Research Article

Kinetics Extraction Modelling and Antiproliferative Activity of *Clinacanthus nutans* Water Extract

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Clinacanthus nutans is widely grown in tropical Asia and locally known "belalai gajah" or Sabah snake grass. It has been used as a natural product to treat skin rashes, snake bites, lesion caused by herpes, diabetes, fever, and cancer. Therefore, the objectives of this research are to determine the maximum yield and time of exhaustive flavonoids extraction using Peleg's model and to evaluate potential of antiproliferative activity on human lung cancer cell (A549). The extraction process was carried out on fresh and dried leaves at 28 to 30°C with liquid-to-solid ratio of 10 mL/g for 72 hrs. The extracts were collected intermittently analysed using mathematical Peleg's model and RP-HPLC. The highest amount of flavonoids was used to evaluate the inhibitory concentration (IC_{50}) via 2D cell culture of A549. Based on the results obtained, the predicted maximum extract density was observed at 29.20 ± 14.54 hrs of extraction ($t_{exhaustive}$). However, the exhaustive time of extraction to acquire maximum flavonoids content exhibited approximately 10 hrs earlier. Therefore, 18 hrs of extraction time was chosen to acquire high content of flavonoids. The best antiproliferative effect (IC_{50}) on A549 cell line was observed at 138.82 ± 0.60 μg/mL. In conclusion, the flavonoids content in *Clinacanthus nutans* water extract possesses potential antiproliferative properties against A549, suggesting an alternative approach for cancer treatment.

1. Introduction

In Malaysia, there were new cases of cancer that have been reported with approximately 30,000 every year most commonly including colorectal cancer, lung cancer, prostate cancer, leukemia, lymphoma, stomach, larynx, liver cancer breast, cervical cancer, and uterine cancer [1]. At the moment, administration of chemotherapy treatment is the best method to be used in reducing human mortality due to cancer prevalence [2]. However, other potential alternative therapeutics via natural product (phytochemicals constituent) are recently being considered as chemopreventive agents in reducing those prevalence. In the past several years, traditional herbs received considerable attention because of their potential to reduce the formation or progression of

certain types of cancer [3]. The valuable constituents in herbs include flavonoid, terpenoid, alkaloids, phenolic, saponins, tannins, and lignin [4]. Among these, flavonoid was highlighted by many researchers because of their biological activities including antioxidant, anti-inflammatory, antiallergic, antimutagenic, antibiotic, and anticarcinogenic properties [5]. Flavonoids are amongst the imminent compounds to date to be classified as diphenolic compounds [6] which are large secondary metabolites in plants [7]. They are categorized into various subclasses including flavones, flavonol, flavanones, isoflavanones, isoflavonoids, anthocyanidins, and catechins [8].

These compounds practically are present in dietary plants, fruits, and roots and should be consumed daily in considerable amounts [9]. One of the recent medicinal plants

TABLE 1: Common names and traditional use of *Clinacanthus nutans* [10].

Country	Common names	Application
Malaysia	Belalai Gajah Sabah Snake Grass	Herbal tea, antioxidant and cancer treatment [20]
Thailand	Phaya po Phaya plontong	Skin rashes, snake bites, herpes simplex virus (HSV), and varicella-zoster virus (VZV), diabetes mellitus, fever, and diuretics [21]
China	E zui hua Qing Jian	Antihepatitis [22]
Indonesia	Dandang gendis	Antimalaria [23]

FIGURE 1: Fresh leaves of *Clinacanthus nutans*.

which has purportedly contained phytochemicals to prevent cancer via tea concoction is *Clinacanthus nutans* or Sabah Snake Grass (Figure 1). *Clinacanthus nutans* is a medical herb which belongs to family *Acanthaceae* [10]. It is a small shrub and has been used traditionally in tropical Asia [11, 12]. This herb is commonly known as "Belalai Gajah" in Malaysia and many other names from different countries (Table 1). This plant is used externally for treatment of skin rashes, insect and snake bites, *diabetes mellitus*, fever, diuretics, herpes simplex virus (HSV), and varicella-zoster virus (VZV) [13]. Besides, it is also reported as having antihepatitis, antiherpes, anti-inflammatory properties [14] and even used to prevent and cure cancer [15].

