

## ESTROGENIC ASSESSMENT OF LABISIA PUMILA EXTRACTS USING A HUMAN ENDOMETRIAL CELL LINE

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### ABSTRACT

**Objective:** *Labisia pumila* (LP) has been used by Malay women for generations to regulate post-menopausal symptoms and contraction of the uterus after child birth. Its activity is due to phytoestrogens which could act like estrogen to bring about an estrogenic effect. This study aims to investigate the estrogenic activity of various LP extracts using an *in-vitro* model.

**Methods:** Various extracts and fractions was obtained from LP and tested for estrogenic activity using an *in-vitro* alkaline phosphatase assay using Ishikawa cells. Highly estrogenic water extract was fractionated using preparative high pressure liquid chromatography (HPLC). The extracts and fractions were also tested for their effect on cell proliferation.

**Results:** Standardized water extract of LP at 100µg/ml was highly estrogenic (2.56 fold of the control) thus was further fractionated. The last fraction eluted from HPLC presented the highest estrogenic activity (2.84 fold of the control), even higher than that of the water extract. Meanwhile, butanol, hexane and dichloromethane solvents did not significantly induce estrogenic activity. All the extracts showed minimal induction of cell proliferation (<20.00%) in comparison to estradiol (a positive control).

**Conclusion:** The high estrogenic activity and lower cell proliferation induction of the water extract provides a good insight for its use as an estrogen replacement agent. Consequently, this provides a respectable outcome towards the commercial products which contains LP extracts.

**Keywords:** *Labisia pumila*, Traditional medicine, Phytoestrogen, Estrogenic activity

### INTRODUCTION

*Labisia pumila* (LP), from the family of Myrsinaceae, is a herb that has been used by Malay women for generations to treat ailments related to the genitourinary system [1]. Traditionally, the plant is boiled, either alone or collectively with other herbs and then drunk. It has been claimed to contain high bioactive compounds that offers health benefits such as anti-dysmenorrhea, contraction of the uterus after childbirth, regulate menstruation, relieve menopausal symptoms and to generally alleviate fatigue [2, 3]. At present the exact mechanism of action of LP is still unclear [4]. However, it was found that LP extract contains ascorbic acid, anthocyanin, beta-carotene, flavanoids, flavonoids and phenolic contents [4, 5]. These flavonoids and phenolics in LP have remarkable pharmacological activity, e.g. anti-inflammatory [6], anti-microbial [7], anti-oxidant [8] and anti-cancer [9] activity. However, these activities may differ with different classes of flavonoids and phenolics present whereby the differences are depending on the variety of LP and the part of the plant. Total flavonoids and phenolics are highest in the leaf extract and lowest in the stem. Leaf extract of LP variety alata (LPva) has the highest amount of phenolics while leaf extract of LP variety pumila (LPvp) has the highest levels of flavonoids [8].

Based on the traditional use of LP in conditions related with lack of estrogen, it had been speculated that they exhibit phytoestrogenic activity [10, 11]. Previously Husniza [12], reported that water extract of LP inhibited 17β-estradiol (17β-E<sub>2</sub>) binding to antibodies raised against it, showing that it has similar activity to natural hormones such as estrone and estriol. Besides that ethanol (EtOH) extract of LP had also been shown to exhibit a weak estrogenic activity at 10-50mg/ml in Ishikawa cells [13]. A later study also showed that LP displayed estrogenic effect on MCF-7 breast cells by increasing cell proliferation [14]. These results further support the claims that LP has phytoestrogenic activities.

