Sains Kesihatan **1**(2003): 53-60

The Oestrogenic and Cytotoxic Effects of the Extracts of *Labisia pumila* var. *alata* and *Labisia pumila* var. *pumila In Vitro*

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ABSTRAK

Efek estrogen dan sitotoksik bagi ekstrak akues, terhidrolisis-asid dan etanol akar dan daun Labisia pumila var. alata dan L. pumila var. pumila telah dikaji ke atas titisan sel Ishikawa dengan masing-masing menggunakan asai alkalin fosfatase Ishikawa dan asai protein secara in vitro. Di antara mereka hanya ekstrak etanol akar L. pumila var. alata didapati menunjukkan aktiviti estrogen yang lemah pada 10-50 μg/ml. Sampel yang menunjukkan kesan sitotoksik secara signifikan terdiri daripada ekstrak etanol akar L. pumila var. alata (IC_{50} 582 μg/ml) dan L. pumila var. pumila (IC_{50} 60μg/ml), dan ekstrak akues akar L. pumila var. alata (IC_{50} 433 μg/ml) dan daun L. pumila var. pumila (IC_{50} 458 μg/ml).

Kata kunci: Labisia pumila, ekstrak etanol, aktiviti oestrogenik, kesan sitotoksik.

ABSTRACT

Aqueous, acid hydrolysed and ethanolic extracts of the roots and leaves of Labisia pumila var. alata and Labisia pumila var. pumila were investigated for their oestrogenic and cytotoxic effects using Ishikawa cell line by performing an in vitro Ishikawa alkaline phosphatase assay and an in vitro protein assay, respectively. Among them, only the ethanol extract of the root of L. pumila var. alata exhibited a weak oestrogenic activity at 10-50 μg/ml. The samples that exhibited significant cytotoxic effect were the ethanol extracts of the roots of L. pumila var. alata (IC_{50} 582 μg/ml) and L. pumila var. pumila (IC_{50} 60 μg/ml), and the aqueous extracts of the roots of L. pumila var. alata (IC_{50} 433 μg/ml) and the leaves of L. pumila var. pumila (IC_{50} 458 μg/ml).

Key words: Labisia pumila, ethanol extract, oestrogenic activity, cytotoxic effect.

INTRODUCTION

Labisia pumila (Bl.) F.-Vill (syn. : *Labisia pothoina* Lindl., Family: Myrsinaceae) is a subherbaceous plant with creeping stems. It is a wild forest herb which is found mainly in the lowland and hill forests of Peninsular Malaysia at an altitude between 300 and 700 m (Sulaiman et al. 1992). It is widely distributed throughout the states of Perak, Pahang, Selangor and Negeri Sembilan. There are three varieties of *L. pumila*, i.e. *L. pumila* var. *pumila*, *L. pumila* var. *alata* (Scheff.) Mez. and *L. pumila* var. *lanceolata* (Scheff.) Mez. (Stone 1988). The first two varieties are most commonly utilised by the traditional healers.

In the Malay traditional medicine, a water decoction of the roots or whole plants of *L. pumila* (also known as *air selusuh*) is often given to a pregnant woman between one and two months before she is due to give birth, as this is believed to induce and expedite labour (Burkill 1966). The plant is also used in post-partum medication as mixed preparation to help contract the birth channel, to delay fertility and to regain body strength (Zakaria & Mohd 1994). Its other folkloric uses include treatment of flatulence, dysentery, dysmenorrhoea, gonorrhoea and "sickness in the bones" (Burkill 1966).

This study was carried out to determine the oestrogenic and cytotoxic effects of the extracts of the roots and leaves of *L. pumila* var. *alata* and *L. pumila* var. *pumila* using Ishikawa cell line. Based on the folkloric uses, the oestrogen-like effect of compounds was considered as a basis by which plants might initiate labour. In this study, an indirect oestrogenic assay was carried out based on the stimulation of alkaline phosphatase (AlkP) enzyme produced by the Ishikawa cells upon activation by oestrogens, or compounds that produce oestrogen-like activity (Littlefield et al. 1990). An *in vitro* protein assay was performed to discriminate the possibility that any reduction of the absorbance measured for the plant extracts in the Ishikawa AlkP assay was not due to the reduction of AlkP activity but caused by the lysis of the cells.