Previous study has shown that the aerial part of the plant such as stem and leaves contains high amount of bioactive constituents when it is being extracted using specific organic solvents [16]. *Clinacanthus nutans* leaves can also be consumed as raw material, mixed juice, tea, and fresh drink [10, 17]. Fresh leaves of this herb are highly demanded for patient with cancer, diabetes, and general ailments [18]. The major principle phytochemical constituents of *Clinacanthus nutans* are stigmasterol, β-sitosterol, lupeol, betulin, six known C-glycosyl flavones, vitexin, isovitexin, schaftoside, isomollupentin, 7-O-β-gluco pyranoside, orientin, isoorientin, two glycoglycerolipids, a mixture of nine cerebrosides and a monoacylmonogalactosylglycerol, and five sulfur-containing glucosides [19]. With all of those unknown phytochemicals beneficial available in the plant therefore, the objectives of this research are to determine the maximum yield and time of exhaustive flavonoids extraction using Peleg's model and to evaluate the potential of antiproliferative activity on human lung cancer cell (A549).

2. Materials and Methods

2.1. Plant Material. *Clinacanthus nutans* fresh leaves were collected from "Pusat Pertanian Pantai Baru," Seremban, Malaysia. The plant was harvested right after it has reached a month old (young leaves) [18].

2.2. Chemicals and Cell Line. PrestoBlue (Invitrogen™) was used to evaluate the cell viability. The cell culture medium, phosphate buffer saline (PBS), trypsin-EDTA, fetal bovine serum (FBS), RPMI-1640, and antibiotics were purchased from Gibco™. Cancer cell line A549 was purchased from America Type Culture Collection (ATCC). Analytical chemical standard (orientin and vitexin), methanol, and glacial acetic acid (HPLC grade, Sigma Aldrich brand) were purchased from Euroscience, Kuala Lumpur.

2.3. Normal Soaking Extraction (NSE). All fresh leaves were washed with distilled water to remove all dirt and separated into two parts. There were two types of preparation: (1) fresh and (2) dried samples. For fresh sample, tissue paper or clean cloth was used to remove any residues and excess water on the leaves. Meanwhile, the dried leaves were put onto the aluminum tray and oven-dried at 50°C for 24 hrs. Both samples were then ground and kept in bottles prior to extraction process. 30 g of dried and fresh samples was wrapped with muslin bag and the extraction was carried out in 500 mL beakers (solid-to-solvent ratio of 1 : 10 (w/v)) [24] for 72 hrs. Next, every 2 hrs interval, 1 mL of extract was collected using a micropipette and weighed for the extract density measurement (g/mL) and flavonoids content analysis by means of RP-HPLC ($n = 3$).

2.4. Kinetic Extraction Model. Peleg's model is mathematical modelling and a useful engineering tool able to predict the maximum concentration/yield of the extract at the exhaustive time point [25]. Two constant K in this model show that K_1 is a value for y-intercept and K_2 is the gradient value through kinetics curves and plateau to the linear graph of time (t)/concentration (ρ) versus time (t) [26]. Peleg's model is shown as

$$\rho(t) = \rho_0 \pm \frac{t}{[K_1 + K_2 \cdot t]}, \tag{1}$$

where ρ is the extract concentration (g/mL) at t (min), ρ_0 is the initial concentration at $t = 0$ (g/mL), K_1 is the constant value for Peleg 1 (min·g/mL), and K_2 is the constant value

for Peleg 2 (mL/g). The initial concentration (ρ_0) at $t = 0$ is assumed as 0, so the linear regression of (2) is derived as

$$\frac{t}{[\rho(t) - \rho_0]} = K_1 + K_2 \cdot t. \tag{2}$$

Peleg 1 constant value (K_1) is related to the rate of extraction (B_0) at specific time p ($t = t_0$) as shown in

$$B_0 = \frac{1}{K_1}. \tag{3}$$

Meanwhile, the Peleg capacity constant K_2 relates to the maximum extraction capacity. When $t \to \infty$, the extraction reaches equilibrium between the dissolved substances in a sample of bulk volume of the extract. Finally, (4) gives essential relationship between the equilibrium of the extract concentration and the constant value of K_2:

$$\rho t \longrightarrow \infty = \rho = \frac{1}{K_2}. \tag{4}$$

2.5. Flavonoids Identification Using HPLC Analysis. Further analysis of liquid extract using reverse-phase high performance liquid chromatography (RP-HPLC, Shimadzu) equipped with solvent (LC-20AD), column oven (SPD-20AV), UV/VIS detector (SPD-20AV), autosampler (SIL-20AC), and LC solution Workstation was done. The chromatographic separation was performed using a XBridge C_{18} (4.6 mm × 250 mm, 5 μm, Waters, Ireland) at 30 ± 1°C. The solvent system consisted of 2 solvents: A: 0.1% glacial acetic acid and B: MeOH. The flow rate and injection volume were adjusted at 10 μL and 0.8 mL/min, respectively. The detection was monitored at 280 nm. Gradient elution was performed as follows: 100% A for 1 min; 90% A for 15 mins; 80% A for 20 mins; 60% A for 28 mins; 20% A for 30 mins. Individual orientin and vitexin contents were determined by internal standard response factor method:

$$\text{Response Factor (RF)} = \frac{\text{Peak standard area } (\mu\text{v}*\text{s})}{\text{Standard conc. (mg/mL)}},$$

$$\text{Sample Concentration (mg/mL)} \tag{5}$$

$$= \frac{\text{Peak sample area } (\mu\text{v}*\text{s})}{\text{RF } (\mu\text{v}*\text{s}/(\text{mg/mL}))}.$$

2.6. IC_{50} Value of Clinacanthus nutans Extract. Serial concentrations of dried leaves extract were tested on human lung cancer cell (A549) for its antiproliferative properties. Briefly, about 1.0×10^4 cells of A549 were seeded into 96 well-plate and then incubated for 24 hrs at 37°C in 5% CO_2 incubator. After overnight incubation, serial dilutions of the extract ranging from 10 to 1000 μg/mL were treated onto A549 cell lines that had been seeded earlier in the 96-well plate. The incubation process continued for 24, 48, and 72 hrs to observe the treatment effect on the cells growth. After 24 hrs of posttreatment, 10 μL of PrestoBlue solution was added into well plates and incubated for 1 hr in 5% CO_2
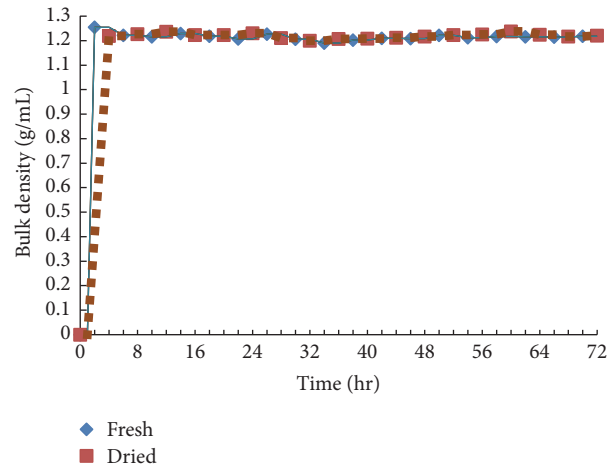

FIGURE 2: Conventional kinetic equilibrium extraction of *Clinacanthus nutans* fresh and dried leaves ($n = 3$).
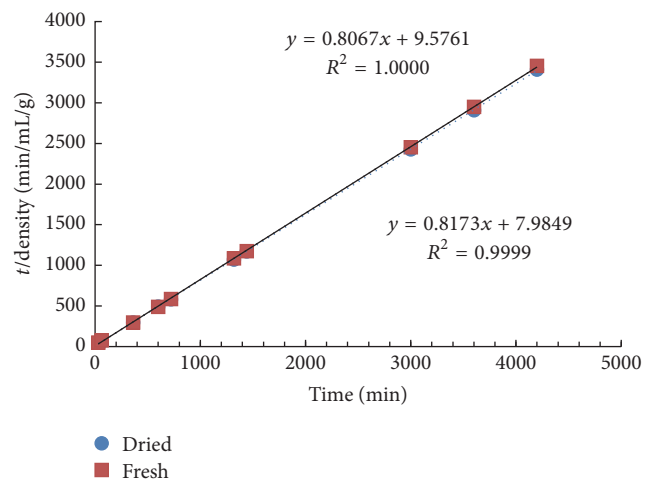

FIGURE 3: Peleg model kinetic regression line of *Clinacanthus nutans* fresh and dried leaves water extract ($n = 3$).

incubator. The colour changes from well plates were read on a micro-ELISA reader using a wavelength of 570 nm. The IC_{50} values were determined based on log concentrations of the extract against probit value (data not shown).

