

## Original Research Article

# A novel anticancer effect of *Licuala longecalyculata* Furtado extracts on lung cancer cell line

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### Abstract

**Purpose:** This study was designed to investigate anticancer effect of ethanol and aqueous extracts of *Lygodium microphyllum* (Cav.) R.Br., *Lygodium salicifolium* Presl, and *Licuala longecalyculata* Furtado on human lung adenocarcinoma A549 cell line, and to determine total phenolic and flavonoid contents of those medicinal plants.

**Methods:** The three medicinal plants were extracted with ethanol and water using a rotary evaporator and freeze dryer, respectively. Cytotoxicity of the crude extracts at concentrations ranging from 3.125 to 200 µg/ml on A549 cells was determined using MTT assay at various times. Aluminium chloride colorimetric assay and the Folin-Ciocalteu colorimetric method were used to analyze total flavonoid and phenolic content, respectively.

**Results:** *L. longecalyculata* Furtado extracted with ethanol strongly inhibited A549 cell proliferation in a dose- and time-dependent manner with an IC<sub>50</sub> value of 12.00±0.41 µg/ml, whereas *L. microphyllum* (Cav.) R.Br. and *L. salicifolium* Presl did not. In addition to the morphological changes, an anticancer effect was observed in *L. longecalyculata* Furtado-treated A549 cells. *L. longecalyculata* Furtado showed the presence of phenolic and flavonoid compounds.

**Conclusion:** These results indicate that in accordance with local wisdom, *L. longecalyculata* Furtado crude extract can empirically inhibit lung cancer cell line, but *L. microphyllum* (Cav.) R.Br. and *L. salicifolium* Presl cannot. However, further study of *L. longecalyculata* Furtado is needed to identify phytochemical compounds and their mechanism of anticancer action, in order to develop them as a new therapeutic agent for lung cancer management.

**Keywords:** Anticancer, Cytotoxicity, *Licuala longecalyculata* Furtado, Lung cancer

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## INTRODUCTION

Cancer currently ranks as a “leading cause of premature death in 134 of 183 countries in the world”, including Thailand [1]. According to

GLOBOCAN 2020 estimates, Asia accounts for 49.3% of global cancer incidence and 58.3% of cancer mortality. The predicted global burden of cancer in 2040 is 28.4 million cases, a 47% increase from 2020. Worldwide, lung cancer is a

leading cause of cancer mortality—ranking highest for both incidence and mortality in men, but third and second, respectively, in women [2]. Meanwhile, lung cancer ranks second in terms of both cancer incidence and mortality in Thailand [3]. Also, bronchial and lung cancer were reported as one of ten ranking causes of death in Narathiwat province [4].

In our increasingly globalized world, natural products are popular and widely used as complementary and alternative medicine (CAM). Reasons given for use of CAM include a belief in its effectiveness, dissatisfaction with conventional medicine, and perceived safety [5]. In the United States, the National Center for Complementary and Integrative Health (NCCIH) has classified herbal products as the most popular biologically-based therapies [6, 7]. As with previous reports, natural-derived compounds, including phytochemicals, from medicinal plants play a significant role in cancer prevention and treatment. Several are undergoing clinical trials and clinical use, including curcumin (Zingiberaceae), camptothecin (Nyssaceae), vincristine sulfate (Apocynaceae), and paclitaxel (Taxaceae) [8-10]. Narathiwat is located in southern Thailand and has a unique natural resource in Sirindhorn peat swamp forest. It is Thailand's largest peat swamp forest and has high biodiversity. According to local wisdom in the Sirindhorn peat swamp forest area, *Lygodium microphyllum* (Cav.) R.Br., *Lygodium salicifolium* Presl, and *Licuala longecalyculata* Furtado are used as medicinal plants for cancer treatment [11]. In a traditional medicine practice [11], stems of *L. microphyllum* (Cav.) R.Br. or *L. salicifolium* Presl were boiled and drunk thrice a day after meals by early-stage cancer patients. Young shoots of *L. longecalyculata* Furtado, raw or cooked, were eaten at least once a day with meals to treat cancer patients. However, anticancer activities and medical properties of the three medicinal plants have never been scientifically reported. *L. microphyllum* (Cav.) R.Br. has only been reported to affect the hepatoprotective mechanism, mitigating carbon tetrachloride-mediated oxidative stress by maintaining antioxidant enzyme activity [12]. In the same family as *L. longecalyculata* Furtado, *Licuala spinosa* has been reported to be active against *Mycobacterium tuberculosis* H37Rv [13], *Candida albicans*, and malaria parasite *Plasmodium falciparum* [14]. *L. microphyllum* (Cav.) R.Br. and *L. salicifolium* Presl are in family Lygodiaceae, which are native to Asia, Africa, Australia, and United States [15, 16]. *L. longecalyculata* Furtado, in family Arecaceae, is distributed throughout the peat swamp forest of

