Majalah Kedokteran Gigi Indonesia
Vol 9 No 3 – December 2023
ISSN 2460-0164 (print), ISSN 2442-2576 (online)Available
online at https://jurnal.ugm.ac.id/mkgi
DOI: http://doi.org/10.22146/majkedgiind.89452

RESEARCH ARTICLE

Effect of hexane extract of *Clinacanthus nutans* leaves on HSC-3 cells migration *in vitro*

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Submitted: 2nd October 2023; Revised: 1st November 2023; Accepted: 29nd November 2023

ABSTRACT

Cancer is a major health issue and considered the most life-threatening disease worldwide. The oral cavity is one of the top ten locations in the body most frequently affected by cancer. The most common type of oral cancer (90%) is squamous cell carcinoma. Leaves of *C. nutans* have many uses and benefits, one of which is its potential as an anticancer agent. This study aims to determine the effect of C. *nutans* leaves hexane extract on HSC-3 cell strain migration. The leaves of *C. nutan*s were extracted using a hexane solvent with the maceration method. Various concentrations (500, 250, 100, 50 and 25 mg/mL) were assessed to identify the effect of *C. nutan*s leaves hexane extract on HSC-3 cells migration at 0, 6, 12, 24 and 48 time point. The concentration of 500 µg/mL was found to possess the ability to inhibit migration and induce apoptosis by the 6th hour. The concentration of 250 µg/mL induced apoptosis by the 24th hour. Concentrations of 100 µg/mL and 50 µg/mL were capable of inhibiting cell migration, and the concentration of 25 µg/mL could only delay cell migration up to the 12th hour. In conclusion, hexane extract of *C. nutans* leaves can inhibit HSC-3 cells migration.

Keywords: *Clinacanthus nutans* leaves; hexane extract; HSC-3; scratch assay

INTRODUCTION

Cancer is a major health problem and the most life-threatening disease in all corners of the world.[1] Oral cancer is the 12th most common cancer in the world, and is the eighth most common cancer in developing countries.[2] Areas of the oral cavity are one of the ten locations of the body most commonly affected by cancer. There are several types of oral cancer, and the most common type (90%) is squamous cell carcinoma.[3] HSC-3 cell is tongue squamos carcinoma derived from tongue carcinoma.[4] In the United States, one person dies in an hour from cancer of the oral cavity that spreads easily.[5] In India especially in Kerala, the incidence of oral cancer is very high at about 20% of all malignancies.[6] In Indonesia, oral cancer cases range from 3-4% of all cancer cases.[5] According to research in the United States, about 7,900 deaths in 2011 were from malignant tumors of the oral cavity with more than 90% being squamous cell carcinoma (SCC).[7,8]

The cause of SCC is still not known with certainty until now because the cause of cancer is multifactorial and complex.[9] However, there are several factors that may influence the occurrence of oral cancer, namely local factors, external factors, and host factors. Local factors include poor hygiene of the oral cavity, dental caries, and chronic irritation from restorations. External factors include drinking alcohol and smoking, while host factors are age, sex, nutrition, immune system, and genetics.[5]

The pathogenesis of SCC is known to be multifactorial.[10] There are progressive changes in SCC at the cellular and genetic levels that program cells to proliferate uncontrollably, causing malignant masses.[11] Management of tongue cancer until now is still done by surgery

Majalah Kedokteran Gigi Indonesia. December 2023; 9(3): 245-252
ISSN 2460-0164 (print)
ISSN 2442-2576 (online)

accompanied by radiation and chemotherapy.[12] However, these actions can cause various side effects to normal tissues with various symptoms, such as nausea, vomiting, anorexia, diarrhea, oral mucositis, and numbness.[13] Considering the side effects of surgery and therapy, as well as the relatively expensive cost, research on cancer therapies from natural sources with relatively inexpensive synthetic drugs and with minimal cytotoxicity effects to normal cells has been continuously conducted.[14] These alternatives are expected to have fewer side effects and have better treatment effectiveness.[15] Eighty percent of the world's population is still dependent on traditional medicine.[16]