MATERIALS AND METHODS

PLANT MATERIALS

The roots and leaves of *L. pumila* var. *alata* were collected at Pasoh Forest Reserve, Negeri Sembilan, Malaysia (voucher specimen number, FRI 39261) and that of the *L. pumila* var. *pumila* at Krau Game Reserve, Pahang, Malaysia. Both voucher specimens were sampled at the Herbarium of Forest Research Institution Malaysia, Kepong. The plant materials were air-dried, ground to powder and kept at room temperature in the dark until extraction.

PREPARATION OF SAMPLES

Aqueous extract - Dried roots and leaves (60 g) of *L. pumila* var. *alata* and *L. pumila* var. *pumila* were separately extracted with distilled water (750 ml) by decoction until the water was reduced to 500 ml. The resultants were lyophilized.

Acid hydrolysed water extract - Freeze-dried water extract (500 mg) was refluxed with 50 ml of sulphuric acid (10%) for 20 minutes and the resultant was extracted twice with ethyl acetate (50 ml). The ethyl acetate layer was collected, dried with anhydrous sodium sulphate and filtered. The extract was evaporated to dryness under vacuum.

Ethanol extract - Dried roots and leaves (60 g) of *L. pumila* var. *alata* and *L. pumila* var. *pumila* were separately extracted with ethanol (1000 ml) by soxhlet extraction for about 72 hrs. The extracts were taken to dryness under vacuum.

IN VITRO OESTROGEN BIOASSAY

The bioassay was performed according to the method described by Littlefield et al. (1990) and Markiewicz et al. (1992). The Ishikawa cells maintained in a oestrogen-free basal medium (EFBM) were seeded in 96-well microtitre plates (100 μl cell solution/well) and 50 μl aliquots of each concentration of the plant extracts (1 - 1000 μg/ml in EFBM) and negative control solution (EFBM) were delivered to each well. 17β-Oestradiol (E_2 Sigma) (1 - 1000 pg/ml in EFBM) was used as a positive standard in the bioassay. After incubation of the cell cultures for 72 hrs, cells were washed with phosphate buffer solution (PBS) and frozen for 15 min. Then 50 μl of ρ-nitrophenyl phosphate was added to each well and the plate was further incubated for 1 to 1.5 hrs to allow production of ρ-nitrophenol as a result of hydrolysis catalysed by AlkP. The optical density of the AlkP activity at an absorbance of 405 nm was determined using an assay plate reader.

IN VITRO PROTEIN ASSAY

The assay was carried out using a commercial BCA protein assay kit (Pierce Biotechnology, USA). The Ishikawa AlkP assay was repeated as described above, involving cells seeding, introduction of E_2 (1 - 1000 pg/ml), plant extracts (1 - 1000 μg/ml) and negative control solution (EFBM) in appropriate wells but leaving the outer perimeter wells free of cells and medium, washing, freezing and thawing of the plates. Then 100 μl of PBS was dispensed to the wells with cells (leaving the outer perimeter empty). Subsequently, 100 μl of albumin standards (bovine serum albumin fraction V, 2.0 mg/ml in 0.9% aqueous sodium chloride solution containing sodium

azide) of concentrations 10 to 40 μg/ml was added to the empty outer wells with one empty gap between the concentration and then 100 μl of the working reagent (50 parts of Bicinchoninic Acid Protein Assay Reagent A to 1 part of Bicinchoninic Acid Protein Assay Reagent B; stabilised at room temperature prior to assay) was delivered to all the 96-wells. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 30 min to allow the colour development. The optical density of the protein at an absorbance of 562 nm was determined using an assay plate reader.

STATISTICAL ANALYSIS

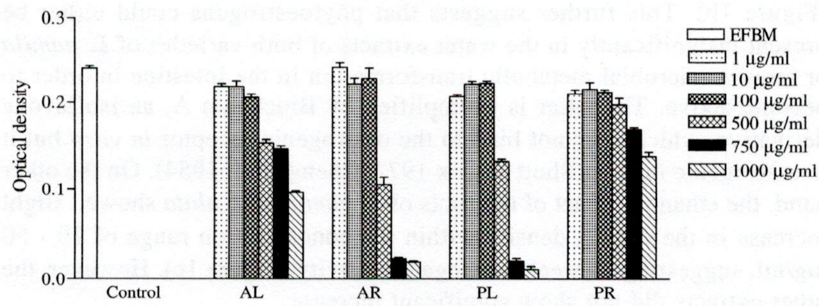
Data are expressed as mean ± S.E.M., obtained from separate experiments, and statistical significance of differences is determined using Student's *t*-test ($p < 0.05$) in Microsoft Excel 2000.

