

Research Report

Inhibitory effect of *n*-hexane: ethyl acetate fraction from *Artemisia vulgaris* L. on cell culture of oral epithelial carcinoma

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ABSTRACT

Background: Sudamala herb (*Artemisia vulgaris* L.) is often used in society as an anti tumor for organs of digestive system including oral cavity. Nevertheless, there are still no further scientific researches on active materials which can be used as anti carcinogen in oral cavity. Most of anti carcinogens are actually obtained from the genus *Artemisia*. Moreover, in Indonesia, the species of the genus *Artemisia* that grows the most is *Artemisia vulgaris* L. The problem of this research, however, is that the effect of *n*-hexane fraction: ethyl acetate from *Artemisia vulgaris* L. towards the decreasing of oncogene in oral squamous cell carcinoma is still indefinite. **Purpose:** The objective of this research is to explain the effect of giving *n*-hexane : ethyl acetate (3:7) fraction containing terpenoid from *Artemisia vulgaris* L. towards the decreasing of oncogene in oral epithelial carcinoma cell line. **Methods:** The method of this research is laboratory experimental research by using squamous cell carcinoma of oral cavity on cell culture. The inhibitory percentage test *in vitro*, furthermore, is taken during the analysis. The result then is analyzed by probit analysis with drawing relation curve between the inhibitory percentage and concentration. **Result:** The result of *n*-hexane : ethyl acetate (3:7) fraction containing terpenoid from *Artemisia vulgaris* L. has the smallest IC50, 3.902 µg/ml, less than 20 µg/ml suitable with NCI criteria; thus, it can potentially be used as anti carcinogen. **Conclusion:** There is the decreasing of oncogenes after being given *n*-hexane : ethyl acetate (3:7) fraction containing terpenoid from *Artemisia vulgaris* L. towards oral epithelial carcinoma cell line.

Key words: *n*-hexane: ethyl acetate fraction, *Artemisia vulgaris* L., oral squamous cells carcinoma

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INTRODUCTION

Carcinoma in oral cavity is one of the tumor cases that often occur around the world. The tumor in oral cavity and oropharynx is found in about 3% of one million tumor cases detected in the United States during one year.¹ In Indonesia, on the other side, the rate of carcinoma cases in oral cavity is high enough which is on the sixth rank of all carcinoma cases that often occurs and increases in every year. Oral squamous cell carcinoma (OSCC) in oral mucosa epitel is a kind of cancer often found in oral cavity about 90%.²

In addition, some herbal medicine discoveries showing pharmacology effects encourage researchers to exploit bioactive materials from herbs. The basic reason of this research is that herbs are often used by the society for curing cancer since they are safer, cheaper, and more available.

Sudamala herb (*Artemisia vulgaris* L.), for instance, is often used in the society as anti tumor for organs of digestive system including oral cavity. Nevertheless, there are still no further scientific researches on active materials which can be used as anti carcinogen in oral cavity.

In Indonesia the species of the genus *Artemisia* that grows the most is *Artemisia vulgaris* L. *Artemisia vulgaris* L. called sudamala. This herb can be found growing wildly in fields, in forests, and in humid areas which are rich of humus. A lot of Sudamala herbs used in the society is empirically useful as anti inflammation, analgesic, and anti cancer for digestive system and breast.³ In fact, there is no a lot of researches on *Artemisia vulgaris* L. as anti carcinogen. However, with ethnopharmacology and chemotaxonomy approach it can be proved *Artemisia vulgaris* L. as anti carcinogen. Ethnopharmacology, moreover, is theoretical

approach using empiric indications about the use of herbal materials as medicine. On the other hand, chemotaxonomy is theoretical approach using another herb from the same genus proven that it contains active extracts. Artemisinin, furthermore, is an isolate active material from *Artemisia annua* L. used as anti carcinogen.⁴ Artesunate derivative Artemisinin from *Artemisia annua* L. is also proved that it can prevent the growth of cancer cell in colon.⁵ The herbal extract of *Artemisia argyi* L. containing terpenoid and flavonoid can also prevent cervix carcinoma and has cytotoxic effects on He La cell culture.⁶ Some natural compounds, moreover, are proven that they can prevent the interaction between BP-7,8-diol-9,10-oxide carcinogen and DNA through some mechanisms. Those compounds have polyphenol structure or are classified into flavonoid and terpenoid groups found a lot in some kinds of herbs.⁷