Clinacanthus nutans is one of alternative medicinal plants that is being developed recently. The plant originates from Southeast Asian countries, especially Indonesia, Malaysia, Thailand, and Vietnam. In Indonesia the plant is known as ki tajam (Sundanese) or dandang gendis (Javanese). *Clinacanthus nutans* is also known as Sabah snake grass because it is found in Sabah, Malaysia.[17] In Thailand and Malaysia *C. nutans* leaves are traditionally used to treat skin diseases, snakebites, scorpion stings, and insect stings and bites.[18] In China the whole *C. nutans* plant is used in various ways to treat inflammatory conditions such as hematomas, bruises, injuries, rheumatism and anxiety.[19] In Indonesia, *C. nutans* leaf tea is used for the treatment of diabetes, dysuria, and dysentery. The leaf extract has many health benefits such as treating lesions caused by herpes simplex virus. It also has anti-inflammatory, anticancer, antibacterial, and anti-toxic activities.[20]

In previous studies, among four organic solvent extracts (hexane, chloroform, ethanol, and methanol), only hexane extracts had a significant antiproliferative effect on *C. nutans* because hexane extracts could induce apoptosis in all cell types in this study. These are A549 cell strain (non-small cell lung cancer), CNE1 cell strain (nasopharyngeal cancer), and HepG2 cell strain (liver cancer).[21] These observations suggest that the nonpolar phytochemical constituents of *C. nutans* could be used as an additional alternative for patients at risk of cancer.[22] Given this background, we are interested in examining the effect of *C. nutans* leaves hexane extract on HSC-3 cell line migration.

MATERIALS AND METHODS

This research was conducted at the BioCORE Laboratory of the Faculty of Dentistry, Universitas Trisakti, Grogol, West Jakarta and was divided into two stages. The first stage was extracting *C. nutans* leaves by maceration method, and the second stage was observing the effect of *C. nutans* hexane extract on HSC-3 cell line migration with scratch assay method. In this study, the treatment group was divided into seven groups. The first group was a negative control without *C. nutans* leaves hexane extract, the second group was a positive control (3 mM doxorubicin), and the third group was given *C. nutans* leaves hexane extract with concentrations of 500, 250, 100, 50, and 25 mg/mL. The number of repetitions of the study according to the Federer's formula was 4 times for each group.

A total of 100 g of leaves powder of *C. nutans* was soaked in 500 mL of hexane solvent for 3 x 24 hours, and agitation was carried out every 15 minutes. Next, hexane containing extract was filtered with Whatman filter paper, and the filtrate was evaporated using a rotary evaporator to separate the hexane from the extract at a set temperature of 69 °C. This process was repeated 3 times.

A qualitative phytochemical screening was conducted to evaluate the components in the hexane extract of *C. nutans* leaves. In this process, 5% sodium hydroxide (NaOH) solution was mixed with 1 mL of the crude extract, and yellow color appeared. The presence of flavonoids was confirmed when hydrogen chloride (HCl) was added, causing the yellow colour to fade away. To detect steroids, 1 milligram of the extract was dissolved in 10 mL of chloroform, and an equal volume of sulfuric acid (H_2SO_4) was added. The H_2SO_4 layer showed yellow hue with green fluorescence, and the layer above it turned red,

indicating the presence of steroids. To detect terpenoids, the crude extracts were blended with 1 mL of chloroform and H_2SO_4. Red-brown color appeared, which was indicative of the presence of terpenoids. To identify tannins, 50 grams of the crude extract was combined with 5% ferric chloride ($FeCl_3$). The development of bluish-black coloration indicated the presence of tannins. Lastly, Dragendorff's solution was added to 0.1 g of the extract, and formation of an orange-red precipitate confirmed the presence of alkaloids.

HSC-3 was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, New York, USA) supplemented with 20% fetal bovine serum (FBS) (Gibco), 1% amphotericin B, 1% penicillin-streptomycin (APS) (Invitrogen, Massachusetts, USA) at 37 °C in a 5% CO2 incubator. The fibroblasts were sub-cultured when cells reached 80%-90% confluency in the flask.