Phytoestrogens are a diverse group of non-steroidal plant-derived compounds that structurally or functionally mimic mammalian estrogens [15, 16]. They exhibit estrogenic activity in the body by binding weakly to ER to induce transcription of estrogen-responsive target genes in a dose-dependent manner [17, 18]. The relative affinities of phytoestrogens for ER are more than 10<sup>2</sup> to 10<sup>5</sup>-fold lower than 17β-estradiol (17β-E<sub>2</sub>) [19], which is an important factor

when considering dietary intake of phytoestrogens and their subsequent circulating concentrations [10]. Overall, data on the estrogenic actions of phytoestrogens are perplexed by multiple factors such as the chemical structure of phytoestrogen, site of action, cell type, route of administration, metabolism, presence of endogenous estrogens and other treatments used in conjunction with phytoestrogens [18, 20, 21]. Presently, there is no journal which compared the estrogenicity of various LP extracts. Therefore, the objective of this study is to compare the estrogenicity of various LP extracts and sub-fractions, as well as to evaluate their effect on cell proliferation.

### MATERIAL AND METHODS

#### Chemicals

Minimum Essential Medium (MEM) and dextran-coated charcoal (DCC) were purchased from Sigma, USA. Fetal bovine serum (FBS), fetal calf serum (FCS), antibiotic-antimycotic and L-glutamine were obtained from Gibco, USA. Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) was sourced from Nacalai Tesque, Japan. All other chemicals were purchased from Merck, Germany.

#### Cell culture and maintenance of Ishikawa cells

Ishikawa cells (99040201, Sigma Aldrich), which are human endometrial adenocarcinoma cells, were maintained in MEM supplemented with 10% FBS, 1% antibiotic-antimycotic and 1% L-glutamine. Cells were passaged twice weekly. Two days before the start of the experiment, near-confluent cells were changed to an estrogen-free basal medium (EFBM). The EFBM consist of DMEM/ F-12 media supplemented with 5% DCC-stripped FCS, 1% antibiotic-antimycotic and 1% L-glutamine. After 24 h, the cells were harvested with 0.25% EDTA-trypsin and seeded in 96-well flat-bottomed microtiter plates, in 100 µl of EFBM/ well.

#### Preparation of test compounds

All the LPvp extracts and fractions used in this experiment were extracted in Universiti Sains Malaysia (USM), Penang, Malaysia. First, LP root and leaf (leaf: root; 3:2) was cut and blended before boiling in water (plant: water; 1:10). The decoction was then filtered and

freeze-friend to produce a standardized (KF1) extract. Butanol (B<sub>4</sub>OH), dichloromethane (DCM), hexane (Hex) and water (H<sub>2</sub>O) extract were then obtained from the KF1 extract. The solvents Hex, DCM and B<sub>4</sub>OH were mixed with 1g of standardized extract in that order one after the other and then separated by solvent-solvent extraction method. The remaining liquid after extraction with the three solvents was the H<sub>2</sub>O extract. After solvent-solvent extraction, the extracts were then dried in a rotary evaporator to concentrate the samples and to remove any trace of solvent present.

The LP extracts were tested against 10<sup>-8</sup>M 17β-E<sub>2</sub> (SIGMA, USA), dissolved in ethanol (EtOH) (Merck) as the carrier. KF1 and H<sub>2</sub>O extract of LP were prepared as a 1mg/ml stock solution in water while the solvent extracts were dissolved as 1mg/ml stock solution in EtOH. The stock solutions were then filtered with a 0.22μm filter to remove any precipitate. The stock solutions were then diluted to appropriate concentrations in media for use. Concentration of EtOH for all samples used was maintained at 0.1%. All test compounds were stored at -20°C.

### Alkaline Phosphatase Activity Assay

For this study, the alkaline phosphatase assay (ALP) was used as the main assay in evaluating the estrogenicity of LP in Ishikawa cells. Ishikawa cells were seeded at a density of 2.5×10<sup>4</sup>cells/100 μL in EFBM/well in a 96-well flat bottomed plate. After incubation with test compounds for 72 h, the wells were washed twice with ice-cold phosphate buffered saline (PBS) (OXOID). The plate was then frozen in -80°C for 20min before being thawed at 37°C. After that, 50μL of ice-cold *p*-nitrophenyl phosphate (pNPP) (SIGMA) solution was added and the ALP enzyme activity was monitored for 1.5 - 3h by reading the plate periodically at 405nm until maximally stimulated cells show an absorbance of about 1.2 [22]. All experimental conditions were assayed in triplicate.