Narathiwat, and its shoots are eaten both raw and cooked as part of the local diet [17].

The objectives of this study were to investigate anticancer effect of ethanol and aqueous extracts of *L. microphyllum* (Cav.) R.Br., *L. salicifolium* Presl, and *L. longecalyculata* Furtado on lung cancer cell line, and to determinate total phenolic and flavonoid contents of those medicinal plants. These exploratory findings may lead to the development of a new therapeutic drug for lung cancer management.

## EXPERIMENTAL

### Preparation and extraction of medicinal plants

The stems and leaves of *L. microphyllum* (Cav.) R.Br. and *L. salicifolium* Presl, as well as the young shoots of *L. longecalyculata* Furtado were obtained from Sirindhorn Peat Swamp Forest Nature Research and Study Centre, Narathiwat, Thailand. These were identified by Mr. Jarearnsak Sae Wai and the specimens were deposited at Prince of Songkla University (PSU) Herbarium.

The aforementioned parts of those medicinal plants were cleaned and cut into small pieces and then dried in a hot air oven (Memmert) at 45 °C for 3 days. Maceration ratio of each dried plant to absolute ethanol (Merck KGaA; Darmstadt, Germany) was 1:4 (g: ml) for 72 h at room temperature while decoction ratio of each dried plant to distilled water was 1:20 (g: ml) for 15 minutes [18]. After maceration and decoction, the supernatants were filtered using Whatman filter paper (Grade 1). Subsequently, ethanol crude extract was concentrated in a vacuum below 45 °C using a rotary evaporator (Heidolph/Hei-VAP Precision) and aqueous crude extract was lyophilized using a freeze dryer (Labconco/ FreeZone Plus-84C). The crude extracts obtained were kept in humidity controllers (Weifo/DRY-70) at 40% RH and 25 °C until further analysis. Percentage yield of extraction was determined as follow:

$$\text{Percentage yield} = \left[ \frac{\text{weight of crude extract}}{\text{weight of dried plant}} \right] \times 100$$

### Cytotoxic assay

Two cell lines were used in this assay, namely A549 cells (human lung carcinoma) with L929 cells (Mouse fibroblast) as non-cancerous control cells. A549 cells were maintained in RP10 (RPML-1640 medium containing L-glutamine [Gibco; New York, USA] supplemented with 10%

heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin sulfate). L929 cells were maintained in DM10 (DMEM [Gibco; New York, USA] with the same supplements as RP10).

Cytotoxicity of ethanol and aqueous crude extracts in A549 and L929 cells was assessed by MTT assay (Invitrogen; Oregon, USA). This assay measures metabolic activity as a proxy for cell viability. Briefly, the cells were seeded at a density of  $1 \times 10^4$  cells/well into 96-well flat-bottomed plates in the absence or presence of various concentrations of each crude extract, in triplicate, and incubated in a humidified incubator with 5% CO<sub>2</sub> and 37 °C for 24, 48 and 72 h. Each concentration of crude extract was used in two-fold serial dilutions from 3.125 to 200 µg/ml. The cell control wells without crude extract were used as control cells. In conditions without cells, a control blank was set without each crude extract, and the treated blank was set with each concentration of crude extracts. Both conditions were used as negative controls to determine background absorbance. Doxorubicin and curcumin served as positive controls. Subsequently, the supernatant in each well was gently discarded. Next, 100 µl of MTT reagent (5 mg/ml) was added to each well, and the plates were incubated at 37 °C for 2 h. After incubation, the supernatant was gently discarded, and then insoluble purple formazan sediment was dissolved completely with 100 µl DMSO (Sigma-Aldrich; Missouri, USA) for 30 minutes. Finally, OD of absorbance was measured at 570 nm for test wavelength and 630 nm for reference wavelength. Results were recorded as mean ± SD of triplicate cultures of three separate experiments. Percentage of cell viability determined as follow:

$$\text{Cell viability} = \frac{[\text{OD treated cells} - \text{OD treated blank}]}{[\text{OD control cells} - \text{OD control blank}]} \times 100$$

