant powdered raw material was never been reported.

Natural products have widely been marketed as herbal supplemets and drugs for treating diseases. Scientific research is crucial in order to provide additional evidence of its safety and efficacy. Therefore, physico-chemical and phytochemical standardization is important for the useful application of the plant parts in modern medicine (Ismail et al., 1999).

Table 1; Physicochemical analysis of *L. pumila* powdered raw materials. Results displayed as means ± SD (n=3).

Parameter	Mean value ± SD (% w/w)
Foreign matter	1.01 ±0.02
Total ash (%)	9.583 ±0.17
Acid insoluble ash (%)	1.724 ±0.12
Water soluble ash (%)	9.513 ±0.10
Loss on drying	1.772 ±0.16
Water extractive value (cold method)	9.349 ±0.13
Water extractive value (hot method)	12.577 ±0.17
50% Ethanol extractive value (cold method) 50% Ethanol extractive value (Hot method)	8.734 ±0.08 10.500 ±0.22

Analysis of metabolites in *L. pumila*

Both extracts exhibited varying degree of metabolites (Table 2). Total phenolics and flavonoids were found as the major phytochemicals in this plant. LPEW showed the highest phenolics and flavonoids contents compared to LPW. Total proteins were found at highest amount in LPW extracts compared to LPEW. Total phospholipids, glycosaponins, and polysaccharides were found at lesser amount in both extracts. These findings suggested that the plant could be a potential source of bioactive compound having therapeutic potentials.

Table 2; Analysis of metabolites in *L. pumila* extracts. Results displayed as means ± SD (n=3).

Test of Metabolites (% w/w)	LPEW (mg/g)	LPW (mg/g)
Total proteins	0.452 ±0.13	1.100 ±0.16
Total phenolics	5.305 ±0.21	1.077 ±0.18
Total flavonoids	8.489 ±0.15	0.114 ±0.19
Total phospholipids	0.009± 0.004	0.003 ±0.001
Total polysaccharides	0.013 ±0.003	0.023 ±0.20
Total glycosaponin	0.313 ±0.26	0.066 ±0.21

LPEW=*Labisia pumila* ethanol water extract,
LPW=*Labisia pumila* water extract

Plant contain mixture of phytochemical constituents including primary metabolites such as carbohydrates, protein, lignins, nucleic acid and lipids; secondary metabolites such as terpenoids, alkaloids, glycosides, flavonoids and tannins (Ibrahim, 2006). Primary metabolites usually involved in normal development and reproduction of the cell. Secondary metabolites are synthesized during secondary metabolism of plants. They play a role in defense mechanism of the plant against predators. Secondary metabolites are the basic source for the establishment of several pharmaceutical industries since they have enormous medicinal properties (Lingarao and Savithramma, 2011). The phytochemical screening and quantitative estimation of the percentage crude yields of chemical constituents showed that the *L. pumila* were rich in flavonoids and phenolic as reported by Chua et al., (2011) and Karimi et al., (2011) Both phenolic acids and flavonoids are believed to be responsible for the wide spectrum of pharmacological activities attributed to the herb (Zhang and Ye, 2008). The presence of phenolic compounds might also contribute to the anticarcinogenic activity of *L. pumila* (Pandey and Madhuri, 2009). These primary and secondary metabolites have made natural products as a popular and effective source of remedy to treat various diseases and disorder.

In this study, water and ethanol water extract of *L. pumila* whole plant (roots and leaves) were used for the analysis. Traditionally, solvents used include water, ethanol, a mixture of water and ethanol, glycerine, acetic acid and vegetable oils (Mills, 1991). Ismail et al., (1999) stated that water extracts of *L. pumila* have long been used for gynecological issues in traditional medicine. The preparation of *L. pumila* in water extract is needed in order for this extract to be consumed by subject

i*n-vivo* studies (Norhanisah et al. 2013). Water extract also used to evaluate toxicity level in this plant in term of genotoxicity or teratogenic and reproductive toxicity (Zaizuhana et al. 2006; Ezumi et al, 2005; Singh et al., 2009). A study done by Murkish et al. (2012) indicated that the standardized water extracts of *L. pumila* provides better effect on collagen synthesis in human skin fibroblast (HSF1184) cells. Ethanol has been known as a good solvent for polyphenol extraction and is safe for human consumption (Dai and Mumper, 2010). According to Grodowska and Parczewski (2010), ethanol is less toxic in acute or short-term studies, shows negative in genotoxicity studies and normally accepted to be use in pharmaceuticals. Study done by Azimahtol et al., (2012) showed that ethanol extract of *L. pumila* and its active fraction showed antiproliferative and proapoptotic effect in HM3KO cells while having reduced effects on non-malignant cells.