The effects of the hexane extract from *C. nutans* leaves on the migration of HSC-3 cells were assessed using the *in vitro* scratch assay. Cells (1 x 10^6 cells/well) were cultivated in 6-well plates with DMEM, supplemented with 10% FBS and 1% penicillin-streptomycin. Once they reached 90% confluence, scratches were made both vertically and horizontally at the center of each well's monolayer. To remove cellular debris from the scratch, phosphate-buffered saline (PBS) was utilized. Subsequently, cells in the wells were treated with various concentrations (500, 250, 100, 50, and 25 µg/mL) of *C. nutans* leaves hexane extract. The scratches were periodically observed under an inverted microscope at intervals of 0, 6, 12, and 24 hours. The negative control was of DMEM without any extracts, while the positive control was of 3 µM doxorubicin. The gap area in the monolayers was calculated using Image J software (National Institutes of Health, Bethesda, MD) after capturing images. The formula to calculate gap closure[23] is:

$$\% \text{ cell gap closure} = [(A_{t=0h} - A_{t=xh})/A_{t=0h}] \times 100$$

$A_{t = 0h}$: area measured immediately after scratching
$A_{t = xh}$: area measured after x hours of scratching

Data were reported as mean values ± standard deviation. The Shapiro–Wilk test was used to assess normality. Data distribution was considered normal at $p < 0.05$. Significant differences were determined using a one-way analysis of variance test and the post-hoc Tukey test. Results were considered significant at $p < 0.05$.

RESULTS

The data in Table 1 shows the results of phytochemical tests conducted by qualitative methods, which showed that *C. nutans* leaves hexane extract had active compounds, namely flavonoids, terpenoids, triterpenoids, tannins, alkaloids, and quinones.

Table 2 shows the measurement of the ability of various concentrations of *C. nutans* leaves hexane extract on cell migration using the scratch assay method. It was photographed under an inverted microscope, and the area of scratches was measured using ImageJ software. The normality test analysis revealed that the data from the migration test of HSC-3 cells treated with the hexane extract from *C. nutans* leaves exhibited normal distribution with a $p > 0.05$. According to the two-way ANOVA analysis, a significant difference was observed among the groups of HSC-3 cell scratch assay test materials treated with the hexane extract from *C. nutans* leaves, with $p < 0.05$.

The outcomes of the Tukey post-hoc analysis revealed a significant impact of the hexane extract from *C. nutans* leaves on the migration of HSC-3

Table 1. Phytochemical test results

Sample	Test type	Test results	Method
Hexane extract of *C. nutans* leaves	Flavonoids	+	Qualitative
	Terpenoids	+	
	Steroids	+	
	Tannins	+	
	Alkaloids	+	
	Quinon	+	

Table 2. Scratch assay results (in percentages)

Time in hour	Positive control	Negative control	500 µg/mL	250 µg/mL	100 µg/mL	50 µg/mL	25 µg/mL
0	100	100	100	100	100	100	100
6	23.46 ± 54.12 *a,b,c,d,e	45.76 ± 40.57 *e,f,g	Cannot be counted due to cell apoptosis	125.01 ± 26.08 *a,f,h,i	103.44 ± 5.072 *b,g,j	79.77 ± 29.44 *c,h	54.52 ± 37.88 *d,i,j
12	Cannot be counted due to cell apoptosis	20.61 *a,b	Cannot be counted due to cell apoptosis	152.13 ± 26.08 *c	111.09 ± 5.072 *a,c,d,e	63.72 ± 29.44 *b,d,f	16.51 ± 37.88 *d,e,f
24	Cannot be counted due to cell apoptosis	Cannot be counted because the space is already closed	Cannot be counted due to cell apoptosis	Cannot be counted due to cell apoptosis	111.54 ± 5.072 *a	40.15 ± 29.44 *a	Cannot be counted because the space is already closed
48	Cannot be counted due to cell apoptosis	Cannot be counted because the space is already closed	Cannot be counted due to cell apoptosis	Cannot be counted due to cell apoptosis	108.94 ± 5.072 *a	26.96 *a	Cannot be counted because the space is already closed