3. Results and Discussion

3.1. Peleg's Model (Extract Bulk Density). The conventional kinetic of extraction (Figure 2) shows there was insignificant difference ($p > 0.05$) in its extract density profile between both dried and fresh leaves sample. For that reason, the Peleg mathematical model ((2), (3), and (4)) was implemented to determine the maximum extract density (g/mL) and exhaustive time of extraction (hr) theoretically. The regression line from Figure 3 was plotted to determine the maximum yield/extract density of extraction (K_1). The essential constant K_1 (y-intercept) is related to the extract mass/density. The lower the K_1 value, the higher the extraction yield/density [25]. Finally, the logarithm equation in Figure 4 was used to
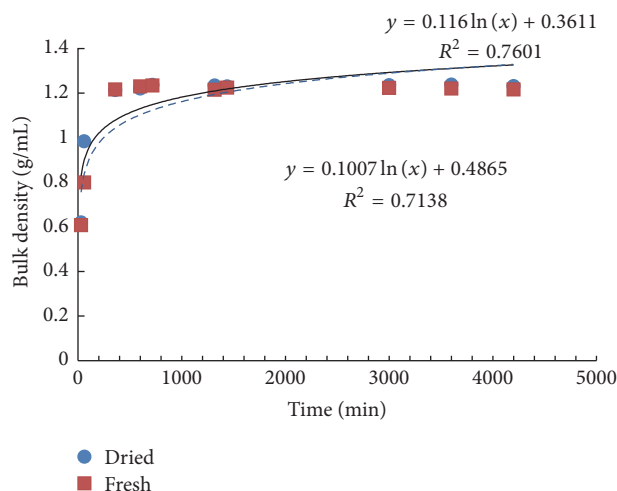
FIGURE 4: Peleg model kinetic equilibrium extraction of *Clinacanthus nutans* fresh and dried leaves ($n = 3$).

TABLE 2: Peleg's maximum extract bulk density (g/mL) and exhaustive time (hr) of fresh and dried *Clinacanthus nutans* leaves water extract.

Peleg response variables	Sample (leaves)	
	Fresh	Dried
*Rate of extraction (g/mL·hr)	1.18 ± 0.02	1.22 ± 0.01
*Max extract density (g/mL)	1.22 ± 0.02	1.24 ± 0.22
*Extraction time (hr)	28.45 ± 13.07	29.20 ± 14.54

*$p > 0.05$: insignificantly different between fresh and dried samples.

determine the exhaustive time of extraction ($t_{\text{exhaustive}}$) based on the K_1 value.

Table 2 represents the Peleg response variables between fresh and dried leaves extracts. Both extracts did not demonstrate any significant differences on all responses. Both extracts exhaustively produced maximum density ranging from 1.20 to 1.46 g/mL after 29 hrs ($t_{\text{exhaustive}}$) of extraction attained.

However, contrary to most of the tea manufacturer's recommendation on the brewing time (not more than 3 mins/serve), it shows that tea sachet itself in fact can be used several more times as the longer the extraction time occurred, the greater the essence can be extracted literally [27]. For the first 30 mins of the extraction process, almost 80% of the essence has been extracted rapidly with a slower rate for the next 30 mins onwards (Figure 2). For that reason, as both fresh and dried leaves extracts produced more or less the same extract density (maximum), dried leaves have been chosen for next Peleg's model analysis by means of flavonoids concentrations. Dried leaves were selected as to replicate the condition of the commercially available *Clinacanthus nutans* dried leaves tea and in fact more shelf-stable.

3.2. Peleg's Model (Flavonoids Concentration).

The extraction of high content of flavonoids in dried leaves was carried out for 72 hrs ($n = 3$). The normal soaking water extraction was
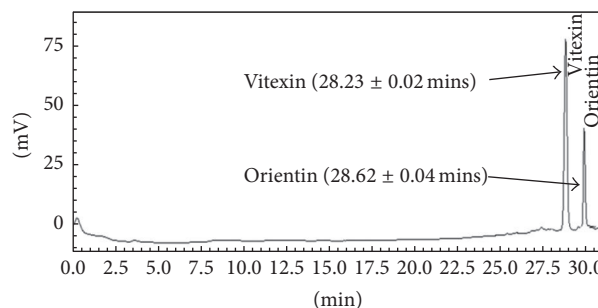

FIGURE 5: HPLC-UV/DAD chromatogram profile of both flavonoids compounds (vitexin and orientin).

carried out due to the fact that it is nontoxic and environmentally friendly solvent as it is universally suitable for natural product applications [28]. Peleg model was utilized ((2), (3), and (4)) in order to determine the optimum flavonoids yield (mg) via internal standard method RP-HPLC and exhaustive time of extraction (hr) for both orientin and vitexin. Figure 6 shows that the regression line was plotted to determine the maximum yield (mg) (K_1). Finally, the logarithm equation (Figure 7) was used to determine the exhaustive time of extraction ($t_{\text{exhaustive}}$) which was based on the K_1 value in Figure 6.