RESULTS AND DISCUSSION

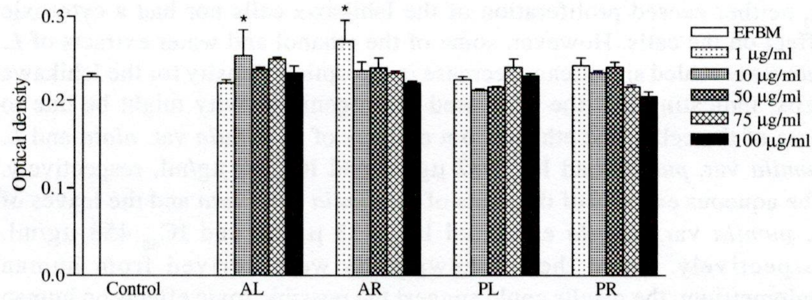
The *in vitro* oestrogen bioassay is based on the oestrogen-specific enhancement of alkaline phosphatase (AlkP) activity in human endometrial adenocarcinoma cells of the Ishikawa-Var I Line grown in 96-well microtitre plates (Markiewicz et al. 1993). Some phytoestrogens are reported to compete with radiolabelled oestradiol for binding to oestrogen receptors and bring about oestrogenic responses in the oestrogenic-responsive tissues and cells suggesting phytoestrogens and traditional oestrogens share a common mechanism of action (Shemesh et al. 1972). Therefore, any increase in the reading of optical density unit in the assay may indicate increase in oestrogen level.

The response of the Ishikawa AlkP assay to the extracts of the roots and leaves of *L. pumila* var. *alata* and *L. pumila* var. *pumila* is shown graphically in Figure 1(a-c). Aqueous extracts of the plants using decoction method were made to mimic the traditional preparation of *L. pumila* for use in childbirth. However, from the experiment, Figure 1a reveals that the hot water extracts of both *L. pumila* var. *alata* and *L. pumila* var. *pumila* roots and leaves do not show any significant increase in the optical density unit indicating the extracts may not have oestrogenic activity. The absence of oestrogenic activity in the water extracts could be explained by the fact that mostly known phytoestrogens are flavonoids, which occur in plant as glycosides. Previous studies had discovered that only their aglycones were found to exert the oestrogen-like activity in animals (Miksicek 1995). Since glycosides are extracted in water, the extracts could not show any activity. Acid hydrolysis of the water extracts was performed to break down any glycosides to their respective aglycones. However, only

(a) H_2O extract



(b) Hydrolysed H_2O extract



(c) EtOH extract

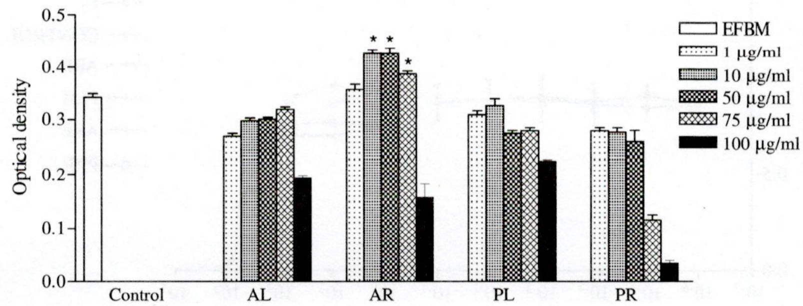


FIGURE 1. Response of the Ishikawa alkaline phosphatase to extracts of H_2O (a), hydrolysed H_2O extract (b) and ethanol (c) of leaves and roots of *L. pumila* var. *alata* and *L. pumila* var. *pumila*. * $p < 0.05$ [AL : *L. pumila* var. *alata* leaves; AR : *L. pumila* var. *alata* root; PL : *L. pumila* var. *pumila* leaves; PR : *L. pumila* var. *pumila root*]

very slight oestrogenicity of the hydrolysed water extracts are observed for the leaves and roots of *L. pumila* var. *alata* and *L. pumila* var. *pumila* (Figure 1b). This further suggests that phytoestrogens could either be present insignificantly in the water extracts of both varieties of *L. pumila* or require microbial metabolic transformation in the intestine in order to become active. The latter is exemplified by Biochanin A, an isoflavone derivative, which does not bind to the oestrogenic receptor *in vitro* but it is oestrogenic *in vivo* (Shutt & Cox 1972; Cheng et al. 1954). On the other hand, the ethanol extract of the roots of *L. pumila* var. *alata* showed slight increase in the optical density within the concentration range of 10 - 50 mg/ml, suggesting its weak oestrogenic activity (Figure 1c). However, the other extracts did not show significant increase.

