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The Cytotoxic Effect of *Eurycoma longifolia* Jack Root Extract on The Prostate Adenocarcinoma PC-3 Cells through Apoptosis Enhancement

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Abstract

BACKGROUND: Prostate cancer is the second most common malignancy in men and has become the sixth leading cause of death in males worldwide. *Eurycoma longifolia* Jack root has active compounds, namely, quassinoids, eurycomanone, and canthine, which have potential as detoxicants, free radical antioxidants, and anticancer.

AIM: This study aimed to analyze the potential of the active compounds in *E. longifolia* Jack root in induce apoptosis in the prostate adenocarcinoma PC-3 cells.

METHODS: *E. longifolia* root active compounds were obtained by extracting them using ethanol solvent. The culture of prostate cancer PC-3 cell line was obtained from androgen-independent prostate adenocarcinoma with bone metastasis use as subject. Examination of the potency of *E. longifolia* root extract was conducted by observing the cells undergoing apoptosis with TUNEL assay.

RESULTS: One-way ANOVA test showed that the increase in apoptotic cells was associated proportionally with the concentration levels of *E. longifolia* root extract and showed a significant difference ($\alpha < 0.05$).

CONCLUSION: The higher the dose of *E. longifolia* root extract, the higher will be the apoptotic level of adenocarcinoma cells PC3. *E. longifolia* extract is potentially used in the treatment of prostate cancer by inducing apoptotic mechanisms.

Background

Prostate cancer is the second most frequent cancer diagnosis made in men and the fifth leading cause of death worldwide. Prostate cancer may be asymptomatic at the early stage and often has an indolent course that may require only active surveillance. Based on GLOBOCAN 2018 estimates, 1,276,106 new cases of prostate cancer were reported worldwide and causing 358,989 deaths (3.8% of all deaths caused by cancer in men) in 2018, with higher prevalence in the developed countries [1], [2]. Based on the 2011 Indonesian Society of Urologic Oncology (ISUO) data during 2006–2010, there were 971 prostate cancer patients. The average age was 68.3 years old, most were between 70 and 79 years (37.6%). The most common stage was Stage 4 (490 patients, 50.5%) [3].

Eurycoma longifolia Jack is a tropical plant from Simaroubaceae family distributed in Southeast Asia [4]. The root of *E. longifolia* Jack has several active compounds. *E. longifolia* Jack has been used as detoxicants, free radical antioxidants, and anticancer [5], [6]. Compounds in *E. longifolia* Jack are quassinoids [7], [8], [9]), alkaloid 9-methoxycanthine-6-one [10], [11], and alkaloid canthinone [12]. Quassinoids have cytostatic effect on colon cancer, breast cancer, pulmonary cancer, skin cancer (melanoma), and fibrosarcoma [13].

The objective of this study was to analyze the potency of active compounds of *E. longifolia* Jack root in induced apoptosis in prostate adenocarcinoma PC-3 cells by TUNEL assay.

Methods

This study was experimental *in vitro* with post-test control group design. The subject of this study is

prostate cancer PC-3 cell line which was obtained from androgen-independent prostate adenocarcinoma with bone metastasis.

Species identification of E. longifolia Jack

E. longifolia Jack roots were obtained from Great Jungle Park of Sultan Adam, Mandiangin Village, Banjar Regency, South Kalimantan. Morphological identification to determine *E. longifolia* Jack species was conducted in the UPT Plant Conservation Center, LIPI, Purwodadi.

E. longifolia Jack root extract

E. longifolia Jack roots were sliced thinly and then dried indirectly under the sun or aerated completely if there is no difference in weight during weighing. After they were dried, *E. longifolia* Jack roots were pulverized with blender and then weighed with analytical balance, until 100 g of powder was obtained. *E. longifolia* Jack root powder was wrapped in filter paper, and then, it was immersed with ethanol in the tubes until all powders in the filter paper were immersed in ethanol solvent for around 1 week. The result was evaporated using rotary evaporator and nitrogen gas to separate *E. longifolia* Jack roots from the ethanol solvent.

PC-3 cell line culture

The culture of prostate cancer PC-3 cell line was obtained from androgen-independent prostate adenocarcinoma with bone metastasis (PC-3 cell line ATCC^{R} CRL-1435^{TM}). The basic medium used was the Eagle's Minimum Essential Medium (MEM). Complete growth medium was made by adding fetal bovine serum 10% and 2 mM L-glutamine into the basic medium.