### MTT Cell proliferation Assay

The effect of the extracts on cell proliferation were estimated using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) assay, Ishikawa cells were seeded at a density of 1.5×10<sup>4</sup>cells/100μL in EFBM/well in a 96-well flat bottomed plate for 24h before addition of test compounds [23]. After 72h of incubation with test compounds, the 10μL of 5mg/ml MTT (Amresco, USA) was added to each well. The formazan produced was then dissolved by dimethyl sulfoxide (DMSO) and read at 595nm [24]. The percentage of cell proliferation of control cells is 100.00% and any increase or decrease in cell proliferation of treated cells was compared to that of control cells. Therefore the untreated control is represented by the baseline of 0% cell proliferation above control. All experimental conditions were assayed in triplicate.

### Statistical analysis

All data were expressed as mean + SEM (Standard Error of Mean). Data collected were analyzed using student's t-test via a statistical software SPSS version 16.0 (SPSS Inc., USA), where \* P<0.05 and \*\* P<0.01.

## RESULTS AND DISCUSSIONS

### Ishikawa cells represent a suitable model for studying estrogen effects on endometrial epithelium

Ishikawa cells respond to both estrogens and anti-estrogens at a concentration approximating physiological levels [23]. They are very sensitive to estrogens; detecting 17β-E<sub>2</sub> at concentration as low as 10<sup>-12</sup> M [22] and is also dose dependent [25]. Past studies had demonstrated that Ishikawa cells responded to estrogen administration with an increase in cell number and placental alkaline phosphatase (ALP) activity, which can be inhibited by anti-estrogens [22, 25]. This ALP expression and activity is under strict estrogenic control at the transcriptional level [26] and that other steroids such as androgens, glucocorticoids or progestins do not produce a similar effect [22].

For this study, ALP assay had been used as the main experiment design for accessing estrogenic effect while MTT assay had been used to study cell proliferation. Based on the results, stimulation of

ALP activity by 17β-E<sub>2</sub> was similar to past studies [25, 27] where the effect is dose dependent and the maximum response was found at 10<sup>-8</sup> M 17β-E<sub>2</sub> (1.22 + 0.00A) which was 5-fold of that induced by the untreated control (0.25 + 0.01A) (Figure 1).

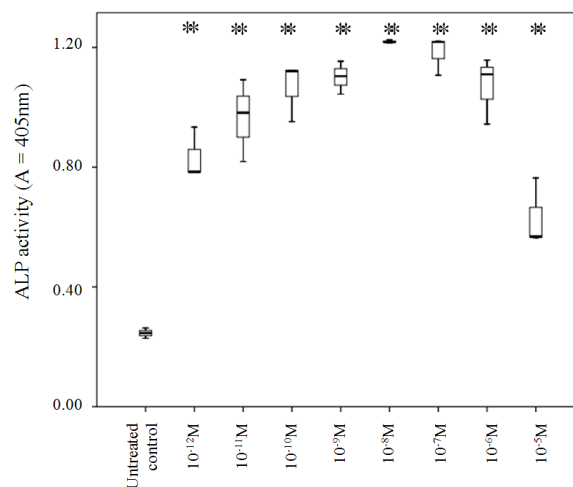


Fig. 1: Induction of ALP activity in Ishikawa cells by 17β-E<sub>2</sub>.

### Standardized extract of LP exhibited high estrogenic activity

Similar to the phytoestrogen genistein and plant extracts with estrogenic activity such as from hop and red clover [28], LP extracts also displayed estrogenic activity. KF1 and H<sub>2</sub>O at 100μg/ml significantly induced ALP activity with P<0.01 and P<0.05 respectively. The maximum ALP induction is at 100μg/ml of KF1 (0.64 + 0.02A), which was almost 3-fold of the untreated control (Figure 2). KF1 increased ALP activity in a dose-dependent manner from 1μg/ml to 100μg/ml, with an EC<sub>50</sub> of 76.14μg/ml. Conversely, B<sub>4</sub>OH, Hex and DCM extracts of LP did not induce ALP activity in Ishikawa cells at the concentrations tested. This is because the bioactive compound responsible for estrogenic activity is more polar in nature and therefore located in the highly polar fractions.

