Toxicity Test n-Hexane Ethyl Acetate (37) Fraction of Sudamala (Artemisia vulgaris L.) - Copy

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Submission date: 18-Jun-2018 09:29AM (UTC+0800)
Submission ID: 976499715
File name: Acetate_37_Fraction_of_Sudamala_Artemisia_vulgaris_L._-_Copy.pdf (202.36K)
Word count: 2920
Character count: 15805

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Procedia Chemistry 18 (2016) 174 – 178

Procedia Chemistry

Molecular and Cellular Life Sciences: Infectious Diseases, Biochemistry and Structural Biology 2015 Conference, MCLS 2015

Toxicity Test n-Hexane : Ethyl Acetate (3:7) Fraction of Sudamala (*Artemisia vulgaris L.*)

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Abstract

Sudamala (*Artemisia vulgaris* L.) is commonly used in the community as anti-tumor in digestive organ, including in oral cavity. However, there have been no scientific studies about oral anti-carcinogenic active substances of Sudamala. The purpose of this study was to analyse the effect of per oral administration of n-hexane : ethyl acetate (3:7) fraction of *Artemisia vulgaris* L. on healthy mice. This experimental study used male Swiss Webster (Balb C) strain mice (*Mus musculus*) 2.5 months old, 20-30 grams by weight. Group 1, the mice received 0.5% CMC- Na fraction solvent, as a control. Group 2, the mice received 200 mg/kgbw n-hexane : ethyl acetate (3:7) fraction of Sudamala (*Artemisia vulgaris* L). The fraction was given once daily for 8 weeks per oral according to the mice's weight. At the end of 8th week, the mice were killed and oral mucosal tissue was taken for biopsy specimens. Tunnel assay for Apoptosis and immunohistochemistry were undertaken for PCNA. T test analysis showed there was no significant difference between control and treatment groups. The fractions of n-hexane : ethyl acetate (3:7) of Sudamala (*Artemisia vulgaris* L) was not toxic , it was not harmful to living tissue in healthy mice.

Peer-review under responsibility of the organizing committee of the Molecular and Cellular Life Sciences: Infectious Diseases, Biochemistry and Structural Biology 2015 (MCLS 2015)

Keywords: ethyl acetate fraction; *Artemisia vulgaris* L.; toxicity

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Peer-review under responsibility of the organizing committee of the Molecular and Cellular Life Sciences: Infectious Diseases, Biochemistry and Structural Biology 2015 (MCLS 2015)
doi:10.1016/j.proche.2016.01.027

Nomenclature

CMC-Na	Carboxyl Methyl Cellulosa – Natrium	NFkB	Nuclear factor kappa Beta
DAB	Diamino Benzidine	PCNA	Proliferating cell nuclear antigen
ERK	Extracellular signal-regulated kinases	SCCOC	Squamous Cell Carcinoma of Oral Cavity
IC 50	The half maximal inhibitory concentration	TDT	Terminal Deoxynucleotidyl Transferase
MMP	Matrix metallopeptidase	TPA	12-o-tetradecanoylphorbol-13-acetate

1. Introduction

The species of *Artemisia* genus is obtained abundantly and In Indonesia, the most growing species is *Artemisia vulgaris* L, named Sudamala. Sudamala is widely used in society because it is empirically efficacious as anti-inflammatory, analgesic, and anti-cancer of the gastrointestinal tract and breast. Sudamala plant (*Artemisia vulgaris* L.*)* is often used in public as an anti-tumor on the digestive organs including the oral cavity, but research on the active ingredient which acts as an anti-cancer in the oral cavity has not been done yet[1]. The main factors of malignancy in the oral cavity include medical history, the habit of consuming tobacco, alcohol and smoking. Benzopirene (B (a) P) is mostly found in cigarette smoke, fumes, smoke from the burning of organic matter and smoked or baked foods. B (a) P can cause gene mutations resulting in the transformation of normal cells into cancer cells[2] . The oral cavity cancer is one of the worldwide malignances frequent cases. In a year in the United states, 3% of the approximately one million more cases of detected malignancy is malignancy in the oral cavity and oropharynx. The incidences of oral cancer in Indonesia are quite high and in the 6th position of the whole most common cancer in the world which is increasing annually. Squamous Cell Carcinoma of the Oral Cavity (SCCOC) is derived from oral mucosal epithelium which is a type of cancer that is most commonly found, approximately 90%, in the oral cavity[3] .