The percentage of cell viability values and concentration of crude extracts were plotted in order to determine the median inhibition concentration (IC<sub>50</sub>) values of each extract. Based on IC<sub>50</sub> values, criteria of cytotoxic activity for the crude extract according to National Cancer Institute guideline and Nguyen et al [19] are as follows: ≤20 µg/ml considered highly active, 21-200 µg/ml considered moderately active, 201-500 µg/ml considered weakly active, and >501 µg/ml considered inactive.

#### Determination of total phenolic content

Total phenolic content was analyzed using Folin-Ciocalteu colorimetric method with modification according to Yildirim et al [20]. Briefly, 20 µl of

crude extract at a 1 mg/ml concentration was added to a 96-well flat-bottomed plate, and 100 µl of tenfold diluted 2M Folin-Ciocalteu's reagent was added into each well. Then 80 µl of 7.5% Na<sub>2</sub>CO<sub>3</sub> was added to the mixture, and the plate was incubated for 30 minutes in the dark at room temperature. After incubation, absorbance was detected with a microplate reader (Bio-Rad; California, USA) at 765 nm. The total phenolic content was compared with gallic acid standards curve. Data were calculated as mean ± SD (n=3) and showed as milligrams of gallic acid equivalents (GE) per gram of crude extract.

#### Determination of total flavonoid content

Total flavonoid content was determined by aluminium chloride colorimetric assay with modification according to Yildirim et al [20]. Briefly, 500 µl of 1 mg/ml extract were mixed with 75 µl of 5% NaNO<sub>2</sub> for 6 minutes. 150 µl of 10% AlCl<sub>3</sub> were added to the mixture, and then 500 µl of 1M NaOH and 275 µl of distilled water were added. The mixture was incubated at room temperature for 15 minutes. After incubation, absorbance was detected with a microplate reader (Bio-Rad; California, USA) at 510 nm. The total flavonoid content was compared with quercetin standards curve. Data were calculated as mean ± SD (n=3) and showed as milligrams of quercetin equivalents (QE) per gram of crude extract.

#### Statistical analysis

Results were recorded as mean ± SD. The data were analyzed using Microsoft Excel and GraphPad Prism Software 9 (GraphPad Software; California, USA). An unpaired t-test was performed for cytotoxicity to determine differences between treated A549 cells and treated L929 cells. *P*-value of less than 0.01 was considered statistically significant.

## RESULTS

The percentage yield of aqueous extracts of *L. microphyllum* (Cav.) R.Br., *L. salicifolium* Presl, and *L. longecalyculata* Furtado were 11.10%, 11.74%, and 13.89%, respectively. These yields were higher than those of ethanol extracts (6.16%, 3.14%, and 0.96%, respectively) as shown in Table 1.

#### Cytotoxicity of crude extracts

To investigate cytotoxicity of crude extracts, A549 cells were treated with various concentrations of each crude extract for 72 h and its cytotoxicity was assessed by MTT assay. The

**Table 1:** Percentage yield and cytotoxicity of medicinal plant extractions. The IC<sub>50</sub> results on A549 cells for 72 h were mean ± SD of three independent experiments

Plant	%yield (w/w)		IC <sub>50</sub> value ± SD (µg/ml)	
	Ethanol extract	Aqueous extract	Ethanol extract	Aqueous extract
<i>Lygodium microphyllum</i> (Cav.) R.Br.	6.16	11.10	N/A	N/A
<i>Lygodium salicifolium</i> Presl	3.14	11.74	N/A	N/A
<i>Licuala longecalyculata</i> Furtado	0.96	13.89	12.00±0.41 <sup>a</sup>	181.40±5.19

<sup>a</sup>According to the National Cancer Institute guideline for a natural product extract, criteria of highly cytotoxic activity for the crude extract is an IC<sub>50</sub>≤20 µg/ml [19]. N/A is not available

cytotoxic effect of each crude extract is shown in Table 1. Regarding *L. longecalyculata* Furtado, the ethanol extract strongly inhibited A549 cell proliferation with an IC<sub>50</sub> value of 12.00±0.41 µg/ml, whereas the aqueous extract was moderately active (181.40±5.19 µg/ml) against cancer cells. The ethanol and aqueous extracts of *L. microphyllum* (Cav.) R.Br. and *L. salicifolium* Presl at concentrations ranging from 3.125 to 200 µg/ml showed no inhibitory effect on the proliferation of lung cancer cells.