The prostate cancer PC-3 cells were washed with saline buffer, and the suspension was poured through 100 mm mesh. The cells were cultured in MEM prepared for fetal bovine serum growth. The culture was incubated in atmospheric humidity at 37°C with 95% air and 5% CO_2. The cells were left adherent for <1 week, while kept changing the medium every 2–3 days. Cell harvest was conducted after 80% cells were confluent. The cells were washed twice with phosphate buffer saline (PBS), and trypsin-ethylenediaminetetraacetic acid (EDTA) was added. The cells were incubated in the incubator for 3 min. A 5 mL complete Roswell Park Memorial Institute (RPMI) was added to inactivate trypsin. Cell resuspension was conducted with pipette until each cell was separated. Separated cells were transferred into new sterile conical tubes.

Assessment of apoptosis of prostate adenocarcinoma PC-3 cells using the TUNEL assay

Cells were seeded at 6 × 10 cells/well in 24-well culture plate. The cells are exposed to *E. longifolia* Jack extract with various doses (100, 50, 25, 12.5, and 6.25 µg/ml) and time (24 h and 48 h) and stored again in 5% CO_2 incubators, 37°C. After the incubation process is complete, the plate is removed from the incubator, the media are removed carefully, and the staining process of tunnel apoptosis is performed. Apoptotic cell was observed in prostate cancer cell samples with the terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end labeling (TUNEL) method using *in situ* cell detection kit from the manufacturer. Cultured cells were washed three times with PBS at pH 7.4, then cells were incubated with 20 µg/mL proteinase-K for 15 minutes at 37°C. After that, the cells were washed 3 times with PBS at pH 7.4 for 5 min each, and then, they were incubated with 3% H_2O_2 for 15 min. The cells were washed 3 times with PBS at pH 7.4 for 5 min each; then, they were incubated with TUNEL fragmented DNA labeling for 60 min at 37°C. The cells were washed 3 times with PBS at pH 7.4 for 5 min each, and then, they were incubated with diaminobenzidine (DAB) substrate for 40 min at 37°C. The cells were washed 3 times with PBS at pH 7.4 for 5 min each, and then, the cells were counterstained with Mayer's hematoxylin, incubated for 10 min, and washed with tap water. The cells were rinsed with dH20 and air-dried. Mounting was conducted with Entellan and covered with the cover glass. Samples were examined with a light microscope. Apoptotic index was counted and stated as a percentage by dividing the TUNEL-positive cell count with the total prostate cancer cells (300–500). Positive immunostaining was evaluated randomly for three visual fields, and the average was counted with ImageJ program.

Results

The percentages of apoptotic adenocarcinoma cells after the administration of *E. longifolia* Jack root extract in various concentrations for 24 h are shown in Table 1.

Table 1: Percentage of apoptotic adenocarcinoma cells assessed with TUNEL assay, 24 hours after the administration of *E. longifolia* Jack root extract

Treatment	Apoptotic cells (%)					
	1	2	3	4	5	Mean ± SD
Control	12.63	14.55	12.19	15.86	15.35	14.1 ± 1.63
6.25 µg/ml	18.36	20.20	16.67	14.6	11.76	16.3 ± 3.28
12.5 µg/ml	14.19	17.42	27.73	16.18	26.07	20.3 ± 6.15
25 µg/ml	23.12	17.01	27.85	18.90	28.27	23.0 ± 5.10
50 µg/ml	23.30	24.63	23.39	19.31	29.97	24.1 ± 3.83
100 µg/ml	54.18	47.99	33.64	20.90	40.15	39.4 ± 12.93

The percentage of apoptotic adenocarcinoma cells was analyzed with ANOVA test after the data normality and homogeneity assumption tests were conducted. The normality assumption was tested with Kolmogorov–Smirnov test, while the homogeneity test was conducted with Levene test. Data normality and

homogeneity assumptions are fulfilled when p-value is the same or higher than α (0.05). p-values for apoptosis variable are 0.098 and 0.135, more than α (p > 0.05). It means that the data normality and homogeneity assumptions have been fulfilled, and the hypothesis testing can be continued with one-way ANOVA.

