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Effects of *Vetiveria zizanioides* on Cytotoxicity of Human Epithelial Cervical Cancer Cells

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Abstract: The potential of *Vetiveria zizanioides* or *Chrizopogon zizanioides*, known as "akar wangi" in Indonesia, has not been much studied as an anticancer ingredient. Moreover, the effect of vetiver compounds on cervical cancer cells is still unknown. This research was conducted to determine the effect of *V. zizanioides* or vetiver oil on the cytotoxicity of HeLa cells (continuous cell line from cervical cancer epithelial cells) for a further finding of the potential anticancer natural compound. The administration of *V. zizanioides* extract in essential oil was done in dose-dependent. The 3‐(4,5‐Dimethylthiazolyl‐2‐yl)-2,5-diphenyltetrazolium bromide (MTT) assay as cytotoxicity assay was used to analyze the cytotoxicity effects of vetiver oil for 24 hours to in vitro culture of HeLa cells by determining the percentage of proliferation inhibition. The in vitro HeLa cell culture was treated with several dilutions of vetiver oil. IC50 concentration of 0.02% to 0.00125% vetiver oil against HeLa cells was recorded. The vetiver oil showed cytotoxicity effects against the HeLa cells for 24 hours in a concentration-dependent manner. The highest concentration of vetiver oil (0.02%) showed the highest cell proliferation inhibition (96.304%). The IC50 value was at 0.05% dilution of vetiver oil. In addition, morphological alterations, including cytoplasm shrinkage. Together these findings indicated the active potential anticancer nature of *V. zizanioides*, especially in human cervical cancer.

Keywords: anticancer, cytotoxicity, HeLa cells, vetiver oil, *Vetiveria zizanioides.*

香根草对人上皮宫颈癌细胞细胞毒性的影响

摘要：香根草或茼蒿，在印度尼西亚被称为“香根”，作为抗癌成分的潜力尚未得到太多研究。此外，香根草化合物对宫颈癌细胞的影响仍然未知。这项研究旨在确定香根草或香根草油对海拉细胞（来自宫颈癌上皮细胞的连续细胞系）的细胞毒性的影响，以进一步发现潜在的抗癌天然化合物。香根草提取物在精油中的给药以剂量依赖性进行。采用3-(4,5-二甲基噻唑基-2-基)-2,5-二苯基溴化四唑试验作为细胞毒性试验，通过测定香根草油24小时对海拉细胞体外培养的细胞毒性作用进行分析。增殖抑制百分比。用几种稀释的香根草油处理体外海拉细胞培养物。记录了0.02%至0.00125%香根草油对海拉细胞的抑制浓度50浓度。香根草油在24小时内以浓度依赖性方式显示出对海拉细胞的细胞毒性作用。最高浓度的香根草油（0.02%）表现出最高的细胞增殖抑制作用（96.304%）。抑菌浓度50值是香根草油稀释0.05%时的值。此外，形态学改变，包括细胞质收缩。这些发现共同表明了香根草具有积极的潜在抗癌特性，尤其是在人类宫颈癌中。

关键词：抗癌、细胞毒性、海拉 细胞、香根草油、香根草。

1. Introduction

Cervical cancer is the second most common type of cancer in women in Indonesia after breast cancer [1]. Data from the Global Cancer Observatory (GCO) in 2018 showed that new cases of cervical cancer in Indonesia reached 32,469 cases or 17.2% of the

ReceivedReceived: November 16, 2021 / Revised: December 01, 2021 / Accepted: January 02, 2022 / Published: February 28, 2022

Fund Project: PUTI (Publikasi Terindeks Internasional) Grant, contract number NKB-1009/UN2.RST/HKP.05.00/2020, on behalf of S. Si Fadhillah, provided by Directorate of Research and Development, Universitas Indonesia

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prevalence of female cancer in Indonesia [2]. The limitations of effective drugs which can cure cancer, the high cost of chemotherapy, and the side effects of using anticancer drugs are some causes of high mortality rates due to cancer [3]. Mortality from cervical cancer even reaches 18,279 per year [2, 3]. Several attempts were made to find natural anticancer ingredients to overcome, slow down, or even stop cancer development [4].

Many anticancer studies have been carried out using traditional medicinal plants to find new therapeutic agents with no side effects [5]. *Vetiveria zizanioides* or *Chrizopogon zizanioides*, called akar wangi in Indonesia, is a plant that has active compound ingredients with various pharmacological activities, such as an anti-oxidant, anti-bacterial, anti-fungal, and anticancer [6-9]. Previous studies by Chitra et al. showed that aqueous crude extract from vetiver plants caused cytotoxicity in the human breast cancer cell line (MCF-7 cell line) [10]. Meanwhile, V. zizaniodes has more than 100 active compounds, named sesquiterpene compounds and their derivatives [11]. Sesquiterpene constitutes a diverse group of bioactive compounds isolated from several plant families (including Asteraceae) exhibiting cancer cell cytotoxicity and anti-neoplastic efficacy and are used to treat a large number of cancers [12]. Sesquiterpene lactone, such as deoxyelephantopin and isodeoxyelephantopin extracted from *Elephantopus carolinianus* and *Elephantopus scaber*, have been shown to induce apoptosis via multiple mechanisms. Such as comprised induces ROS, mitochondrial dysfunction, modulated Bcl-2 family protein, arrest cell cycle, inhibit NF-kB, and STAT3 activation [13, 14].