The purpose of this research, in addition, is to prove the inhibitory effect of *n*-hexane: ethyl acetate fraction from the *n*-hexane extract of *Artemisia vulgaris* L. containing terpenoid towards oral epithelial cell line carcinoma. In order to measure the inhibitory effect, the formulation of inhibitory percentage is used in analyzing the result. Afterwards, probit analysis is used for measuring IC50 by drawing relation curve between inhibitory percentage and concentration. IC50 is a measure of the effectiveness of a compound which inhibitory percentage of cancer cell is 50%. The research result, finally, can be used as therapy development base using active materials of terpenoid compound from *Artemisia vulgaris* L. in order to cure cancer, especially oral mucosa carcinoma.

MATERIALS AND METHODS

The material of this research is *Artemisia vulgaris* L. herb obtained and determined in Balai Konservasi of Botanical Garden Purwodadi, Pasuruan. This herb is taken from a plant about 24 months old and 50 cms tall. The herb, taken from the edge of the leaves to the tip of the leaves, is on 800 ms above the sea level. The herb then was cleaned from other herbs and sludge, washed, and dried in open air without being exposed to the sun directly. After being dried, the herb was milled by miller, and sifted by a powder sifter in order to make the active essence of the herb could easily react with solvent so that it could completely extracted. The powder of the herb then was saved into a closed vessel.

The extract of *Artemisia vulgaris* L. herb was made by maceration, a process of soaking *Artemisia vulgaris* L. powder into *n*-hexane solvent in a closed vessel at ambient temperature for 2 x 24 hours, and stringing it at the same time. It then was sifted by *buchner* sifter, and its filtrate and pellet were macerated again for 6 times with new solvent. Maceration would be stopped if after the orientation of terpenoid concentration with Thin Layer Chromatography (TLC) using mobile phase (eluen) *n*-hexane : ethyl acetate = 1:2, the anisaldehyde stain of sulfate acid was not red

purple on TLC pellet. The result of the maceration was collected and evaporated with rotavapor at low pressure until it could not be evaporated again. Thus, the mass of condensed extract was obtained. The remains of solvent in the condensed extract then were evaporated in acid cabinet so that its result called as dried extract of *n*-hexane.⁸

The extract of *n*-hexane identified contains terpenoid, moreover, was fractionated by using Column Vacuum Chromatography. The stationery phase of silica gel 60 (Merck) was put into a dried *sintered glass*. The filling process was done until reaching 4–5 cm tall for each column with 2.5–3 cm diameter. Hexane extract then was mixed with silica gel and poured on to *sintered glass* which had been watered by solvent. Afterwards, it was closed again with silica gel 60, and then was eluted by mobile phase *n*-heksan-ethyl acetate with polarity increasing. Mobile phase used was *n*-hexane : ethyl acetate (10:0, v/v), *n*-hexane : ethyl acetate (9:1, v/v), *n*-hexane : ethyl acetate (8:2, v/v), *n*-hexane : ethyl acetate (7:3, v/v), *n*-hexane : ethyl acetate (6:4, v/v), *n*-hexane : ethyl acetate (5:5, v/v), *n*-hexane : ethyl acetate (4:6, v/v), *n*-hexane : ethyl acetate (3:7, v/v), *n*-hexane : ethyl acetate (2:8, v/v), *n*-hexane : ethyl acetate (1:9, v/v), *n*-hexane : ethyl acetate (0:10, v/v). Those 11 fractions produced then will be tested *in vitro* for their capabilities as anti carcinogen.⁸