In the results based on Table 3, the yield of orientin (133.97 ± 0.04 mg) shows 1.8-fold higher than vitexin (76.55 ± 0.013 mg) as the extraction process reaches more than 18 hrs as both flavonoids coexisted in the dried leaves extract ($p < 0.05$). There was not much difference in the rate of extraction between orientin (3.25 ± 0.0001 mg/hr) and vitexin (3.58 ± 0.03 mg/hr) ($p > 0.05$). However, it took approximately 18 hrs (17.72 ± 0.70 hrs) to acquire maximum yield of orientin. As for the maximum vitexin content, it will theoretically remain from 7.55 ± 0.72 hrs up to 18 hrs of extraction period. For that reason, the best period to produce exhaustive extraction condition is approximately 18 hrs as both prominent flavonoid compounds were at its maximum range of acquisition.

Meanwhile, based on the Peleg model analysis, 18 hrs of extraction extract (the exhaustive time to produce the highest amount of orientin) was selected and undergone for the flavonoids compounds identification and verification via external standard method by comparing the retention times of flavonoids analytical standards with the peak obtained from the extract (Figure 5). Only 2 peaks were detected in the extract and verified using those analytical standards. For that reason, it was confirmed that both retention times were for orientin (28.62 ± 0.04 mins) and vitexin (28.23 ± 0.02 mins).

3.3. Antiproliferative Activity (IC_{50}) via Probit Analysis.

Based on the Peleg kinetic extraction model, the ground dried leaves were extracted in distilled water for 28 hrs (Table 3) presumably that, at the highest extract density used, both flavonoids (orientin and vitexin) were still in their constant maximum range of acquisition. The maximum extract density (whole extract) was used as a point of reference prior to dilution (Table 2: initial extract density, 1.24 ± 0.22 g/mL). Therefore,
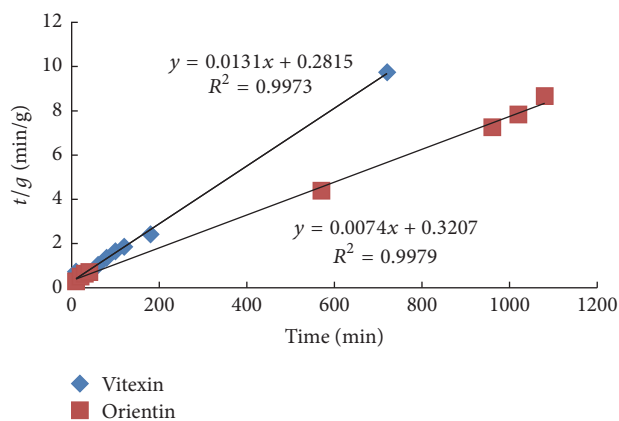
FIGURE 6: Peleg model kinetic regression line of orientin and vitexin in dried leaves extract ($n = 3$).
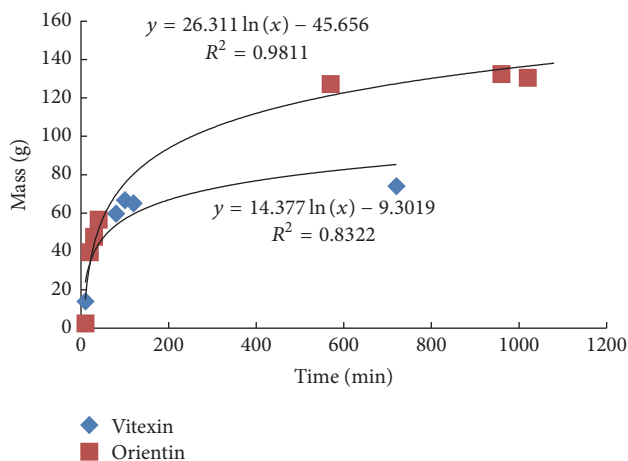

FIGURE 7: Peleg model kinetic equilibrium extraction of orientin and vitexin dried leaves extract ($n = 3$).