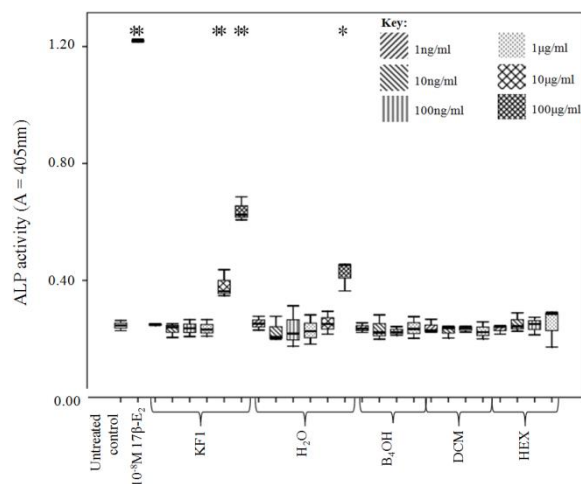


Fig. 2: Effect of various LP extracts on ALP activity in Ishikawa cells in comparison to the untreated control and 10<sup>-8</sup>M 17β-E<sub>2</sub>.

B<sub>4</sub>OH, DCM and Hex represent the solvents used for the extraction of less-polar bioactive compound/s. Among these three solvents, B<sub>4</sub>OH represented the most polar, followed by DCM and Hex, being the least polar solvent. Jamal *et al.* [13] had showed that EtOH extract of LP exhibit estrogenic activity; however, EtOH represents a more polar solvent in comparison to B<sub>4</sub>OH, Hex and DCM, but a less polar solvent compared to water. Thus some of the more polar bioactive compound/s could have been present in the EtOH extract. Besides the above mentioned, Hex extract was also fractionated via reverse phase preparative HPLC into 17 different fractions in which none of the fractions present any ALP activity in Ishikawa cells.

The polarity of the solvent used for extraction determines the type of flavonoid extracted. For less polar flavonoids such as isoflavones and flavonones, dichloromethane or ethyl acetate is usually used. Meanwhile, more polar flavanols such as catechins and tannins can be extracted with water. The composition of the extract will differ depending on the type of solvent used for extraction [29]. Overall, the results suggested that the main bioactive compound/s responsible for causing estrogenic activity in LP could belong to the more polar flavanols.

**Least polar reverse phase HPLC fraction induced highest estrogenic activity**

KF1 was the main extract which induced the highest ALP activity, thus suggesting high estrogenic activity. Therefore KF1 was further fractionated using reverse phase preparative HPLC into 4 sub-water fractions (Figure 3): P1-P4 according to their retention time. P1, P2 and P4 induced ALP activity in Ishikawa cells at a dose-dependent effect which is maximal at 100µg/ml. As with 17β-E<sub>2</sub> and KF1, P1, P2 and P4 also induced dose-dependent increase in ALP activity in Ishikawa cells. P4 at 100µg/ml exhibited the highest ALP activity (0.71 ± 0.02A) among all the extracts and fractions assayed (Figure 4). P3 was the only fraction which did not significantly induce any ALP activity. Based on the results, P4 was the fraction with the greatest estrogenic activity, with an EC<sub>50</sub> value of 42.77µg/ml.

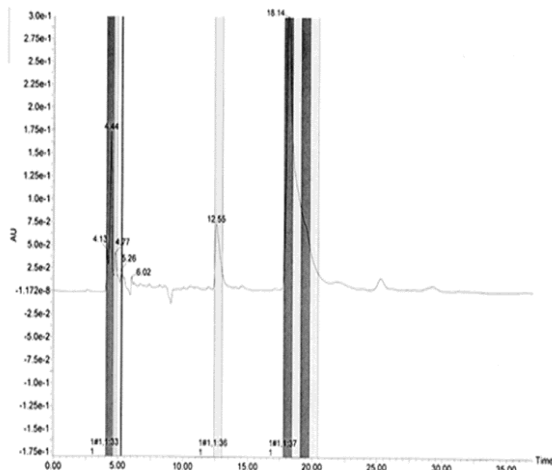


Fig. 3: Chromatogram of the reverse phase preparative HPLC of KF1.