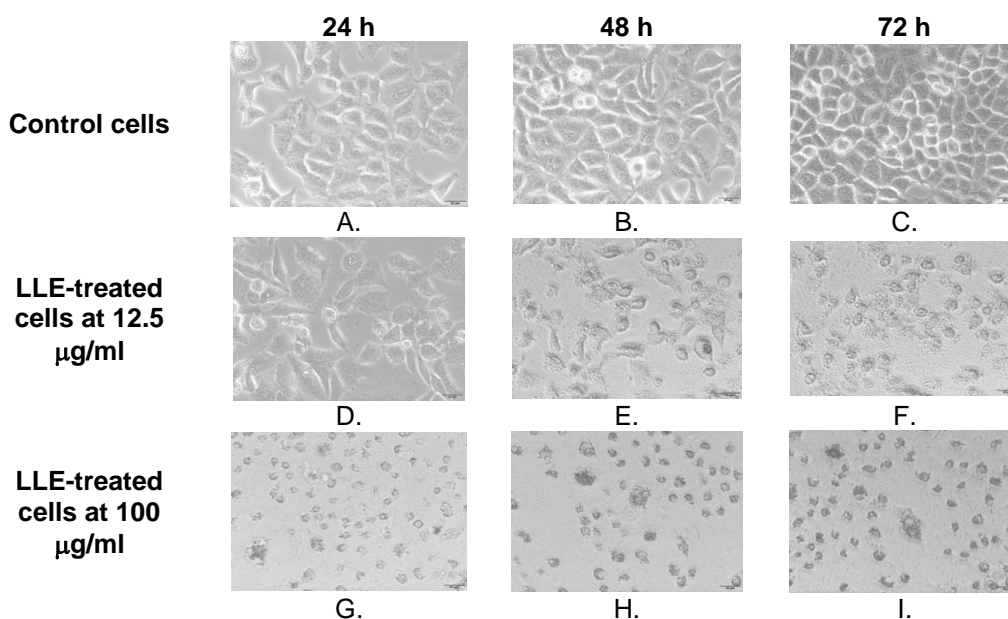
The ethanol extract of *L. longecalyculata* Furtado was highly cytotoxic activity against A549 cell for 72 h. To investigate the anticancer effect of this extract on lung cancer cells (A549), MTT assays were performed at intervals. L929 cells were taken as non-cancerous control cells.

As Table 2 shows, *L. longecalyculata* Furtado ethanol extract had a statistically-significant difference ( $P<0.01$ ) in inhibition of A549 cells and L929 cells at 24 and 72 h. The *L. longecalyculata* Furtado extracted with ethanol inhibited A549 cell

proliferation in a time-dependent manner, in which extract-treated cells were highly active at 48 and 72 h, with IC<sub>50</sub> values of 15.45±0.45 and 12.00±0.41 µg/ml, respectively. Also, extract-treated A549 cells at 24 h showed moderate inhibitory activity (IC<sub>50</sub>=22.04±1.20). In addition to morphological changes, an anticancer effect was observed in the extract-treated A549 cells at 24, 48, and 72 h (Figure 1). The extract-treated L929 cells exhibited highly-inhibitory activity (IC<sub>50</sub>=16.61±0.91) at 48 h and moderate inhibitory activity (IC<sub>50</sub>=24.13±1.21) at 72 h (Table 2).

**Table 2:** Cytotoxicity of *L. longecalyculata* Furtado extracted with ethanol on A549 and L929 cells at 24, 48, and 72 h. Results were mean ± SD of triplicate cultures of three independent experiments. \*indicates a P-value of less than 0.01.