TABLE 3: Peleg's maximum yield (mg) of orientin and vitexin and exhaustive time (hr) of dried leaves *Clinacanthus nutans* water extract.

Peleg response variables	Orientin	Vitexin
Rate of extraction (mg/hr)	3.25 ± 0.0001	3.58 ± 0.03
*Max yield (mg)	133.97 ± 0.04	76.55 ± 0.013
*Extraction time (hr)	17.72 ± 0.70	7.55 ± 0.72

*$p < 0.05$: significant difference between both flavonoids compounds.

serial dilution was prepared ranging from 10 to 1000 μL/mL. As shown in Figure 8 and Table 4, 50% of cell mortality (IC$_{50}$) and antiproliferative activity was observed in day 3. The respective IC$_{50}$ values were determined based on log concentration versus probit value: 260.38 ± 0.36 (24 hrs), 138.82 ± 0.60 (48 hrs), and 817.71 ± 0.19 (72 hrs) μg/mL. Based on the previous study carried out by Yong et al. [14], the IC$_{50}$ of *Clinacanthus nutans* was observed at 41.88 ± 2.81 μg/mL concentration after 72 hrs of treatment using the methanolic extract.
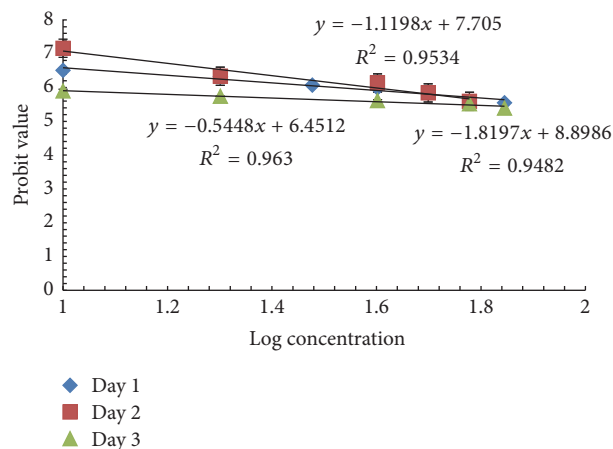

FIGURE 8: Determination of IC$_{50}$ values on various concentration of *Clinacanthus nutans* extracts (24, 36, and 48 hrs of treatment).

TABLE 4: Inhibitory concentration (IC$_{50}$) of the extracts (24, 36, and 48 hrs of treatment).