This research aimed at proving the toxicity based on changes in the expression of PCNA and apoptosis in mice oral mucosal cells from the oral treatment of n-hexane : ethyl acetate (3:7) fraction of *Artemisia vulgaris L.* The result of this research could be used as the basis of therapy development with appropriate dose through the exploration of the active ingredient compound of terpenoids Sudamala (*Artemisia vulgaris L.*) on cancer prevention, especially in the oral mucosa.

2. Methods

The material of the research is Sudamala obtained and determinated in Plants Conservation Center in Purwodadi Pasuruan Botanical Garden.

The extract preparation of Sudamala is made by maceration which is done by soaking the powder of Sudamala in the solvent n-hexane for 4 x 24 hours in a closed vessel, often stirred, at room temperature[4]. The extract of identified n-hexane contains terpenoids in which fractionation is done by using vacuum column chromatography. The mobile used phase was n-hexane : ethyl acetate with increasing polarity. From 11 fractions produced, their anti-cancer was tested *in vitro* using *Carcinoma cell line* of the oral cavity. Next, the result analysis is done by using percentage barriers. To determine the IC50 score, probit analysis is done by making curve relationship between the percentage of obstacles with the content of tested material. The IC50 score is the content of tested material in which the percentage of obstacles on cancer cells is 50%. The obtained n-hexane fraction : ethyl acetate (3:7, v/v) has the lowest IC50 score among 11 fractions which is 3.902 μg/ml[5].

At first, given fraction n-hexane : ethyl acetate (3:7, v/v) Sudamala dose 2 g/kg bb orally once a single dose, observed for 7 days, to the weight of 5 male and 5 female Strain Swiss Webster Mices *(Mus musculus*), 2.5 month, 20-30 gr uses the sonde mice. Observed aims to explain the effects of the material test by an autopsy if there were dead, to see whether there is any indication of organ damage adjusted for pharmacological effects.

The n-hexane : ethyl acetate (3:7, v/v) fraction of Sudamala is dissolved in *Carboxyl Methyl Cellulosa* (CMC)-Na 0.5% (500 mg CMC-Na of 100 ml sterile distilled water). Giving control (0.1 ml / 10 gr bb CMC-Na 0.5%) and fraction dose 200 mg/kg bb orally once daily for 8 weeks according to the weight of 10 male Strain Swiss Webster Mices *(Mus musculus*), 2.5 month, 20-30 gr uses the sonde mice. At the end of 8 week, all mices are sacrificed in order to take their tissue mucosa of oral cavity as the biopsy specimens, coloring tissue is done by using

immunohistochemistry *Biotin Streptavidin Amplivied* technique. The examination is done under a light microscope with 400 times magnification. Next step is counting the number of oral cavity squamous cells undergoing apoptosis with TUNEL *assays* and expresses PCNA per 100 cells with immunohistochemistry. Each of stocks is observed on 4 visual fields 3, 6, 9 and 12 clockwise. Each visual field is observed and counted on two places 6 and 12 clockwise by using *grateculae* and *counter*. Then, the result is averaged. The collected data is analyzed by using T test[6].

3. Results and discussion

From 1 kg of Sudamala (*Artemisia vulgaris L.*) powder are extracted by maceration for 6 times using n-hexane solvent resulting hexane extract weighing 88.4 grams. Sudamala hexane extract was fractionated using vacuum column chromatography with silica gel stationary phase and a mobile phase mixture of n-hexane : ethyl acetate gradient. Result 11 fractions of Sudamala, after in vitro test on oral carcinoma cell line, obtained fraction of n-hexane : ethyl acetate (3: 7, v/v)[5].

From the results test in 10 mice (*Mus musculus*) Swiss Webster strain (Balb / c) 5 males and 5 females aged 2.5 months with a weight of 20-30 grams were given a fraction n-hexane : ethyl acetate (3:7, v/v) of Sudamala dose of 2 g / kg bw orally and observed for 7 days no mice died.