The effect of *E. longifolia* Jack root extract administration at several concentrations on apoptosis in adenocarcinoma cells after 24 h was analyzed with ANOVA and then continued with Tukey HSD.

Based on the analysis results in Table 2, p-value is 0.000, which is lower than 0.05. It can be concluded that there was a significant difference between *E. longifolia* Jack root extract at various concentrations in the apoptosis of adenocarcinoma cells.

Table 2: The comparison of apoptotic adenocarcinoma cells using ANOVA and Tukey HSD tests 24 hours after the administration of *E. longifolia* Jack root extract

Treatment	Mean ± SD	p-value
Control	14.1 ± 1.63	0.000
6.25 µg/mL	16.3 ± 3.28	
12.5 µg/mL	20.3 ± 6.15	
25 µg/mL	23.0 ± 5.10	
50 µg/mL	24.1 ± 3.83	
100 µg/mL	39.4 ± 12.93	

Tukey HSD test results in Table 2 showed that the group given *E. longifolia* Jack root extract at the concentration of 100 µg/mL had the highest average of apoptosis, while the control group showed the lowest average of apoptosis.

The percentages of apoptotic adenocarcinoma cells after the administration of *E. longifolia* Jack root extract in various concentrations for 48 h are shown in Table 3. The effect of *E. longifolia* Jack root ethanol extract administration at several concentrations on apoptosis in adenocarcinoma cells after 48 h was analyzed with ANOVA and then continued with Tukey HSD.

Table 3: Percentage of apoptotic adenocarcinoma cells assessed with TUNEL assay, 48 h after the administration of *E. longifolia* Jack root extract

Treatment	Apoptotic cells (%)					
	1	2	3	4	5	Mean ± SD
Control	18.41	22.63	16.78	16.73	27.97	20.5 ± 4.82
6.25 µg/ml	16.27	26.73	23.12	25.16	25.17	23.2 ± 4.06
12.5 µg/ml	25.65	31.58	29.50	24.05	28.18	27.8 ± 3.00
25 µg/ml	36.7	44.45	17.72	26.05	28.13	30.6 ± 10.27
50 µg/ml	31.87	33.15	40.08	24.62	28.36	31.6 ± 5.78
100 µg/ml	46.72	41.52	45.74	52.87	30.76	43.5 ± 8.21

p-values for apoptosis variable are 0.086 and 0.161, more than α (p > 0.05). It means that data normality and homogeneity assumptions have been fulfilled, and the hypothesis testing can be continued with one-way ANOVA.

Based on the analysis results in Table 4, p-value is 0.000, which is lower than 0.05. It can be concluded that there was a significant difference between *E. longifolia* Jack root extract at various concentrations in the apoptotic adenocarcinoma cells.

Tukey HSD test results in Table 4 showed that the group given *E. longifolia* Jack root extract at the concentration of 100 µg/mL had the highest average of apoptosis, while the control group showed the lowest average of apoptosis.

Table 4: The comparison of apoptotic adenocarcinoma cells using ANOVA and Tukey HSD tests after 48 h after the administration of *E. longifolia* Jack root extract

Treatment	Mean ± SD	p-value
Control	20.5 ± 4.82	0.000
6.25 µg/mL	23.2 ± 4.06	
12.5 µg/mL	27.8 ± 3.00	
25 µg/mL	30.6 ± 10.27	
50 µg/mL	31.6 ± 5.78	
100 µg/mL	43.5 ± 8.21	

Based on apoptotic adenocarcinoma cell data, the values of IC_{50} for *E. longifolia* Jack root extract treatment after 24 and 48 h were counted. The values of IC_{50} are shown in Figure 1.

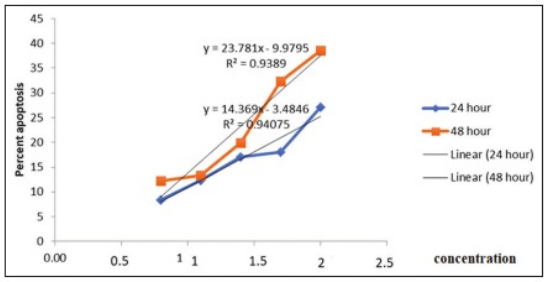

Figure 1: Values of IC_{50} for E. longifolia Jack extract after 24 (7.99 µg/mL) and 48 (7.71 µg/mL) h.