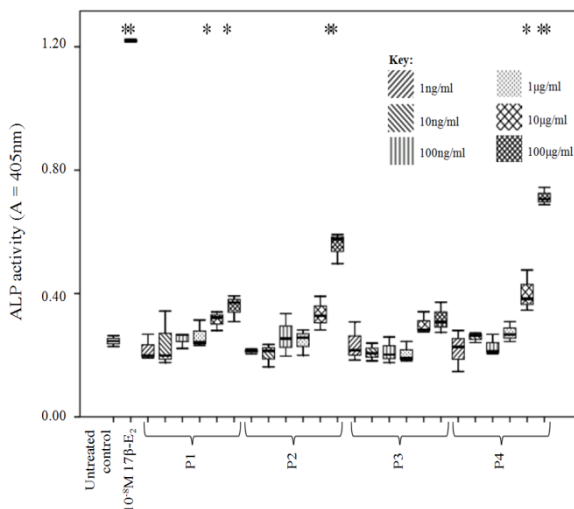


Fig. 4: Effect of LP fractions on ALP activity in Ishikawa cells in comparison to the untreated control and 10<sup>-8</sup>M 17β-E<sub>2</sub>.

**LP extract did not significantly induce cell proliferation in comparison to 17β-E<sub>2</sub>**

For MTT assay of cell proliferation, the percentage of cell proliferation of control cells is 100.00% and any increase or decrease in cell proliferation of treated cells was compared to that of control cells. The highest cell proliferation was achieved at 10<sup>-8</sup>M 17β-E<sub>2</sub>, as shown by Holinka *et al.* [25], in which cell proliferation increased up to 33.33% of the untreated control. At high concentrations, all 5 LP extracts significantly increased cell proliferation. Overall cell proliferation was maintained at about 10% increase from control. The highest induction was at 1µg/ml DCM extract (15.89 ± 1.36% increase). For H<sub>2</sub>O, at 10ng/ml to 100µg/ml it was evident that like 17β-E<sub>2</sub>, an increase in concentration caused an increase in both ALP activity and cell proliferation (Figure 5). The dose-dependent increase in cell proliferation was also observed in DCM extract from 10ng/ml to 1µg/ml, even though the extract did not induce any estrogenic activity in Ishikawa cells. No particular trends were observed for KF1, B<sub>4</sub>OH and Hex extracts. The results demonstrate that unlike in 17β-E<sub>2</sub>, an increase in ALP activity was not related to an increase in cell proliferation.

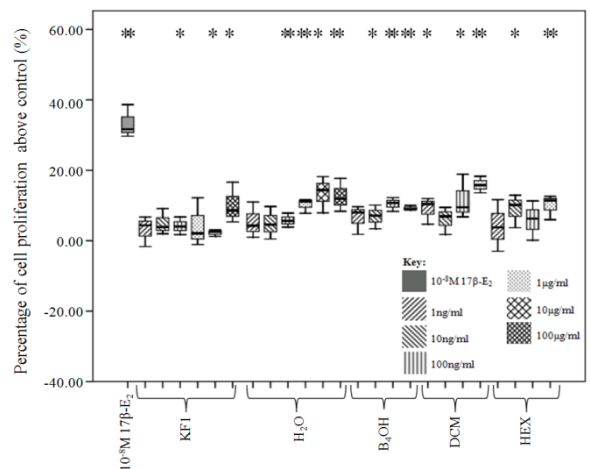


Fig. 5: Effect of various LP extracts on the cell proliferation of Ishikawa cells in comparison to the untreated control.