*letters in the same line indicate a significant difference

cells. Specifically, at 6 hours, notable differences were observed between the positive control group and negative control group, positive control group and 250 µg/mL group, positive control group and 100 µg/mL group, positive control group and 50 µg/mL group, positive control group and 25 µg/mL group, negative control group and 250 µg/mL group, negative control group and 100 µg/mL group, 250 µg/mL group and 50 µg/mL group, 250 µg/mL group and 25 µg/mL group, and 100 µg/mL group and 25 µg/mL group. At 12 hours, significant differences were found between the negative control group and 100 µg/mL group, negative control group and 50 µg/mL group, 250 µg/mL group and 100 µg/mL group, 100 µg/mL group and 50 µg/mL group, 100 µg/mL group and 25 µg/mL group, and 50 µg/mL group and 25 µg/mL group. At 24 hours, a significant difference was found between the groups of 100 µg/mL and 50 µg/mL. Finally, at 48 hours, a significant difference was observed between the 100 µg/mL group and the 50 µg/mL group (Table 2).

At 6 hours, the entire cell population in the 500 µg/mL group had undergone apoptosis. At 12 hours, all cells in both the positive control group and the 500 µg/mL group underwent apoptosis. At 24 and 48 hours, only the 100 and 50 µg/mL groups had not undergone apoptosis, while the space of the 25 µg/mL group was completely covered by cells. Calculation was not performed for the group whose all cells had undergone apoptosis.

DISCUSSION

This study was conducted to investigate how *C. nutans* leaves hexane extract inhibits the migration of HSC-3. Migration testing with scratch assays was carried out by scraping monolayer cells using sterile yellow tips until scratches of a certain size were formed. The determination of the migratory ability of cells was carried out by quantifying the area of strokes at 0 hours and at specified time intervals until the cells migrated to seal the strokes. This measurement could directly determine the

effect of different concentrations on the interaction ability of cells during cell migration.[24] In this study, hexane was used for extraction, which was a nonpolar solvent. Phytochemical tests showed that hexane extract of *C. nutans* leaves had flavonoids, terpenoids, steroids, tannins, alkaloids, and quinones. Each of these compounds was found to possess the ability to inhibit proliferation of cancer cells.

There are various types of flavonoids, and each type has a different level of polarity.[25] Flavonoid compounds have been shown to have antioxidant, anti-inflammatory, antidiabetic, and antiproliferative activities.[25,26] Flavonoids are compounds in plants that have been shown to inhibit the proliferation of some cancer cells that have low toxicity or even non-toxic to normal cells. Antiproliferative activities of flavonoids in cancer cells may involve several mechanisms, including inactivation of carcinogen compounds, cell antiproliferation, inhibition of cell cycle, induction of apoptosis and cell differentiation, inhibition of angiogenesis, and antioxidants.[27]

Terpenoids are bioactive substances with numerous pharmacological properties, such as anticancer properties. Terpenoids, a sizable class of secondary metabolites, are made up of multiple isoprene units and are generated from plant sources. The potential anticancer and pharmacological properties of terpenoids have received interest due to their considerable diversity. Some terpenoids show an anticancer effect by inducing different stages of cancer growth, such as reducing the early stage of carcinogenesis by inducing cell cycle arrest, preventing cancer cell differentiation, and activating apoptosis, to produce an anticancer effect. Certain terpenoids can suppress angiogenesis and metastasis at a late stage of cancer development by altering various intracellular signalling pathways. The number of carbons generated by isoprene units determines how terpenoids are classified. Monoterpenoids possess the ability to alter the expression of the Bax and Bcl-2 proteins. This activates caspases and causes apoptosis. Squamous carcinoma cells (SCC) show signs of mitochondrial damage caused through monoterpenoids. By triggering phospholipase C-dependent Ca^{2+} secretion from the endoplasmic reticulum and Ca^{2+} entry through store-operated Ca^{2+} channels, which may induce apoptosis, monoterpenoids exercise their anticancer action. Terpenoids mainly exert their anticancer effects by targeting various pathways, including mitochondrial death pathway, PI3K/Akt, and NF-$_K$B pathways.[28]