Day	IC$_{50}$ value (μg/mL)
1	260.38 ± 0.36
2	138.82 ± 0.60
3	817.71 ± 0.19

Those IC$_{50}$ values of *Clinacanthus nutans* aqueous extract indicate a great potential in inhibiting cancer cells. The IC$_{50}$ value on day 2 (138.82 ± 0.60 μg/mL) was chosen for further evaluation due to the fact that it has the lowest concentration to inhibit cancer cell (lower IC$_{50}$) [2, 29]. Ideally, the phenolic constituent like flavonoids possesses the antioxidant activity [4, 30] donating electron to react with free radical species and converting them to stable metabolite and terminating the radical chain reaction [15]. This mechanism could possibly lead to the induction of cancer cell further damage and death. Moreover, this antiproliferative activity itself did not show their potential alone but it was due to the presence of combination of few flavonoid compounds [30] which seems to be well correlated with the availability of those 2 eminent flavonoids (vitexin and orientin) in this water extract. In fact, even though other organic solvents (e.g., methanolic leaves extract) were used to extract those important phenolic constituents and produced great responses of killing cancer cells, the subacute oral administration to the rats revealed that no toxicity effect has been recorded/observed [16]. Meanwhile, based on the cell morphology initial posttreatment (Figure 9(b): 24 hrs), the cells started to readapt to a new media condition and poststress symptom seems to develop throughout the whole cell colonies. Meanwhile, after 48 hrs of treatment, the cell shape has shrunk with asymmetric membrane developed (Figure 9(c)). Finally, the cell started to shrink and became clumpy to indicate the cell death occurred after 3 days of incubation with the extract. Therefore, with all the data collected we have concluded that *Clinacanthus nutans* leaves extract demonstrated faster mortality in low
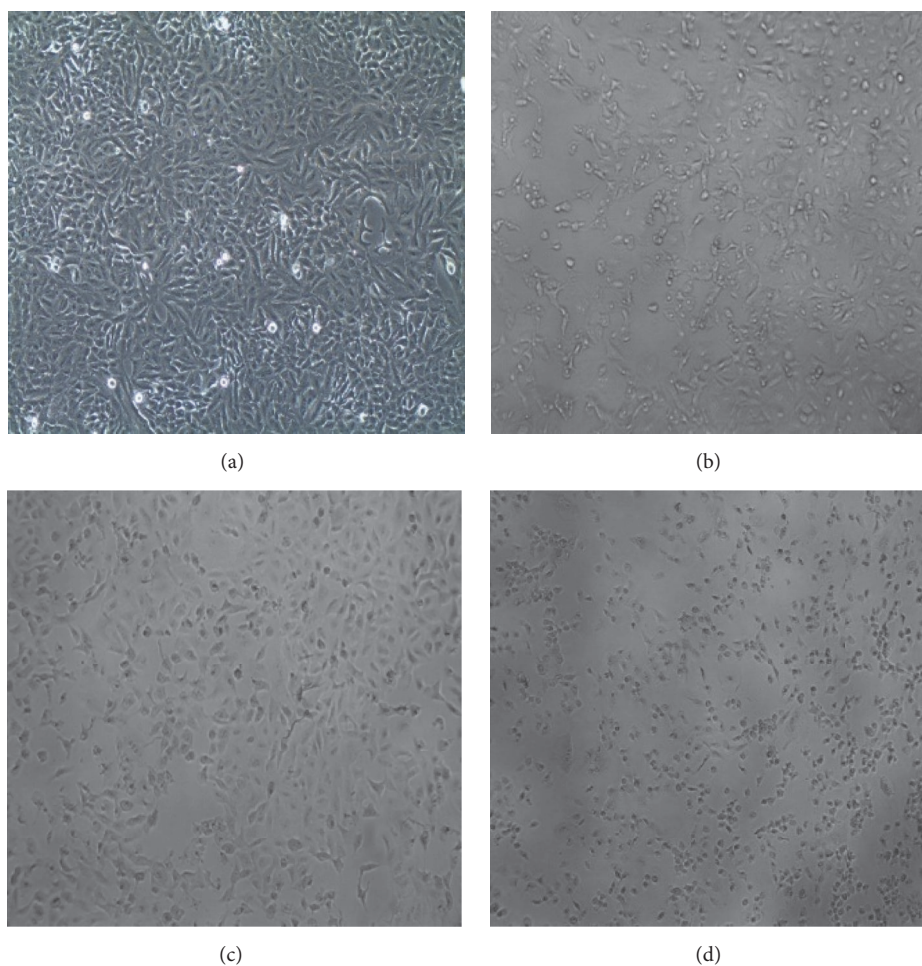
(a)

(b)

(c)

(d)

FIGURE 9: Cell morphology of A549 (a) before treatment; (b) after treatment, day 1; (c) after treatment, day 2; and (d) after treatment, day 3, treated with *Clinacanthus nutans* dried leaves extract. Cells were observed under light microscope at 40x magnification.

concentration and in fact has the capability of inhibiting the proliferation of A549.

4. Conclusion

The application of mathematical Peleg's model has proven that the model has the ability to predict the maximum extraction yield/concentration and exhaustive time of extraction. The model via extract density measurement showed that both fresh and dried leaves extracts produced the same maximum extract density and exhaustive time of extraction ($p > 0.05$). Meanwhile, the exhaustive time of extraction via flavonoids (orientin and vitexin) yield (mg) has proven that those essential bioactive compounds can be extracted much earlier (18 hrs) before the extract reaches its maximum density (29.20 hrs). Therefore, the 18 hrs of extraction time was chosen to acquire high content of flavonoids for the 2D antiproliferative cell culture study and for response surface optimization study. In fact, those conditions will be used as a benchmark for long-term cell culture studies (greater than 2 weeks of incubation). As for the antiproliferative studies

on the whole extract, we have concluded that *Clinacanthus nutans* extract has the ability to inhibit the growth of lung cancer cell lines (A549) and could be used as an alternate adjunctive or chemopreventive for cancer patients. Lastly, further study on the mechanism and in vivo testing of the observed antitumor activity are needed to unveil the potential use in cancer therapy.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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