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Anti-Cancer Effect of Kaffir Lime (*Citrus hystrix* DC) Leaf Extract in Cervical Cancer and Neuroblastoma Cell Lines

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Abstract

Previous study showed that kaffir lime leaf contains alkaloid, flavonoid, terpenoid, tannin and saponin. The objective of this study was to examine the cytotoxic effect of kaffir lime leaf extract on cervical cancer and neuroblastoma cell lines. The method used for this research to determine cell viability was an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results showed that an ethyl acetate extract had an IC50 for HeLa cells, UKF-NB3, IMR-5 and SK-N-AS parental cells of 40.7 $\mu g \cdot mL^{-1}$, 28.4 $\mu g \cdot mL^{-1}$, 14.1 $\mu g \cdot mL^{-1}$, and 25.2 $\mu g \cdot mL^{-1}$ respectively. Furthermore, the IC50 of chloroform extracts for HeLa cells, UKF-NB3, IMR-5 and SK-N-AS parental were 17.6 $\mu g \cdot mL^{-1}$, 18.9 $\mu g \cdot mL^{-1}$, 6.4 $\mu g \cdot mL^{-1}$, and 9.4 $\mu g \cdot mL^{-1}$ respectively. These data showed that kaffir lime extract reduces the viability of cervical and neuroblastoma cell lines and may have potential as anti-cancer compounds.

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Keywords: Cervical cancer; kaffir lime; MTT assay; neuroblastoma.

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Nomenclature	
MYCN	MYCN amplification is a major determinant of high-risk neuroblastoma disease
IC50	concentration of a compound that reduces cell viability by 50 %
IC90	concentration of a compound that reduces cell viability by 90 %
yr	year
d	day

1. Introduction

Natural products are a major source of anti-cancer agents. Natural products are used as therapeutic agents against cancer has become widespread in recent years, particularly considering the toxicity of chemotherapeutics. Natural products are often considered to be safe and some have been shown to have the capability of reducing the mutagenicity in normal cells. Citrus peels and their extracts have been reported to have potent pharmacological activities and health benefits due to the abundance of flavonoids in citrus fruits. Kaffir lime (*Citrus hystrix*, jeruk purut), is a variety of citrus which is native to Indonesia, Malaysia and Thailand. Previous study showed that the leaf of this plant contains alkaloid, flavonoid, terpenoid, tannin and saponin compounds. Therefore further study to determine if extracts of the leaves show a relevant activity against cervical cancer and neuroblastoma cell lines is needed.

Previous studies revealed kaffir lime leaves and fruit extract to have antioxidant activity[1], free radical scavenging ability[2], antimicrobial activity[1], and anti-inflammatory activity[3]. In regard to cancer research, kaffir lime essential oil has been shown to have anti-proliferative activity on human mouth epidermal carcinoma (KB) and murine leukemia (P388) cell lines[4]. Furthermore, extract of kaffir lime leaf showed cytotoxic effect against HL60 (promyelocytic leukemia), K562 (chronic myelocytic leukemia), Molt4 (lymphoblastic leukemia) and U937 (monocytic leukemia)[5] cells.

Cervical cancer remains one of the leading causes of cancers in women from Indonesia[6]. The central cause of cervical cancer is the human papillomavirus (HPV). HPV is sexually transmitted, and chronic infection with high-risk strainscan lead to cervical cancer. From HPV infection to cervical cancer it usually takes 10 yr to 20 yr. As a result, patients with cervical cancer are often only detected when they have already entered an advanced stage of cancer, which is more difficult to treat[7].

Neuroblastoma is the most common extracranial solid tumor of childhood. It displays a unique propensity to undergo spontaneous regression in infants and/or differentiation in some older patients. Unfortunately, 50 % of the neuroblastoma patients are diagnosed with high-risk disease associated with cure rates below 50 %[8].MYCN amplification is a major indicator of high-risk disease in neuroblastoma[9].

The objective of this study was to examine the effect of kaffir lime leaf extract on cancer cell lines viability using the cervical carcinoma cell line HeLa, the MYCN-amplified neuroblastoma cell lines UKF-NB3 and IMR-5 and the non-MYCN-amplified cell line SK-N-AS.

2. Materials and methods

2.1. Extraction of kaffir lime

Samples of leaves are taken in Magelang, Central Java, Indonesia. The samples are then dried until they obtain a constant dry weight and made into a simplicia powder. The powder was then extracted using a maceration method by dissolving into ethyl acetate or chloroform.