Steroids cause apoptosis or autophagy by upregulating or downregulating proteins that are involved in apoptosis (Bax, Bcl2, BclxL, Caspase 3/8/9, PARP, TNFR I/II, Fas, or HER2) and autophagy (LC3, AKT, or mTOR). Another mechanism underpinning their anticancer efficacy is the interaction with CDKs, which inhibits cell cycle progression at the G0/G1 or G2/M phase. Steroids also show anticancer effect by altering the DNA content and cell shape, which compromises the integrity of the cell membrane.[29] Human oral squamous cell carcinoma and salivary gland tumour cell lines are more sensitive to the cytotoxic effects of hydrolyzable tannins than are normal human gingival fibroblasts. Gallic acid, a component of tannins, has significantly weaker selective cytotoxicity. The TUNEL technique and the M30 monoclonal antibody both show that tannins cause apoptotic cell death, which is defined by DNA fragmentation and cytokeratin 18 being cut by activated caspase(s). Quinone stimulates intrinsic and extrinsic mechanisms of apoptosis by activating several caspase cascades, up- and down-regulating apoptotic genes, antitumor cell proliferation, and ROS modulation. This results in a substantially promising anticancer action against oral and lung cancer.[30]

According to previous studies, hexane extract of *C. nutans* leaves have a significant antiproliferative effect, by inducing apoptosis in all cell types in this study, namely A549 cell strain (non-small cell lung cancer), CNE1 cell strain (nasopharyngeal cancer), and HepG2 cell strain (liver cancer).[21] Previous research also found that hexane extract of *C. nutans* leaves can inhibit the proliferation of HONE-1 cell strains (epithelial

tumors) by up to 61% because terpenoids in hexane extract has anticancer effects.[31]

In this study, the concentration of 500 µg/mL was the most effective concentration in inhibiting migration and inducing apoptosis overall as this concentration had an effect by the 6th hour. Compared to the positive control group, the concentration of 500 µg/mL was more effective because by the 6th hour the positive control group had not induced apoptosis. This can be seen from the measurable gap at the 6th hour, which was 23.46 ± 54.12%. The positive control group induced overall apoptosis by the 12th hour.

Meanwhile, the concentration of 250 µg/mL only induced overall apoptosis by the 24th hour. Concentrations of 100 µg/mL, up to the 48th hour, showed inhibitory effects on migration but did not induce overall apoptosis, as observed from the widening gap at the 48th hour. The concentration of 50 µg/mL also exhibited inhibitory effects on migration, but by the 48th hour, the gap narrowed, indicating that there was an inhibitory effect but not as strong as the 100 µg/mL concentration. The concentration of 25 µg/mL only showed migratory inhibition effects up to the 12th hour, and by the 24th hour, the gap had completely closed.

Previous research showed that hexane extract of *C. nutans* leaves had no effect on the migration of human gingival fibroblast cells, but in this study, the hexane extract of *C. nutans* leaves exhibited an inhibitory effect on HSC-3 cell migration. The difference in these results may be attributed to the use of different cell types and possibly variations in the extraction methods. The previous research utilized the Soxhlet method, while this study employed the maceration method.[23]

CONCLUSION

This study has identified that hexane extract of *C. nutans* leaves is effective in inhibiting the migration of HSC-3 cells in the concentrations of 50 µg/mL, 100 µg/mL, 250 µg/mL, and 500 µg/mL, while the concentration of 25 µg/mL is not. Future research could study the effect of hexane extract of *C. nutans* leaves on cancer cell migration with other migration test methods.

CONFLICT OF INTEREST

The authors declare no conflict of interest with the data contained in the manuscript.

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