Furthermore, the inhibitory percentage of those 11 fractions was measured *in vitro* by using epithelial *Carcinoma cell line* in oral cavity from *American Type Culture Collection* (ATCC) no CCL-17. This phase was done into *Laminar Air Flow Cabinet* (LAFC) with three time replication. Oral epithelial *Carcinoma cell line* was cultured into *Dulbecco's Modification of Eagles Medium* (DMEM) 10% *Fetal Bovine Serum* (FBS) 10 ml media, 2 ml penicillin streptomycin, and 0.5 ml fungizone in 100 ml DMEM, and then was harvested by trypsin–EDTA 0.25%. Afterwards, it was put into centrifuge tube and was centrifuged in five minutes, 1500 rpm. The result then was measured with hemocytometer, and was put into 20,000 cells/hole. The cells were put into 100 μ l media in *microwell plate*. Furthermore, 10 mg fraction of solution test (sample) was added and dissolved into 100 μ l methanol through the series of dilution until the concentration becomes 2.5, 5, 10, 20, and 40 μ g/ml after the optimization.⁵ Moreover, incubation process was done by using an incubator, 95% O₂ and 5% CO₂, with temperature at 37° C for 24 hours. Four hours before the incubation period was over, MTT (3-(4,5 Dimethylthiazol-2yl)-2,5 diphenyltetrazolin bromide) had been added about 5 μ g/ml for each holes, and the incubation process then was continued again. After the incubation process had finished, centrifugation was taken. Afterwards, 1 ml isopropil alcohol was added in order to destroy cell. Vortex 30 rpm then was used for 5 minutes. In order to measure the absorbance of supernatant, colorimetry (*Elisa reader*) with 550 nm long wave was used, and then the analysis of the result was taken by using the following formulation:

Table 1. The average percentage of inhibitory of cancer cell in vitro and the value of IC50 after being given *n*-hexane : ethyl acetate fraction

Materials	The Average Percentage of Inhibitory Value						IC50 μg/ml
	Replication	Dosage	Dosage	Dosage	Dosage	Dosage	
		2,5 μg/ml	5 μg/ml	10 μg/ml	20 μg/ml	40 μg/ml	
<i>n</i> -hexane : ethyl acetate(10:0, v/v)	3	31,549	31,590	35,95	36,046	35,960	54,462
<i>n</i> -hexane : ethyl acetate (9:1, v/v)	3	32,510	37,913	38,66	43,850	43,416	38,782
<i>n</i> -hexane : ethyl acetate (8:2, v/v)	3	34,980	39,316	40,297	45,030	44,487	37,160
<i>n</i> -hexane : ethyl acetate (7:3, v/v)	3	25,503	26,149	26,86	46,330	37,220	44,006
<i>n</i> -hexane : ethyl acetate (6:4, v/v)	3	39,800	40,070	47,87	51,050	49,300	29,324
<i>n</i> -hexane : ethyl acetate (5:5, v/v)	3	47,330	50,070	53,853	64,379	61,310	16,936
<i>n</i> -hexane : ethyl acetate (4:6, v/v)	3	42,3630	55,973	64,443	83,050	82,900	9,278
<i>n</i> -hexane : ethyl acetate (3:7, v/v)	3	59,290	64,155	81,675	85,486	83,429	3,902
<i>n</i> -hexane : ethyl acetate (2:8, v/v)	3	44,185	51,241	72,764	83,759	81,600	8,756
<i>n</i> -hexane : ethyl acetate (1:9, v/v)	3	40,792	54,496	60,918	83,143	74,743	10,837
<i>n</i> -hexane : ethyl acetate (0:10, v/v)	3	38,529	46,443	63,48	81,550	68,010	12,934

$$\% \text{ Inhibitory} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100\%$$

Note: OD = *Optical Density*

In order to determine the IC50, probit analysis was used by drawing the relation curve between inhibitory percentage and concentration. The IC50 was a measure of the effectiveness of a compound which inhibitory percentage of cancer cell is 50%.⁹

Among 11 fractions that have been tested for their ability as anti-carcinogen in vitro, it was found that *n*-hexane : ethyl acetate (3:7,v/v) fraction has the lowest value of IC50 i.e. 3,902 μg/ml less than 20 μg/ml which is appropriate with criteria of *National Cancer Institute* (NCI). Therefore, it is potential to be used as anti-carcinogen. Since the value of IC50 is 3,902 μg/ml, this ethyl acetate has the highest possibility as anti-carcinogen

DISCUSSION

Researches of traditional medicines, which studied on herbal plants, are continuously done and recently the numbers of those researches are increased. In the contrary, only a few results of researches studying herbal plants are used as medicines in medical services. Medicines consumed in society must meet some requirements; safe, valuable, and standardized. Thus, pre-clinical and clinical experiments are undergone to examine the herbal plants. The pre-clinical experiment includes utility test based on experimental research that can be undergone either by *in vivo* or *in vitro*.