Time	IC <sub>50</sub> value ± SD (µg/ml)		P-value
	A549 cells	L929 cells	
24 h	22.04±1.20	28.20±1.15	*0.003
48 h	15.45±0.45	16.61±0.91	0.147
72 h	12.00±0.41	24.13±1.21	*0.001

**Figure 1:** Morphology of A549 cells treated with *L. longecalyculata* Furtado ethanol extract (LLE) at 12.5 µg/ml (D, E, F) and 100 µg/ml (G, H, I) versus untreated control cells (A, B, C) for 24, 48, and 72 h, respectively. The cell images were captured using a microscope at 20x objective

### Total phenolic and flavonoid contents of *L. longecalyculata* Furtado ethanol extract

The total phenolic content of *L. longecalyculata* Furtado extracted with ethanol was  $0.138 \pm 0.003$  mg/g, reported as milligrams of GE per gram of *L. longecalyculata* Furtado extract. Total flavonoid content was  $0.117 \pm 0.002$  mg/g, shown as milligrams of QE per gram of this extract.

## DISCUSSION

This study demonstrated that *L. longecalyculata* Furtado crude extract can inhibit lung cancer cell line proliferation, whereas *L. microphyllum* (Cav.) R.Br. and *L. salicifolium* Presl do not. The ethanol extract of *L. longecalyculata* Furtado strongly inhibited A549 cell proliferation with morphological changes in a dose- and time-dependent manner. Additionally, it revealed the presence of phenolic and flavonoid compounds. Non-cancerous cell line treated with *L. longecalyculata* Furtado showed a statistically-significant increase in survival time adaptation.

Our study revealed that *L. longecalyculata* Furtado extracted with ethanol showed high efficacy against lung cancer cell line and was composed of phenolic and flavonoid compounds. In previous reports, natural phenolic compounds like flavonoid (green tea), curcumin (turmeric), cucurbitacin B (Cucurbitaceae family), benzyl isothiocyanate (papaya seeds), and isoflavone (soy) showed potential to inhibit lung cancer. They can affect multiple biochemical pathways involved in lung cancer, for example, inducing apoptosis through downregulation of Bcl-2 and NK- $\kappa$ B pathway or modulation of PI3K/AKT/mTOR signaling pathway, and epigenetic alterations in cells via modulation of DNA methylation and chromatin modeling [21-23]. Consequently, we hypothesize that *L. longecalyculata* Furtado crude extract may have a similar mechanism of anticancer actions. Identification of these inhibitory mechanisms and phytochemical compounds will remain part of our plans for future study.

For normal cells treated with *L. longecalyculata* Furtado, an increase in survival adaptations was observed over time. Interestingly, nanotechnology-based delivery of phytochemicals as chemotherapeutic drugs against cancer has potential to overcome the limiting side-effects on normal cells, due to target-specific manner of delivery [24, 25]. Recent advancements in phytochemical-based nanomedicine approved by Food and Drug Administration (USA) for clinical

trials and clinical use in the treatment of various cancers are commercially available, including paclitaxel (Taxaceae), Abraxane<sup>®</sup>; vincristine (Apocynaceae), Marqibo<sup>®</sup>; and camptothecin (Nyssaceae), L9-NC. Also, paclitaxel and camptothecin are currently under clinical trial as effective treatments for non-small cell lung cancer [10, 26]. Our pioneering research suggests that *L. longecalyculata* Furtado has the potential to enable the development of a new therapeutic drug for lung cancer management.

## CONCLUSION

This study has not only revealed that *L. longecalyculata* Furtado extracted with ethanol can inhibit lung cancer cells with morphological changes in a dose- and time-dependent manner, but also showed the presence of phenolic and flavonoid compounds. The non-detection of cytotoxic effects in the other plants may be attributed to the extraction procedure adopted in this work. However, further studies are needed not only to identify the phytochemical compounds present in *L. longecalyculata* Furtado and their mechanism of anticancer action but on the possible anticancer properties of the other plants. In addition, more studies will be conducted on *L. longecalyculata* Furtado to explore its possible development as a new therapeutic agent for cancer prevention and treatment.

## DECLARATIONS

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### Ethical approval

None provided.

### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Conflict of interest

No conflict of interest is associated with this work.

### Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Aornrutai Promsong designed study, performed experiments, directed project, interpreted results, prepared and revised manuscript. Lanlalin Nasomyon, Saowanee Maungchanburi, Rachata Saksawad, and Arisara Pratakkarn performed experiments. All authors read and approved the final manuscript.

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