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ANTIPROLIFERATIVE PROPERTIES OF AQUEOUS *Labisia pumila* EXTRACT ON PROSTATE CANCER CELL LINES

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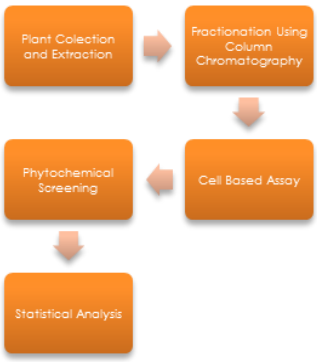
Graphical abstract



Abstract

Labisia pumila or locally known as Kacip Fatimah has been widely used by women in Malaysia to treat post-partum illnesses. This herbaceous undershrub of the Myrsinaceae family has been exploited aggressively as beverages, cosmetics as well as health supplement to promote wellness in the past. This present study was to determine the anti-proliferative properties of *L. pumila* aqueous extract against prostate cancer cell lines and to identify the active compound which may contribute to the anti-proliferative activity of *L. pumila*. In this study, the aqueous extract was prepared by using ultrasound assisted extraction method. The preliminary result revealed that *L. pumila* aqueous extract inhibited the proliferation of the prostate cancer cell lines. The aqueous extract was further fractionated using column chromatography. The result from the scavenging activity test confirmed that *L. pumila* crude extract and its fraction (F2) displayed a strong activity with 70% inhibition. Total phenolic contents assay was carried out to determine the phenolic compound of the extract and its fractions and the results showed the total phenolic content was higher in the F2 (96.82±0.3 GAE mg/g) compared to crude (63.75±0.1 GAE mg/g) and F1 (73.75±0.1 GAE mg/g). In conclusion, these findings suggested that the crude and fraction (F2) of *L. pumila* contain phenolic compounds that act as anti-proliferative agent against proliferation of prostate cancer cell lines *in vitro*.

Keywords: *Labisia pumila*, anti-proliferative, prostate cancer cell lines

Abstrak

Labisia pumila atau dikenali sebagai Kacip Fatimah telah digunakan secara meluas oleh wanita di Malaysia sebagai perawatan selepas bersalain. Herba renek berasal daripada keluarga *Myrsinaceae* ini telah dieksploitasi secara agresif sejak dahulu lagi sebagai minuman, kosmetik dan makanan tambahan kesihatan untuk menggalakkan kesejahteraan. Kajian ini adalah untuk menentukan ciri anti-proliferatif ekstrak akueus *L. pumila* terhadap jujukan sel kanser prostat dan mengenal pasti sebatian aktif yang menyumbang kepada tindakan anti-proliferatif *L. pumila*. Dalam kajian ini, ekstrak akueus diekstrak disediakan menggunakam kaedah pengekstrakan berbantukan *ultrasound*. Keputusan awal menunjukkan bahawa ekstrak akueus *L. pumila* merencat pertumbuhan jujukan sel kanser prostat. Ekstrak ini diperingkatkan lagi dengan menggunakan kromatografi turus. Keputusan yang diperolehi daripada ujian aktiviti memerangkap mengesahkan bahawa ekstrak mentah dan fraksi (F2) L. menunjukkan aktiviti yang kuat dengan nilai perencatan 70%. Jumlah kandungan fenolik telah dijalankan untuk menentukan sebatian fenolik dalam ekstrak dan fraksinya dan keputusan menunjukkan kandungan fenolik adalah lebih tinggi pada F2 (96.82 ± 0.3 GAE mg / g) berbanding ekstrak mentah (63.75 ± 0.1 GAE mg / g) dan F1 (73.75 ± 0.1 GAE mg / g). Kesimpulannya, hasil kajian ini menunjukkan bahawa ekstrak dan fraksi (F2) *L. pumila* mengandungi sebatian fenolik yang boleh bertindak sebagai agent anti proliferatif kepada jujukan sel kanser prostat *in vitro*.

Kata kunci: Kacip Fatimah, anti proliferative, jujukan sel kanser prostat

1.0 INTRODUCTION

Prostate cancer is one of the most common cancers in male of all races in the United States and the second leading killing cancers among American, African American, American Indian and Alaska native. This cancer is also the fourth most deadly cancer killing in Asia [1]. In Malaysia, prostate cancer is also one of top five recurrentcancers [2].

Various kinds of treatments available as options for the patientin treating prostate cancer. For example, the treatment can be done either through radiotherapy [3], surgery or hormonal therapy [4]. Unfortunately, these treatment have side effects. Therefore, phytotherapy or phytomedicine should be considered as an alternative solution for the treatment. Combination of natural sources or phytotherapy and synthetic drug may produce significant synergetic effect in treatment of diseases like prostate cancer [5].