The effect of vetiver extracts on cervical cancer cells is still unknown. In this study, the essential oil of *V. zizanioides*, called vetiver oil and commercially available, was used to test the effect of active compounds in *V. zizanioides* on the cytotoxicity of HeLa cells, which are known as immortal cells because of their ability to grow and divide endlessly. The cytotoxicity effect of vetiver oil in several dilutions for 24 hours on HeLa cell proliferation was analyzed using MTT assay and assessment of cell morphology.

2. Methods

2.1. HeLa Cell Culture

HeLa cells used in this study were obtained commercially. The cells were cultured using Dulbecco's Minimum Essential Medium (DMEM) containing 10% (v/v) heat-inactivated fetal calf serum and 1% penicillin-streptomycin. The cell lines were grown in 95% air and 5% CO^2 and incubated at 37° C. At first, the cells were seeded in a 6-well plate until they reached 80–90% confluency for 2–3 days. The cells were then transferred into a 96-well plate for cytotoxicity analysis by MTT assay. Meanwhile, the cultured cells were passage for the morphology assay and seeded in the plastic disc for further steps.

2.2. Vetiver Oil Preparation

The vetiver oil was prepared in several dilutions. The vetiver oil was diluted in DMSO from 0.02% to 0.00125%. Different dilutions of vetiver oil were assigned as treatment groups. All treatments were performed in triplicates.

2.3. Cytotoxicity Assay

MTT assay was performed as described earlier [15]. Briefly, HeLa cells were aseptically seeded at 5 x 10^4 cells per well in a microtiter plate in a DMEM medium. Then, it was incubated overnight, and various dilutions of vetiver oil were added to respective wells containing the cells. After 24 hours, the cells were treated with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). The cell culture medium was then aspirated from the microtiter plates after 4 hours of incubation. Then the formazan crystal from the microtiter plates was dissolved in DMSO, and the plate was subjected to analysis at 570 nm using a 96 well microplate reader. The cytotoxic effect on inhibition of proliferation (%) was then calculated.

2.4. Assessment of Cell Morphology

The cultured HeLa cells treated with vetiver oil for 24 and 72 hours were fixed using a fixative solution at a dilution of 3:1 methanol and acetic acid at room temperature for 15 minutes. Slides were subsequently stained with Giemsa stain solution [15] at room temperature for 4 minutes and washed in distilled water. The morphology of HeLa cells was observed at 10x40 magnification using a light microscope [16]. The morphological parameters observed include the cell volume, the shrinkage of cytoplasm, and the shape of the nucleus [16, 17].

3. Results and Discussion

Many medicines have been derived from various medicinal plants in recent years, and this process continues. The cytotoxic property of medicinal plants plays a powerful role in treating various cancers [17]. *Vetiveria zizanioides* has been cultivated for many industrial applications, including the production of commercially and medicinally valued volatile oil that can be distilled from its root [18, 19]. Several previous studies reported that *V. zizanioides* showed various pharmacological activities, including an anticancer agent [6-9].

The ethnopharmacological knowledge is helpful to lead the search for plants with potential cytotoxic activity [3, 5]. In the present study, we determined the cytotoxicity effect of *V. zizanioides* in HeLa cells by using vetiver oil on the cell proliferation of HeLa cells.

The present findings showed the cytotoxic effects of vetiver oil in several dilutions on HeLa cells by reducing cell proliferation. The average percentage of proliferation inhibition can be seen in Table 1.

Table 1 The average proliferation inhibition of cultured HeLa cells with vetiver oil addition for 24 hours

Vetiver oil concentration (%)	Average of proliferation inhibition = PI (%)	Standard Deviation
0.00125	8.774	5.344
0.0025	30.373	1.349
0.005	51.973	4.075
0.01	73.572	4.406
0.02	96.304	1.298

In Table 1, the average proliferation inhibition percentage in HeLa cells in addition to vetiver oil ranged from 8.774% to 96.304%. Based on the analysis, the vetiver oil used in the present study produced 50% net killing (IC_{50}) at 0.05% dilution at 24 hours incubation. These results reported that vetiver oil showed cytotoxicity effects on HeLa cells by inhibiting cell proliferation. Further, vetiver oil's highest concentration (low dilution rate) showed the highest proliferation inhibition value. In other words, vetiver oil showed the cytotoxicity effect on HeLa cells in a concentration-dependent manner.

The results of the present study were in agreement with previous reports. Chitra et al. reported that the aqueous extract of *V. zizanioides* exhibited cytotoxicity towards the human breast cancer cell line in a concentration-dependent manner [10]. The cytotoxicity effects of *V. zizanioides* in several concentrations were analyzed against human breast adenocarcinoma MCF-7 cell line, and the maximum concentration inhibition showed in 31µg/mL to 37µg/mL [10]. Meanwhile, Lock *et al.* studied the etoposide cytotoxicity by methylxanthines, including caffeine, in HeLa cells. It is reported that the caffeine enhanced HeLa cell cytotoxicity also in a concentration-dependent manner for 24 hours [20].