2.2. Cytotoxic assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide*(MTT) assay*

Cell lines were cultured in iscove's modified dulbecco's medium (IMDM) supplemented with 10 % fetal bovine serum (FBS) and 100 $IU \cdot mL^{-1}$ penicillin, and 100 $mg \cdot mL^{-1}$ streptomycin. When the cells reached 70 % to 80 % confluence, they were harvested and plated for consequent passages or for kaffir lime leaf fractional extract

treatments. Cells were trypsinised, resuspended in media and counted using a haemocytometer. The cell number was adjusted to 40 000 cells · mL^{-1} with media and added to 96-well. After 5 d incubation at 37 °C in 5 % CO_2, 25 µL of MTT 2 mg· mL^{-1} was added. After an additional incubation time for 4 h at 37 °C in 5 % CO_2, the cells were solubilised using 100 µL of a 20 % sodium dodecyl sulphate (SDS) solution in purified water and dimethylformamide (1 : 1) adjusted to pH 4.7. This step to lyse the cells and dissolve the precipitated formazan. The absorbance was read in a plate reader at 600 nm. The higher the absorbance value, the more converted formazan is present and so the higher the number of viable, proliferating cells. Note the outer edge of the plate is not used to test compounds to avoid edge effects.

MTT assay (Microculture Tetrazolium Salt) is a colorimetric test based on the reduction of yellow MTT to purple formazan by cellular dehydrogenases, which are only active in living, proliferating cells. This test is capable of measuring viability, proliferation, and cell activation. The more converted formazan present, the higher the number of viable and proliferating cells.

3. Results and discussion

The kaffir lime leaf extract concentrations that reduced the viability of the investigated cell lines by 50 % (IC50) or 90 % (IC90) are reported in Table 1.

Table 1. IC50 and IC90 values of several types of cancer cell after treated by kaffir lime leaf extract

No.	Extract	Type of cancer cell	Average of IC50 (µg · mL^{-1})	Average of IC90 (µg · mL^{-1})
1	Ethyl acetate	HeLa	40.8	202.9
		UKF-NB3	28.4	65.9
		IMR-5	14.1	45.4
		SK-N-AS	25.2	91.7
2	Chloroform	HeLa	17.6	99.0
		UKF-NB3	18.9	68.5
		IMR-5	6.4	25.9
		SK-N-AS	9.4	139.4

As described in the introduction, cervical cancer remains one of the leading causes of cancers in women from Indonesia[6] while neuroblastoma is one of the most common extracranial solid childhood malignancy. Many patients have metastatic disease at the time of diagnosis, often followed by tumour progression and fatal outcome[8].

IC50 and IC90 values are the concentration of extract required to inhibit 50 % and 90 % of cell growth and proliferation, respectively.This value determines the level of potency of a compound in inhibiting cell growth. The low the IC50 and IC90 value means the higher potential of a compound in inhibiting cell growth. Based on the IC50 and IC90 values, chloroform extract had the most potential to reduce HeLa cell viability compared to ethyl acetate.

Ethyl acetate and chloroform extracts differed substantially in terms of potency.This suggests that the composition of the extracts differs in dependence on the solvent used. Further work should be undertaken to determine exactly what is extracted under each of these conditions to isolate any ‘active’ compounds for additional studies.

It is interesting to note that the kaffir lime extracts reduced the viability of cervical and neuroblastoma cells although differ cancer cells had different responses. Cancerous cell lines possess differences in their origin, morphology, and genomes resulting in susceptibility differences to the chemotherapeutic agents.The original cells of HeLa came from a visible malignant cervical tumor in the body of a 31 yr old woman. On the other hand, there were two types of neuroblastoma cell lines used in this study: MYCN-amplified neuroblastoma cell lines (UKF-NB3 and IMR-5) and non-MYCN-amplified cell lines (SK-N-AS).The neuroblastoma cell line UKF-NB-3 was established from bone marrow metastasis of a stage IV neuroblastoma patient with MYCN amplification. MYCN and MYC are oncoproteins that play crucial roles in determining the malignancy of unfavorable neuroblastoma[9].

Taken together, kaffir lime leaf extract reduced the viability of cervical cancer and neuroblastoma cells in micromolar concentrations. These results are in concert with aprevious study in which kaffir lime extract was shown to exert anti-cancer effects against human mouth epidermal carcinoma (KB) and murine leukemia (P388) cell lines[4], HL60 (promyelocytic leukemia), K562 (chronic myelocytic leukemia), Molt4 (lymphoblastic leukemia) and U937 (monocytic leukemia)[5] cells. Further work should be undertaken to determine the anti-cancer effects of the investigated extracts *in vivo* and to identify the active ingredient

4. Conclusion

- Kaffir lime extract reduced the viability of cervical and neuroblastoma cells in micromolar concentrations.
- The results warrant the further investigation of kaffir lime leaf extracts for anti-cancer activity.

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