Over the past years, plants has been widely used traditionally by the Malays as remedies for ailments. *Labisia pumila* or locally known in Malaysia as Kacip Fatimah is a popular herb. This plant is consumed to alleviate discomforts caused by menstrual cycle and period pain [6]. Furthermore, the consumptions of this herbal medicine by mothers ameliorates the birth channel after childbirth and heals "sickness of bone" [7]. In addition, Bodoker [8] reported that ingestion of this plant helps to escalate the overall wellness such as sexual health function and vitality.

In the previous few years, *L. pumila* has widely investigated to determine its biological and pharmacological activities either through *in vivo* or *in vitro*. In one of the studies, it was revealed that the dichloromethane extract of this plant and its active fraction demonstrated a concentration-dependent antinociceptive [9]. In another study, various cell lines such as human melanoma HM3KO [10], prostate and colon cancer [11] were tested against *L. pumila* and showed the positive result. In different kind of study, aqueous extract of *L. pumila* was claimed to promote collagen synthesis [12] and contain photoprotective activity against UV induced human fibroblast [13]. However, it was not reported whether the phytochemical compound of this plant were responsible for the claimed activities.

Recently, there has been a revival of interest in plant phytochemicals as potential chemopreventive and chemotherapeutic agents. Several researches were carried out to identify the bioactive compounds of this plant. There were studies reported that *L. pumila* extract is abundance in phenolic compounds such as gallic acid (Figure 1), myrecetin, kaempherol and catechin [14-16]. The presence of phenolic compounds in the plant could leads to anti-proliferative activity of *L. pumila* against prostate cancer. As of now, this current study was the first research of the *L. pumila* tested on the human prostate cancer cell lines using extract that was prepared through ultrasound assisted extraction.

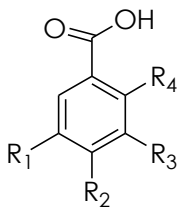


Figure 1 Chemical structure of Gallic acid [14]

2.0 EXPERIMENTAL

2.1 Preparation of Plant Material

Labisia pumila var *alata* was purchased from local supplier. Species authentication and voucher specimen (SK 2253/13) was deposited at Herbarium Unit, Institute of Bioscience, Universiti Putra Malaysia The leaves of the plant was cleaned, air dried and ground into powder form. He ground and powder form of *L. pumila* was kept in a tight container until further used.

2.2 Extraction and Fractionation

Dried and ground leaf of *L. pumila* was extracted using ultrasound assisted extraction in water at ratio of 1:16 [17] as described by Liu. The aqueous extract was evaporated under reduced pressure *via* rotary evaporator. Then, the extract was fractionated *via* column chromatography using gradient eluents of methanol and water. Briefly, *L. pumila* extract (1.0 g) was dissolved in methanol and adsorbed in silica gel (0.063- 0.2 mm mesh) using rotary evaporator. *L. pumila* silica gel mixture was added to the top of a chromatography column (2 cm x 40 cm) packed with silica gel. Fractionation was carried out using eluents comprised of 20, 40, 60, 80 and 100% methanol to water. Five fractions were collected and concentrated under reduced pressure until it became semi solid. The fractions were air dried and ready for further analysis [18].

2.3 Cell Culture

Human prostate cancer cell lines (DU 145) was obtained from the American Type Cell Collection (ATCC). The cell was cultured in Eagle's Minimal Essential Medium (EMEM) supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin. The cell was grown in theT-flask in the humidified incubator with 5% carbon dioxide (CO_2) at 37°C. All of the media were purchased from Life Technologies Bio Diagnostic, Selangor, Malaysia.

2.4 Cell Proliferation Assay

The cell was seeded in the 96 well plate at the density of 5 x 10^4 cells/well in 100 µL per well and

subsequentlyincubated overnight to allow confluence. After that, the medium was discarded and the cell was washed with buffer. Then, various concentrations of plant extract and fractions were added into the well untreated wells were used as a control. The plate was left in the incubator for 48 hours. Each concentration was prepared in triplicate.