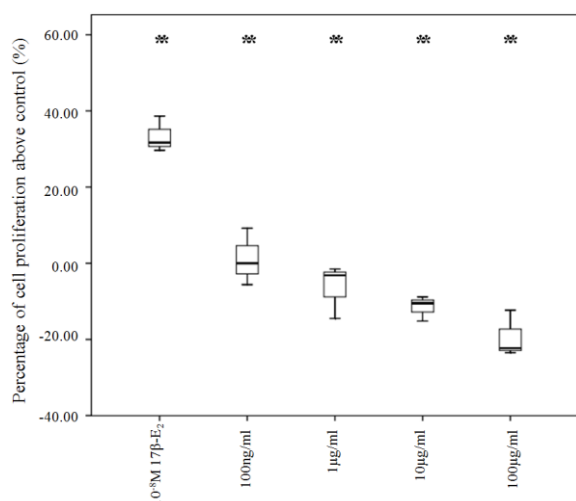
Past study had shown that isoflavones such as genistein and daidzein increased proliferation of Ishikawa cells [27]. As mentioned earlier, less polar isoflavones are usually isolated using less polar solvents such as DCM [29]. Based on the results, less polar solvent extracts such as B<sub>4</sub>OH, DCM and Hex generally induced a greater increase in cell proliferation compared to the water extracts. This increase could be induced by the presence of isoflavones present in the extract, which is similar to soy isoflavones as reported by Kayisli *et al.* [27]. Meanwhile, in animal studies, water extract of LP had been shown to increase uterine weight as compared to control cells [30, 31]. The increase in uterine weight is supportive of its use as an estrogen replacement agent.

Overall, none of the extracts tested resulted in an increase in cell proliferation as great as 10<sup>-8</sup>M 17β-E<sub>2</sub> on Ishikawa cells. This is favorable when considering the use of LP over 17β-E<sub>2</sub> as a possible estrogen replacement agent, as it can bring about an estrogenic effect with a lower increase in cell proliferation, which could lead to endometrial cancers which are common with conventional estrogen replacement drugs.

**Most estrogenic fraction displays anti-proliferative effects**

Unlike KF1, P4 did not induce cell proliferation in Ishikawa cells but instead decreased cell proliferation with an IC<sub>50</sub> of 368.77µg/ml (Figure 6). This showed that the bioactive compound/s present in P4 exhibited high estrogenic activity without the side-effect of increase in cell proliferation. However, as P4 decreased cell proliferation, there is a possibility for cytotoxic effects and therefore IC<sub>50</sub> values were used as parameter for evaluating cytotoxicity whereby a substance with an IC<sub>50</sub> lower than 30µg/ml is considered

to be cytotoxic [9, 32]. Due to the high  $IC_{50}$  of P4, it is considered to be relatively non-toxic.



**Fig. 6: Effect of reverse phase preparative HPLC LP fraction P4 on cell proliferation of Ishikawa cells.**

Many past studies have also showed that LP extracts exhibit anti-proliferative effects. In 2003, Jamal *et al.* reported that EtOH extract of the roots of LPva ( $IC_{50}$  582 $\mu$ g/ml) and LPvp ( $IC_{50}$  60 $\mu$ g/ml), aqueous extract of LPva root ( $IC_{50}$  433 $\mu$ g/ml) and LPvp leaves ( $IC_{50}$  458 $\mu$ g/ml) exhibited cytotoxic activity on Ishikawa cells [13]. With the exception of ethanol extract of LPvp, the other extracts required very high concentrations of extract to cause toxicity on Ishikawa cells which is irrelevant on a therapeutic level and thus does not pose any safety risks. In another study in 2012, Lope Pihie *et al.* found that aqueous, ethanol and hexane extract of LP exhibits anti-proliferative effect on HM3KO, MDBK and Vero cell lines [9]. The results obtained in this experiment were different compared to past studies, as neither aqueous nor hexane extracts tested decrease cell proliferation. This could be because of the difference in the method of extraction resulting in different composition of the extracts and the different cell lines used. In this study, only the last HPLC fraction of the standardized water extract-P4 was found to decrease cell proliferation.