In this research, experiment of *in vitro* using cell culture was applied to determine the fraction material which has the lowest value of IC50. The value of IC50 indicates the level

of material experiment where the percentage of inhibitory towards cancer cell is 50%. In this experiment, radioactive material was not used but reagent that was reduced by metabolite of living cell was used, forming blue formazan called MTT or Thiazolyl Blue (3-(4,5-Dimethylthiazol-2yl)2-5-Diphenyl Tetrazolium Bromide). Process of MTT usage by redox reaction did not use radioactive material and it was more uncomplicated and harmless. Furthermore, the result of measurement was the same as the result using radioactive material. The living cell would engulf the yellow MTT reagent and would be reduced by metabolite of cell which later formed blue crystal. The intensity of its color –blue– would be measured by calorimetry at the wave length 550 nanometer after the cell had been obliterated by isopropyl-alcohol solution. Meanwhile, the intensity for yellow was equal to the number of living cell. The experiment used MTT method, which had to be completed cautiously especially when it came to the process of washing cell otherwise MTT on supernatant or there would be no cells in the fetched. These can cause error on reading Elisa calorimetry.⁹

The experiment result *in vitro* of *n*-hexane : ethyl acetate fraction *Artemisia vulgaris* L. is determined from the value of IC50 using probit analysis by drawing relation curve between the percentage of inhibitory and the concentration. From the analysis, *n*-hexane : ethyl acetate (3:7,v/v) fraction has the lowest value of IC50 of all fractions i.e. 3,902 μg/ml seen in the table 5.1. NCI (*National Cancer Institute*) has determined criteria that a material has characteristics as anti-carcinogen if IC50 is less than 20 μg/ml for extract and fraction.¹⁰ Of 10 fractions, the value of IC50 for *n*-hexane: ethyl acetate (3:7,v/v) fraction from *n*-hexane extract of *Artemisia vulgaris* L. is the lowest so it has the highest ability as anti-carcinogen.

Sudamala herb is frequently used as anti tumor in organs of digestive system including in oral cavity but there has been no research studying active substance which has a role

as anti-carcinogen in oral cavity. Many active substances as anti-carcinogen are found in genus *Artemisia* while the species that mostly grown in Indonesia is *Artemisia vulgaris* L. In this research, extraction process of *Artemisia vulgaris* L applied maceration because the use equipment was simple and did not need any heat in order to avoid the compound being reduced by heat. Extraction using *n*-hexane was applied in isolation of terpenoid compound found in *Artemisia vulgaris* L that has a role as anti-carcinogen. General characteristic of non-polar terpenoid required non polar solution of *n*-hexane so it could take terpenoid compound.⁷ The fractionation process using solution of *n*-hexane: ethyl acetate aimed to obtain terpenoid compound. Terpenoid is a chemical compound derived from plant which has isoprene molecule (C5) and its carbon pattern is composed from connecting 2 or more (C5) units. Terpenoid is classified into several kinds; isoprene (C5), monoterpenoid (10), sesquiterpenoid (C15), diterpenoid (C20), triterpenoid (C30), tetraterpenoid (C40), polyisoprene (Cn). Some terpenoid compounds from genus *Artemisia* that have been studied have a function as anti-carcinogen for example Artemisinin. Artemisinin belongs to *Sesquiterpene lactone* from *Artemisia annua* L. that is able to inhibit breast cancer cell by *in vitro* by means of enhancing the activation of P53 *wild*.¹¹ Sesquiterpene lactone belongs to terpenoid compound that has potential effect of anti-carcinogen and it can induce apoptosis on cancer cell *in vitro*. Besides, it can metastasize various cancers in animal.⁷ Terpenoid and saponin affect the permeability of cell membrane. The increase of its permeability can cause the liquid electrolyte on the outside the cell will be easy to get into cells. As the result, the cell will be separated into several fragments.⁷ This can explain one of mechanisms of terpenoid compounds in obliterating material.

According to this research, it can be concluded that *n*-hexane: ethyl acetate (3:7,v/v) fraction which is derived from extract of *n*-hexane *Artemisia vulgaris* L., has the lowest value of IC50 3,902 µg/ml of 10 fractions. Consequently, it has the highest ability as anti carcinogen. It is suggested that the findings of the research can be used as basis to compose a medicine containing *n*-hexane: ethyl acetate(3:7,v/v) fraction which later is used as anti-carcinogen in oral cavity.

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