The cell proliferation assay was done usingthe 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bro mide (MTT) assay [19]. In this assay, the proliferation of the prostate cancer was quantified when the enzymes from the living cells were able to reduce the yellow MTT dye into the blue coloured insoluble formazan crystal. After the incubation time, an amount of 20 µL of MTT solution (5 mg/mL)were pipetted into each well and the plate was incubated for 4 hours in the humidified incubator with 5% carbon dioxide ($C0_2$) at 37°C. The medium was discarded and dissolved with 150 µL of DMSO producing the purple MTT formazan crystals. The absorbance was measured at 550 nm using ELISA microplate reader. The rate of inhibitory for the cells was determined using the following formula;

Cell viability (%) = (Sample/ Control) x 100

2.5 Evaluation of Antioxidant Activity

DPPH scavenging activity was carried outwith minor modification [20]. Briefly, an amount of 100 µL of the extract and fraction in a various concentration were mixed with methanol and DPPH solution (2.5 mg/mL). The mixture were allowed to react at room temperature for 30 minutes and in the dark environment. The absorbance was measured at 517 nm using ELISA microplate reader. Ascorbic acid was used as a positive control. The scavenging activity was calculated using the formula below:

Scavenging activity (%) = 1-(sample/blank) x 100

2.6 Determination of Total Phenolic Content

Total phenolic content of the plant extract and fraction was determined using Folin-Ciocalteu assay [21]. About 20 µL of the sample consisting of plant extract and active fraction was mixed with 100 µL Folin- Ciocalteu reagent. Then, an amount of 80 µL of 7% sodium carbonate solution was added into the wells. After 2 hours of incubation in the dark environment, the absorbance was measured at 725 nm. Gallic acid was used as the standard reference. TPC was expressed as mg gallic acid equivalents per gram of dried sample.

2.7 Statistical analysis

Statistical significance of differences between control and compound-treated samples were calculated by using the Student's *t*-test. The significant differences between means from triplicate analyses ($p < 0.05$) were determined.

3.0 RESULT AND DISCUSSION

3.1 Effect of *L. pumila* on Anti-Proliferation Activity

In this study, anti-proliferative activity of *L. pumila* extract was carried on the prostate cancer cell lines.

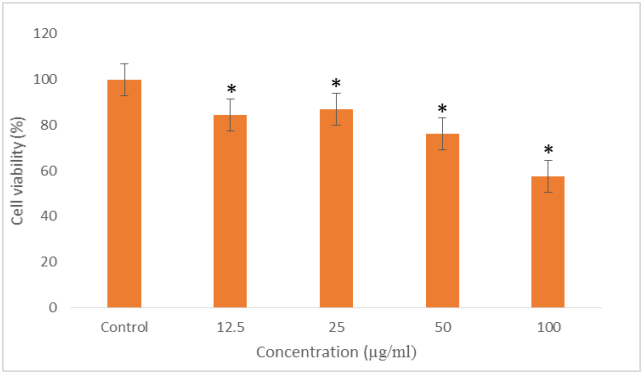


Figure 2 Cytotoxicity of aqueous extract of*L. pumila* against DU 145 cell lines. (All values represent mean ± standard deviation of three different experiments and *p < 0.05 indicate significant difference as compared to the control)

The Inhibition concentration (IC_{50}) was used as the parameter to evaluate the cytotoxicity. As shown in Figure 2, the inhibition concentration (IC_{50}) of the crude extract was 53 µg/mL. Preliminary anti-proliferative screening of 5 fractions (F1-F5) displayed only one fraction produced the most significant inhibition on the DU 145 cell lines. There result are shown in Table 1. F2 recorded the lowest IC_{50} value compared to F1 with 14.76 and 171 µg/mL respectively. According to the US NCI plant screening program, the IC_{50} value (the concentration needed to inhibit cell proliferation by 50%) of a crude extract should be less than 30 µg/mLI, to be considered to have *in vitro* cytotoxicity activity [22].

From the results, crude extract and fractions of *L. pumila* showed significantinhibitions on proliferation of the DU 145 cell lines. Al-Mekhlafi and his colleague in 2012 confirmed that *L. pumila* possessed anticancer type selectivity against prostate cancer cell lines [10]. Nonetheless, different type of prostate cancer, PC3 was used in their study. Although different type of cell lines was used to test in the current study, the anti-proliferative activity was comparatively equivalent. The significant anti-proliferation of crude extract and active fractions of *L. pumila* needs further investigation.

Table 1 IC_{50} values of crude and fractions from *L. pumila*

Fraction	IC_{50} value (µg/ml)
Crude	53 ±3.98
F1 (20% MeOH)	171± 1.29
F2 (40% MeOH)	14.76 ± 4.15
F3 (60% MeOH)	N.D
F4 (80% MeOH)	N.D
F5 (100% MeOH)	N.D

All values were expressed as the means ± SD from three independent experiments. The lower the IC_{50} value (the concentration needed to inhibit cell proliferation by 50%), the higher the potency.
*N.D= Not Detected

3.2 Antioxidant Activity

Generally, there are strong relationship between total phenolic content and antioxidant activity, as phenols possess strong scavenging activity to free radicals due to thepresence of the hydroxyl groups. The scavenging activity of the crude and fractions of *L. pumila* were assessed with the ascorbic acid as the positive control. The activities were presented in Figure 3 respectively.