We also assessed HeLa cell morphology by comparing the morphology of the cells with and without the addition of vetiver oil. The results are shown in Fig. 1: the morphology of HeLa cells in the control group is compared with the morphology of HeLa cells in the treatment group (also vetiver oil for 24 and 72 hours). Through these pictures, the morphology difference between groups was seen. The control group showed a round shape with a clear membrane and nucleus. Meanwhile, the treated HeLa cells for 24 hours showed the shrinkage of cytoplasm and unclear nucleus. Moreover, the treated HeLa cells and vetiver oil for 72 hours showed the damaged structure of cytoplasm and unseen nucleus. Based on these results, we found that the treatment of HeLa cells with vetiver oil induced the morphological changes that confirmed the MTT assay by changing morphological appearance, which indicates the increase in apoptotic cell population [21].

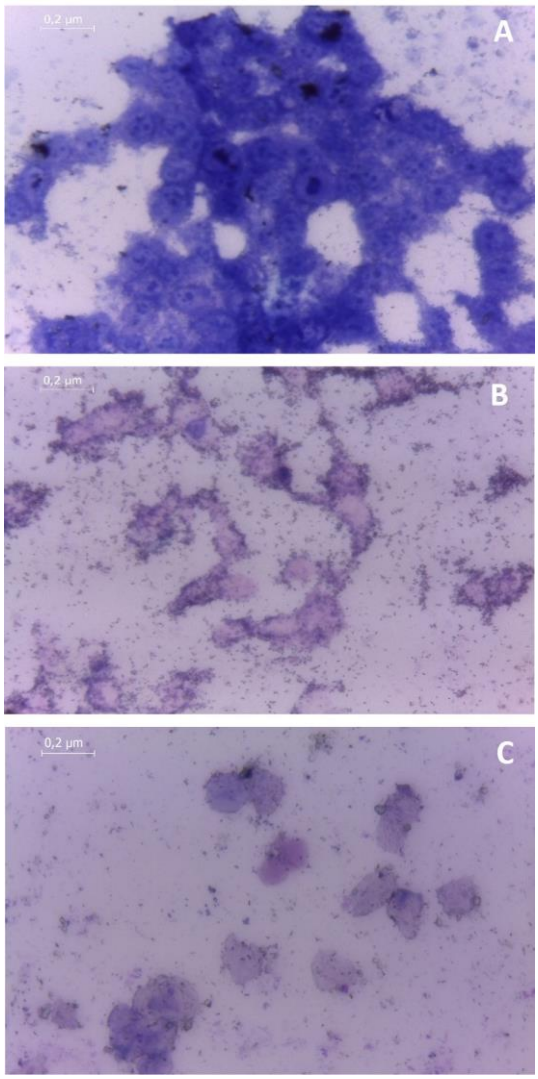

Fig. 1 The morphology of cultured HeLa cells without vetiver oil (A, control) and cultured HeLa cells with vetiver oil for 24 hours (B) and 72 hours (C)

The cellular mechanisms of anticancer agents are known to induce apoptosis and cytotoxicity in cancer cells. They are associated with oxidative stress formation, ROS production, mitochondrial depolarization, and cell cycle arrest [18, 19, 21]. The cytotoxicity effects of vetiver oil in HeLa cells found in the present study may also be related to one or more of those cellular mechanisms.

Sesquiterpene lactones, the active constituents of various medicinal plants for treating inflammatory diseases, which are already known also contained in *V. zizanioides* [11], are reported to play their role in molecular mechanisms involved in the anticancer activity. Namely induction of apoptosis, prevention of metastasis, and inhibition of inflammatory responses such as nuclear transcription factor-kappaβ (NF-kappaβ) and mitogen-activated protein kinases (MAPK). However, the cellular mechanisms of which

active compounds in *V. zizanioides* contributed to the HeLa cells' death remain to be investigated. In addition, *in vivo* study is needed to emphasize these results and further clarify the role of vetiver oil compounds as an anticancer agent.

4. Conclusion

In this study, the vetiver oil, essential oil of *Vetiveria zizanioides*, showed cytotoxicity effects against the HeLa cells through inhibition of cell proliferation and morphology alteration. The highest concentration of vetiver oil inhibited the HeLa cell proliferation at the maximum and induced alteration of HeLa cell morphology. These findings indicated the active potential anticancer nature of *V. zizanioides*, especially in human cervical cancer. Further studies are needed to clarify the most effective bioactive compounds in *V. zizanioides* as anticancer and the mechanism of compounds in apoptosis by investigating the apoptotic factors.

4.1. Acknowledgments

The authors would like to thank the Directorate of Research and Development, Universitas Indonesia, for providing financial support through a PUTI (Publikasi Terindeks Internasional) Grant, contract number NKB-1009/UN2.RST/HKP.05.00/2020, on behalf of S. Si Fadhillah (Agricultural MD, PhD).

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