## CONCLUSION

Based on the results, the sub-fraction of the standardized extract - P4 expressed the highest estrogenic activity. This showed that it is suitable for further study as a possible estrogen replacement agent. Besides that, P4 also induced a lower increase in cell proliferation compared to  $10^{-8}M$  17 $\beta$ -E<sub>2</sub> demonstrating favorable result for its use as a safe estrogen replacement, with a lower risk of endometrial cancer. This decrease in cell proliferation could also be further studied to identify the mechanism and explored as a possible cytotoxic agent. Future studies include further fractionation of P4 to be analyzed by nuclear magnetic resonance (NMR) to identify the main bioactive compound/s which induces estrogenic activity. From there forth the bioactive compound would be studied to identify the mechanism of action.

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## REFERENCES

- Fazliana M, Ramos NL, Luthje P, Sekikubo M, Holm A, Wan Nazaimoon WM, Brauner A: Labisia pumila var. alata reduces bacterial load by inducing uroepithelial cell apoptosis. *J Ethnopharm* 2011; 136:111-116.
- Burkill I. A dictionary of the economic products of the Malay Peninsular: Ministry of Agriculture of Malaysia; 2002.
- Zakaria M, Mohd MA. Traditional Malay Medicinal Plants: Penerbit Fajar Bakti, Kuala Lumpur, Malaysia; 1994.
- Norhaiza M, Maziah M, Hakiman M: Antioxidative properties of leaf extracts of a popular Malaysian herb, Labisia pumila. *J MedPlant Res* 2009; 3: 217-223.
- Chua L, Latiff N, Lee S, Lee C, Sarmidi M, Aziz R: Flavonoids and phenolic acids from Labisia pumila (Kacip Fatimah). *Food Chem* 2011; 127:1186-1192.
- Nadia ME, Nazrun AS, Norazlina M, Isa NM, Norliza M, Ima Nirwana S: The Anti-Inflammatory, Phytoestrogenic, and Antioxidative Role of Labisia pumila in Prevention of Postmenopausal Osteoporosis. *Adv Pharmacol Sci* 2012; DOI 10.1155/2012/706905.
- Karimi E, Jaafar HZ, Ahmad S: Phytochemical analysis and antimicrobial activities of methanolic extracts of leaf, stem and root from different varieties of Labisa pumila Benth. *Mol* 2011; 16:4438-4450.
- Karimi E, Jaafar HZ, Ahmad S: Phenolics and flavonoids profiling and antioxidant activity of three varieties of Malaysian indigenous medicinal herb Labisia pumila Benth. *J Med Plant Res* 2011; 5:1200-1206.
- Lope Pihie AH, Zakaria ZA, Othman F (2012) Antiproliferative and Proapoptotic Effects of Labisia pumila Ethanol Extract and Its Active Fraction in Human Melanoma HM3KO Cells. Evid-based Complement Alternat Med 2012; DOI 10.1155/2012/123470.
- Rice S, Whitehead SA: Phytoestrogens and breast cancer--promoters or protectors? *Endo Relat Can* 2006; 13: 995-1015.
- Chlebowski RT, Kuller LH, Prentice RL, Stefanick ML, Manson JE, Gass M, *et al.*: Breast cancer after use of estrogen plus progestin in postmenopausal women. *N Engl J Med* 2009; 360: 573-587.
- Husniza H. Estrogenic and androgenic activities of kacip fatimah (Labisia pumila). In: Abstracts of Research Project. Kuala Lumpur: Institute for Medical Research, Ministry of Health Malaysia, 2002. p. 8.
- Jamal J, Houghton P, Milligan S, Ibrahim J: The estrogenic and cytotoxic effects of the extracts of Labisia pumila var. alata and Labisia pumila var. pumila in-vitro. *Malays J Health Sci* 2003; 1:53-60.
- Jamal JA, Ramli N., Stanslas J, Husain K: *Estrogenic activity of selected Myrsinaceae species in MCF-7 human breast cancer cells.* *Int J Pharm Pharm Sci* 2012; 4: 547-553.
- Mense SM, Hei TK, Ganju RK, Bhat HK: Phytoestrogens and breast cancer prevention: possible mechanisms of action. *Environ Health Perspect* 2008; 116: 426-433.
- Kalita J, Milligan S: In vitro estrogenic potency of phytoestrogen-glycosides and some plant flavanoids. *Indian J Sci Tech* 2010; 3:1142-1147.
- Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson JA: Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endo* 1997; 138: 863-870.
- Moutsatsou P: The spectrum of phytoestrogens in nature: our knowledge is expanding. *Horm (Athens)* 2007; 6:173-193.
- Schmitt E, Dekant W, Stopper H: Assaying the estrogenicity of phytoestrogens in cells of different estrogen sensitive tissues. *Toxicol In Vitro* 2001; 15: 433-439.
- Ososki AL, Kennelly EJ: Phytoestrogens: a review of the present state of research. *Phytotherapy Res* 2003;17: 845-869.
- Poh SWM, Visweswaran N, Chia YY: Phytoestrogenic property of Labisia pumila for use as an estrogen replacement therapy agent. *Afr J Biotechnol* 2012; 11:11053-11056.
- Littlefield BA, Gursipde E, Markiewicz L, McKinley B, Hochberg RB: A simple and sensitive microtiter plate estrogen bioassay based on stimulation of alkaline phosphatase in Ishikawa cells: estrogenic action of delta 5 adrenal steroids. *Endocrinol* 1990; 127: 2757-2762.
- Liu W. Traditional Herbal Medicine Research Methods. In: Identification, Analysis, Bioassay and Pharmaceutical and Clinical Studies:Wiley; 2011.
- Jo YH, Choi YJ, Kim HO, Yoon KS, Lee BY, Jung MH, Huh CY: Corticotropin-releasing hormone enhances the invasiveness and migration of Ishikawa cells, possibly by increasing matrix