Determination of Marker Compounds by HPTLC analysis

High performance thin layer chromatography (HPTLC) serves as quality assessment tool, which helps in identification of variation in chemical composition plants. Qualitative HPTLC analysis was conducted to screen the compounds that present in *L. pumila* extracts. Two reference markers were used in this study include gallic acid and caffeic acid. Among bioactive compounds naturally occurring phenolic flavonoids have gained a particular interest because of their broad pharmacological activity. Figure 1 shows the HPTLC profiles of *L. pumila* extracts with the reference markers visualized under 254 nm and 366 nm. Reference markers was observed at Rf value of 0.28 for GA and 0.48 for CA. Under 254 nm, GA and CA were well spotted and appear as dark band. In LPEW, GA was present but GA was not clearly visible. However, in LPW, both markers were not visible. Under 366 nm, CA was present as a vibrant blue band, GA was not visible. Both marker were not visible in both extract. Reference marker was prepared at varied concentration and are displayed in the densitogram profile. Results showed that gallic acid was present in both extracts whereas other markers were not clearly visible. This result indicated that gallic acid is the major compound compared to others and can be applied as chemical markers for the profiling and standardization of *L. pumila* extracts.

Phytochemical screening is useful tools in the detection of the bioactive compounds and drug discovery and development. In this study, gallic acid and caffeic acid were used as reference markers. Among the phenolic and flavonoid acid compounds profiled, gallic acid was found as the prominent marker in *L. pumila*.

Cytotoxicity effect of *L. pumila* on uterine leiomyoma cancer cell SK-UT-1

Cancer is a group of diseases characterized by cells that grow out of control. In most cases, they formed masses of cells, or tumors that infiltrate, crowd out, and destroy normal tissue. According to the US NCI plant screening program, a crude extract is generally considered to have *in vitro* cytotoxic activity if the IC_{50} value was less than 30 µg/mL (Boik, 2001; Karimi et al. 2013). The result for anticancer activity of *L. pumila* extracts was presented in Figure 2. LPEW appeared to be more cytotoxic compared to LPW with IC_{50} 22 and 28 µg/mL respectively. Doxorubicin was used as a positive control in this study.

Both extracts showed possible anticancer activity. In relation with the marker compounds determined in previous section, gallic acid was found present in both extract. Faried et al., (2007) indicated that gallic acid isolated from Phaleria macrocarpa induced cancer cell death in various cancer cells such as breast cancer (MCF-7), gastric cancer (MKN-28) and colon cancer (HT-29, colon 201 and colon 26). Gallic acid also showed a pro-apoptotic activity mediated, at least in part, by the production of H_2O_2 in leukemia cancer cells (Fabiani et al., 2011).

Numerous researches have been carried out to test this plant on various cancer cell lines. Saxena et al., (2012) revealed that ethanol extract from *L. pumila* was more cytotoxic against HM3KO cells. Karimi et al. (2013) found that all leaf and root extracts of three varieties showed anticancer activity at various concentrations when tested on MCF-7 and MDA-MB-231. Isolation from leaves of *L. pumila* found that, 1-O-methyl-6-acetoxy-5-(pentadec-10Z-enyl) resorcinol1 and 1-O-methyl- 6-acetoxy-5-pentadecylresorcinol exhibited strongest cytotoxic activity against the PC3, HCT116 and MCF-7 cell lines (IC_{50} values ≤ 10 µM) (Al-mekhlafi et al., 2012). This showed that, *L. pumila* has a possible anticancer property.