The results of the TUNEL assay as a marker of adenocarcinoma apoptotic cells after 24 and 48 h are shown in Figures 2 and 3.

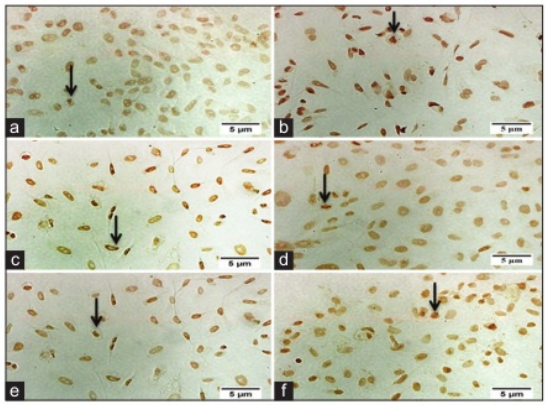

Figure 2: Apoptotic adenocarcinoma cells were characterized by brown color in the nucleus, shown by the TUNEL assay after 24 h. (A) Control, (B) adenocarcinoma cells given E. longifolia Jack root extract at the concentration of 6.25 µg/mL, (C) adenocarcinoma cells given E. longifolia Jack root extract at the concentration of 12.5 µg/mL, (D) adenocarcinoma cells given E. longifolia Jack root extract at the concentration of 25 µg/mL, (E) adenocarcinoma cells given E. longifolia Jack root extract at the concentration of 50 µg/mL, (F) adenocarcinoma cells given E. longifolia Jack root extract at the concentration of 100 µg/mL. Apoptotic cell area was analyzed with ImageJ program.

Figures 2 and 3 show apoptosis in adenocarcinoma cells after the administration of *E. longifolia* Jack root extract. Apoptotic cells were

characterized by dark brown nuclei, shown by arrows.

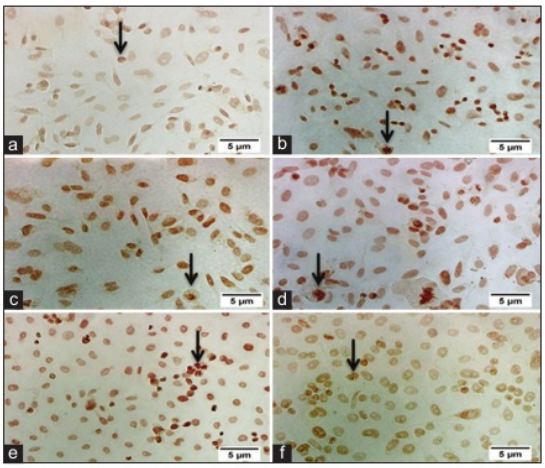

Figure 3: Apoptotic adenocarcinoma cells were characterized by brown color in the nucleus, shown by the TUNEL assay after 48 h. (A) Control, (B) adenocarcinoma cells given E. longifolia Jack root extract at the concentration of 6.25 µg/mL, (C) adenocarcinoma cells given E. longifolia Jack root extract at the concentration of 12.5 µg/mL, (D) adenocarcinoma cells given E. longifolia Jack root extract at the concentration of 25 µg/mL, (E) adenocarcinoma cells given E. longifolia Jack root extract at the concentration of 50 µg/mL, (F) adenocarcinoma cells given E. longifolia Jack root extract at the concentration of 100 µg/mL. Apoptotic cell area was analyzed with ImageJ program.

Figure 4 shows the comparison of the average percentage of apoptosis in adenocarcinoma cells. The highest average apoptotic cell percentage was given by the treatment with *E. longifolia* Jack root extract at the concentration of 100 µg/mL, with average percentages of 39.4% (24 h) and 43.5% (48 h). Based on the analysis, *E. longifolia* Jack root extract at the concentration of 100 µg/ml had the highest effect on the increase apoptotic cells.