- metalloproteinase-2 and matrix metalloproteinase-9. *J Intl Med Res* 2011; 39: 2067-2075.
25. Holinka CF, Hata H, Kuramoto H, Gurpide E: Effects of steroid hormones and antisteroids on alkaline phosphatase activity in human endometrial cancer cells (Ishikawa line). *Can Res* 1986; 46: 2771-2774.
  26. Bhat KP, Pezzuto JM: Resveratrol exhibits cytostatic and antiestrogenic properties with human endometrial adenocarcinoma (Ishikawa) cells. *Can Res* 2001; 61: 6137-6144.
  27. Kayisli UA, Aksu CA, Berkkanoglu M, Arici A: Estrogenicity of isoflavones on human endometrial stromal and glandular cells. *J Clin Endocrinol Metab* 2002; 87: 5539-5544.
  28. Overk CR, Yao P, Chadwick LR, Nikolic D, Sun Y, Cuendet MA, Deng Y, et al.: Comparison of the in vitro estrogenic activities of compounds from hops (*Humulus lupulus*) and red clover (*Trifolium pratense*). *J Agric Food Chem* 2005; 53:6246-6253.
  29. Andersen OM, Markham KR. *Flavonoids: Chemistry, Biochemistry and Applications*: CRC Press; 2005.
  30. Fazliana M, Wan Nazaimoon WM, Gu HF, Ostenson CG: *Labisia pumila* extract regulates body weight and adipokines in ovariectomized rats. *Maturitas* 2009; 62:91-97.
  31. Manneras L, Fazliana M, Wan Nazaimoon WM, Lonn M, Gu HF, Ostenson CG, Stener-Victorin E: Beneficial metabolic effects of the Malaysian herb *Labisia pumila* var. *alata* in a rat model of polycystic ovary syndrome. *J Ethnopharmacol* 2010; 127:346-351.
  32. Suffness M, Pezzuto JM. *Assays related to cancer drug discovery*. In: *Methods in plant biochemistry: assays for bioactivity*: London: Academic Press; 1990.