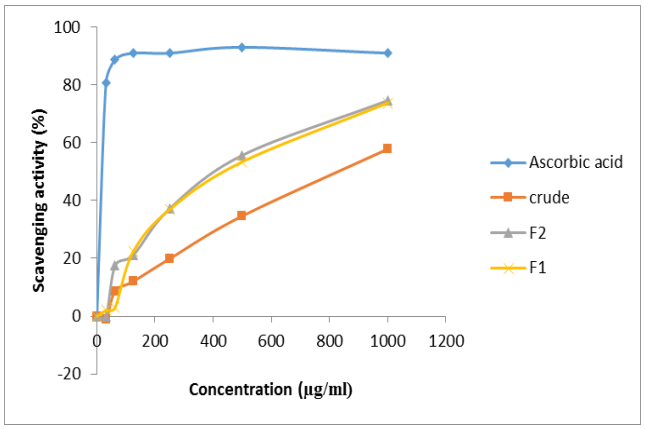


Figure 3 Scavenging activity of crude and fractions of *L. pumila*

The crude extract of *L. pumila* exhibited significant antioxidant activity (57.82%) at the concentration of 1000 µg/ml. The result was comparable to ascorbic acid which exhibited the scavenging activity with the value being approximately more than 80% at the concentration of 31.25 µg/mLl. Furthermore, F2 (40% MeOH) from *L. pumila* displayed strong scavenging activity with the inhibition value of 74.62% at concentration of 1000 µg/mL. Likewise, the scavenging activity of F1 (20% MeOH) was 73.78%. The scavenging activity increased proportionally with the amount of crude and its fractions.

This result are in agreement with the previous studies which reported that *L. pumila var alata*demonstrated higher total antioxidant compared to *L. pumila var pumila* for all the tested methods [15]. Previous study confirmed that *L. pumila var alata* methanolic leaves extract have the highest concentration of different phenolic compounds compared to other varieties [23]. These results were in agreement with another study which stated that high polarity extract such as methanol extract had produced high antioxidant activity than other extracts [24].

3.3 Total Phenolic Content

The Folin-Ciocalteu assay was the most commonly used method to determine the content of total phenolic compound. In this current study, the total phenolic compound from crude extract and fractions were analysed. Results are shown in the Table 2.

Table 2 Total Phenolic Content (TPC) of crude and fractions of *L. pumila*

Sample	GAE (mg/g)
Crude	63.75±0.1
F1	73.75±0.1
F2	96.82±0.3

TPC values were expressed as Gallic acid equivalents (GAE) mg/g. (All values represent mean ± standard deviation of three different experiments and *p < 0.05 indicate significant difference compared to the standard (Gallic acid)

As shown in Table 2, F2 contained the highest total phenolic compound with the value of 96.82 mg/g. In contrast, crude *L. pumila* extract possessing the total phenolic content with 63.75 mg/g. Water appeared to be the effective extraction for phenolic compound, while ethanol and *n*-butanol displayed poor extraction capacities [25]. Previously, *L. pumila var alata* was examined based on the spectrophotometric data of total phenolic and flavonoid compounds. Based on the result, the fraction from the methanolic extractof this plant consist of nine flavonols like quercetin, myricetin and kaempferol and two flavanols such as catechin and epigallocatechin. In additions, nine phenolic acids were identified from this active fraction [14].

Phenolic compounds had been reported to possess potent antioxidant, anticancer, anti-inflammatory and anti-bacteria activities [26-27]. *L. pumila* was reported to contain abundance of polyphenol and flavonoid compounds [13-15]. Previous studies suggested that the antioxidant activity of many edible plant extracts increased as the polyphenol content of the extract increased. Besides that, the antioxidant of fruits and vegetables significantly increase as the concentration of total phenolic content increased [28-29].

L. pumila contains bioactive ingredient such as phenolics, flavonoids and saponins [14,23,25]. Therefore, other than polyphenolic and flavonoids, other effective anti -proliferative agent more likely to exits in the *L. pumila* extract that may be responsible to inhibit proliferation of prostate cancer cell lines.

4.0 CONCLUSION

In conclusion, Labisia pumila possessed anti-proliferative activity against prostate cancer cell lines. Strong anti-proliferation exerted by fraction(F2) might be due to the presence of bioactive anti-proliferative agent in *L. pumila*. Phenolic compound such as gallic acid demonstrated chemopreventive and suppressive activities against cancer cell lines particularly prostate cancer cell lines. Further studies are required to identify potential anti-proliferative compound of *L. pumila* extracts and also to study its roles in cellular cancer mechanism.

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