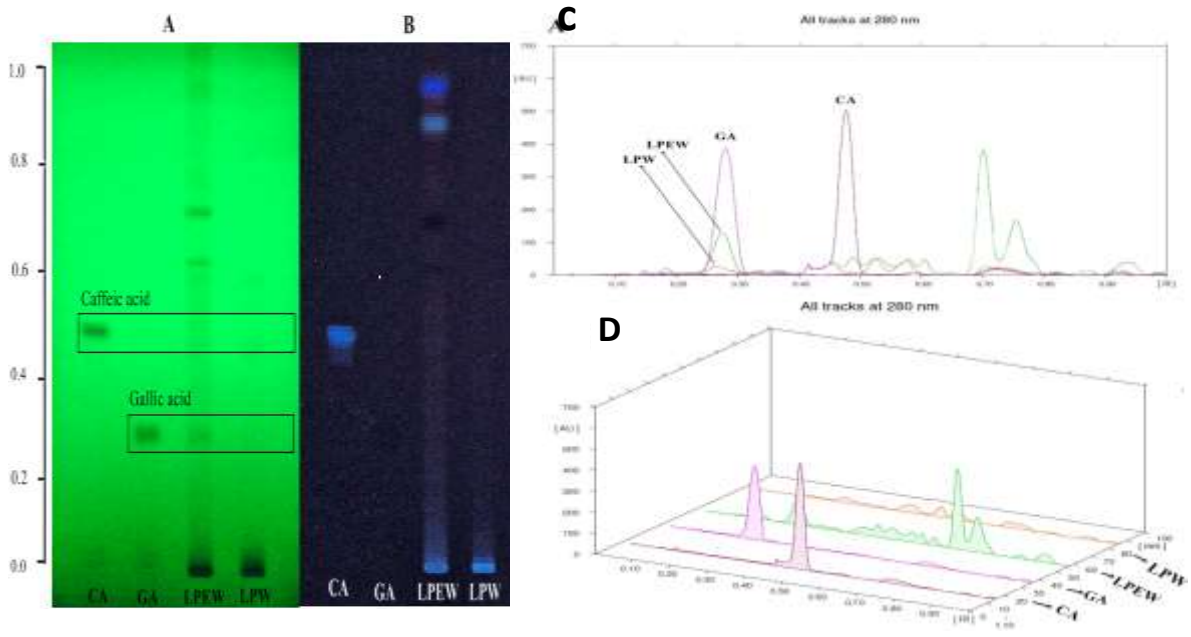

Figure1; HPTLC profile of *L. pumila* extracts visualized under a) 254 nm b) 366 nm c) 2D densitogram c) 3D densitogram. Caffeic acid and gallic acid serve as markers. LPW- Labisia pumila water extract; LPEW- Labisia pumila ethanol water extract; GA- Gallic acid; CA- caffeic acid.

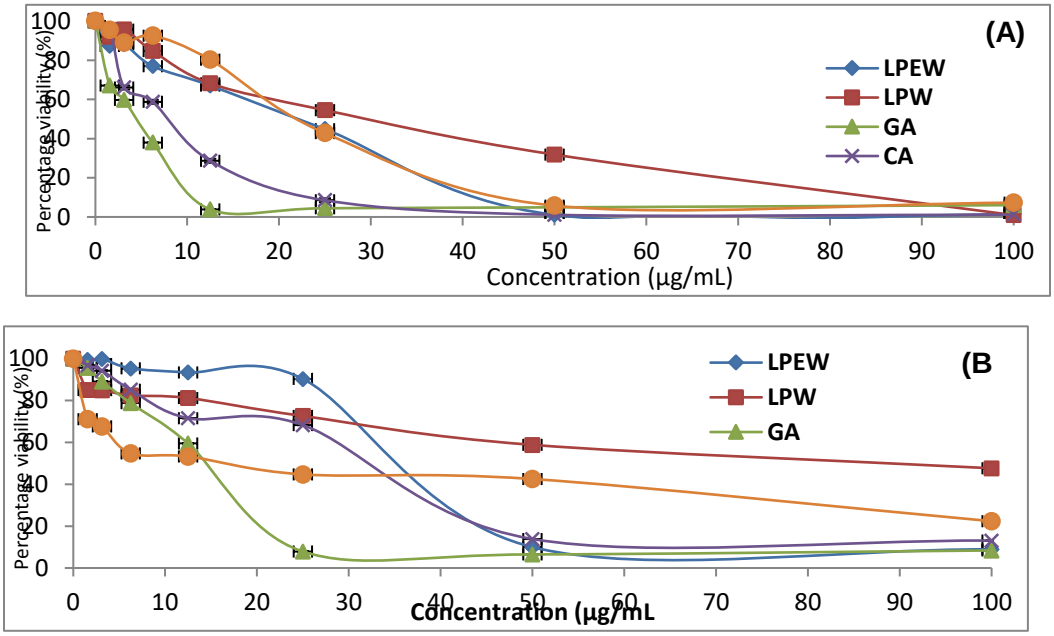

Figure 2; Cytotoxicity of *L. pumila* extracts against SK-UT-1 cells (A) and normal cell, A7R5 (B) for 72H incubation. Data given as means from 3 different experiments. LPEW- ethanol water extract; LPW- water extract; GA- gallic acid; CA- caffeic acid.

CONCLUSION

Standardization is important to ensure the quality, efficacy and safety of herbal materials. The presented physicochemical, metabolites analysis and major active constituents presented in the extract would be an important data for standardization of *L. pumila.* The presented data could be utilized for quality, safety and standardization for their safe use. Present study revealed the possible effect of anti-uterine fibroid activity of *L. pumila.*

CONFLICT OF INTEREST

There was not any conflict of interest in this study.

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AUTHOR CONTRIBUTION

NZ, KSM and ZI contributed with the conception of the study including standardization conception including physicochemical analysis, analysis of metabolites, determination of marker compounds and cytotoxic study. NZ and MAAS were responsible for all the experiments. KSM was contributed in revising this paper.

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