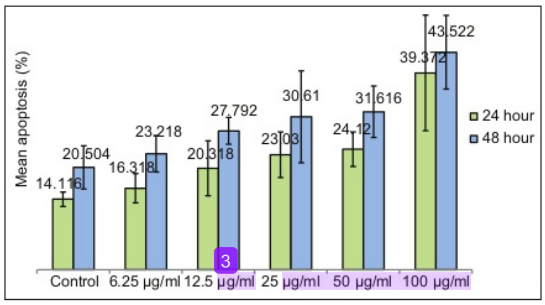

Figure 4: Comparison of average percentage of apoptotic adenocarcinoma cells given E. longifolia Jack root extract at various concentrations after 24 and 48 h.

Discussion

E. longifolia Jack (known as Pasak Bumi) is one kind of endemic plant in South Borneo, Indonesia. It belongs to Simaroubaceae family and believes a function as an aphrodisiac [14]. The plant is reported to be rich in various classes of bioactive compounds such as quassinoids, canthin-6-one alkaloids, β-carboline alkaloids, triterpene tirucallane type, squalene derivatives and biphenyl neolignan, eurycolactone, laurycolactone, and eurycomalactone, and bioactive steroids. Among these phytoconstituents, quassinoids account for a major portion of *E. longifolia* root phytochemicals [15]. Furthermore, *E. longifolia* Jack root known to consist of an essential active compound, such as eurycomanone, quassinoids, and canthine, which have anticancer effect [16]. Hajjouli *et al.* concluded that *E. longifolia* constituents, eurycomanone and eurycomanol, are the regulators of signaling pathways involved in proliferation, cell death, and inflammation [17]. The antitumor activity is one of the most impressive medicinal properties of quassinoids and has been well researched [18,19]. Many quassinoids display antitumor activity in different potencies [20].

In silico study by Rahman *et al* showed that only eurycomanone and quassinoids had Pa > Pi for antineoplastic agonist, and the value was between 0.8 and 1 for apoptosis [21]. The analysis will be resulting probability of active (Pa) and the probability of inactive (Pi) score with a range from 0 to 1. If Pa > Pi mean, it is potential for a specific therapeutic candidate [22]. Canthine showed a lower value. Based on the prediction as anticancer, active compounds, eurycomanone and quassinoids, have potency as therapeutic agents due to their ability as apoptotic agents and proliferation inhibitors by inhibiting active site of RAS protein [21].

Apoptosis is arguably one of the most potent forms of defense against cancer [23]. In cancer therapy, one approach that suppresses the tumor growth is by activating the apoptotic machinery in the cell due to the effect of anticancer agents. Therefore, the induction of apoptosis has been recognized as a strategy for the identification of anticancer drugs [24]. Apoptotic agents that suppress the proliferation of malignant cells by inducing apoptosis may represent a useful mechanistic approach to both, cancer chemoprevention and chemotherapy [25]. The phase of apoptotic execution involves the activation of several series caspases. Upstream caspase of the intrinsic pathway is caspase-9 while the extrinsic pathway is caspase 8. The intrinsic and extrinsic pathways will converge to activate caspase-3 which plays a role in the apoptotic nuclear effector [26]. Direct activation of the execution caspase can be used in anticancer therapeutic strategies by increasing the concentration of procaspase-3 [27].

This study showed that *E. longifolia* Jack root extract increased the apoptotic level of PC3 cells. There was a proportional correlation, where the higher the dose of *E. longifolia* root extract, the higher the apoptotic level of adenocarcinoma cells. In addition, after 24 h of treatment with *E. longifolia* Jack extract, cells showed an increase in the percentage of DNA damaged cells of 39.4% at highest concentration

(100 µg/ml) and 43.5%, after 48 h of incubation. This confirms that *E. longifolia* Jack extract induces early signs of apoptosis after 24 h, but cell death takes longer. This strongly correlates to the study performed by Nurkhasanah *et al*. after exposure of HeLa cells with eurycomanone, the percentage of apoptotic cells in the annexin +/PI- quadrant increased from 24 to 48 h of exposure, thus indicating that eurycomanone induced apoptosis in HeLa cells [28]. Furthermore, it was found that treatment with *E. longifolia* Jack extract induced DNA fragmentation by TUNEL assay in PC3 cells as well, in a time- and concentration-dependent manner.

Conclusion

There is a clear effect of *E. longifolia* root extract administration on the increase in apoptotic prostate adenocarcinoma cells. *E. longifolia* active ingredients are potentially used in the treatment of prostate cancer by inducing apoptotic mechanisms.

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