The effect of 17β-oestradiol (E_2) and plant extracts on Ishikawa cell line is shown in Figure 2. The E_2 solution did not give any significant change in the optical density with respect to the control, suggesting that E_2 neither caused proliferation of the Ishikawa cells nor had a cytotoxic effect on the cells. However, some of the ethanol and water extracts of *L. pumila* revealed significant decrease in the optical density for the Ishikawa cells, indicating that the decreased oestrogenic activity might be due to lysis of the cells. The ethanol root extracts of *L. pumila* var. *alata* and *L. pumila* var. *pumila* had IC_{50} 582 µg/ml and IC_{50} 60 µg/ml, respectively. The aqueous extracts of the roots of *L. pumila* var. *alata* and the leaves of *L. pumila* var. *pumila* exhibited IC_{50} 433 µg/ml and IC_{50} 458 µg/ml, respectively. Since the Ishikawa cells were derived from human endometrium, the results could suggest the possible toxic effects on human uterus. However, information on the exact amount of *L. pumila* water

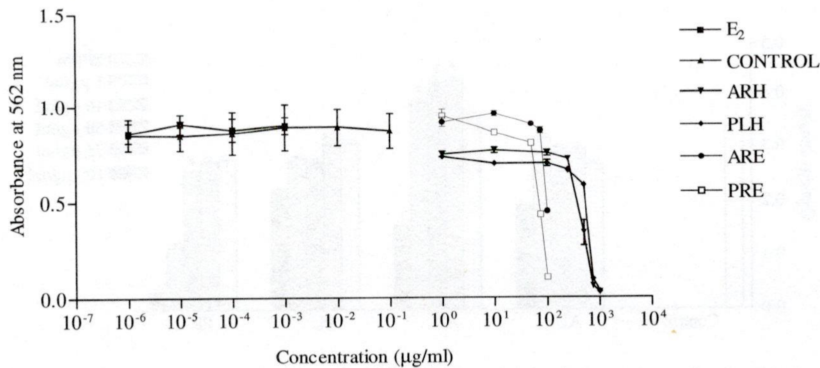


FIGURE 2. Effect of oestradiol and extracts of the leaves and roots of *L. pumila* on Ishikawa cell line. [ARH : water extract of *L. pumila* var. *alata* root; PLH : water extract of *L. pumila* var. *pumila* leaves; ARE : ethanol extract of *L. pumila* var. *alata* root; PRE : ethanol extract of *L. pumila* var. *pumila* root]

extract consumed by pregnant women in terms of weight per volume per consumption and the frequency of consumption is not available, making it difficult to determine whether the concentration of the extracts consumed is higher or lower than that tested in the experiment. On the other hand, the toxic compounds could be metabolised *in vivo*, forming inactive compounds that could be excreted.

CONCLUSION

From the *in vitro* studies, the ethanol extract of the roots of *L. pumila* var. *alata* was found to be weakly oestrogenic but the ethanol root extracts of *L. pumila* var. *alata* and *L. pumila* var. *pumila*, and the water extracts of the roots of *L. pumila* var. *alata* and the leaves of *L. pumila* var. *pumila* were found to be cytotoxic. Therefore, it is recommended that further studies should be performed such as *in vivo* tests using animal experiments to evaluate the oestrogenicity and mechanism of action of the *L. pumila* extracts, as well as *in vitro* and *in vivo* toxicological studies to assess the possibility of toxicity for human consumption.

ACKNOWLEDGEMENTS

The Forest Research Institute of Malaysia and Department of Wildlife and Natural Parks, Malaysia are thanked for providing plant materials, and the Standards and Industrial Research Institute of Malaysia and the Public Services Department of Malaysia for financial support.

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