Test results n-hexane : ethyl acetate (3:7, v/v) fraction of Sudamala dose of 200 mg/kg bw orally for 8 weeks on 10 male mice, no one died. In the T test results obtained proliferation (PCNA) and apoptosis were not significantly different with the results of the control group was given 0.5% CMC-Na orally for 8 weeks. It means giving the n-hexane : ethyl acetate (3:7, v/v) fraction of Sudamala does not damage healthy cells.

Table 1. Results of the T test number of apoptotic cells and PCNA

Group	Mean ± SD	Significantly
Apoptosis (control)	5.85 ± 2.04	P= 0.688
Apoptosis(dose 200 mg/kg bw)	6.26 ± 2.45	no significant difference
PCNA (control)	6.68 ± 2.56	P= 0.177
PCNA(dose 200 mg/kg bw)	5.13 ± 2.36	no significant difference

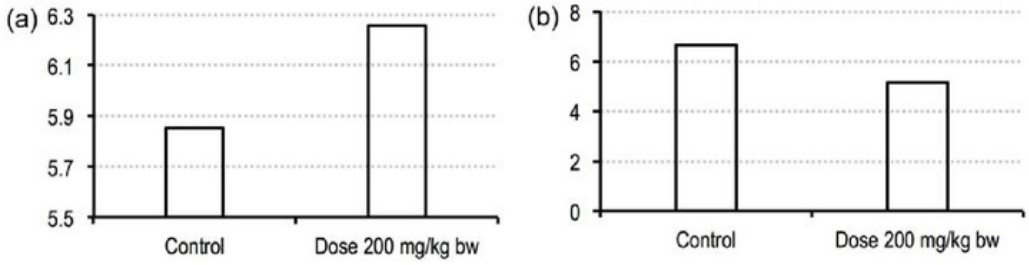

Fig. 1. (a) The average number of apoptotic cells ; (b) The average PCNA protein expression

Research on traditional medicines, especially medicinal plants/herbs, is continually increasing recently. However, until today, research on herbs is not much used as medicines in health service. Used medicines in society must meet the requirements such as safe, beneficial, and standardized. To fulfil those requirements, medicines must pass toxicity tests.

The species of *Artemisia* genus is obtained abundantly. In Indonesia, the most growing species is *Artemisia vulgaris* L, named Sudamala, it could be found in field, forest, and fertile soils which are humid and full of humus. Sudamala is widely used in society because it is empirically efficacious as anti-inflammatory, analgesic, and anti-cancer of the gastrointestinal tract and breast[1].

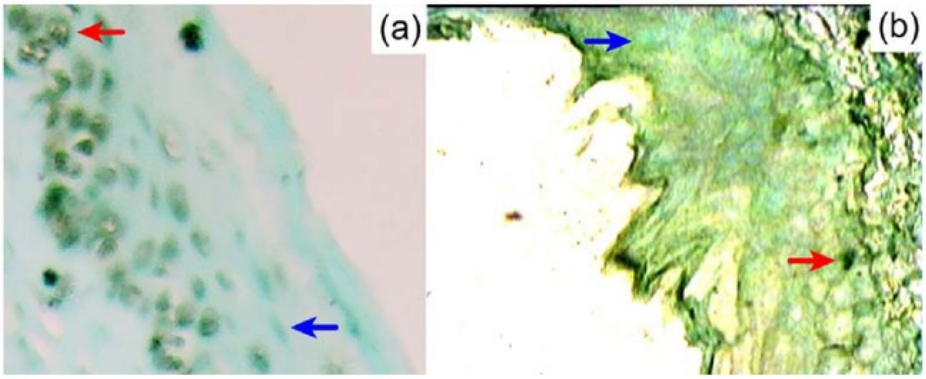

Fig. 2. (a) Results of staining by TUNEL assay; (b) Immunohistochemistry using monoclonal antibodies anti mice PCNA. With 400 x magnification, red arrows = positive reaction; blue arrow = negative reaction

This study uses Sudamala (*Artemisia vulgaris L.)* orally because the more appropriate way to use empirically in the community. In this study the Sudamala extraction process using maceration techniques for the use of simple tools and do not use the heat thus obviating the compound in which degraded by heat. Extraction using n-hexane so that isolation terpenoid compounds contained in Artemisia vulgaris L which has anti-cancer activity. Terpenoids are non-polar and require n-hexane non-polar solvent which is expected to attract terpenoid compounds[7]. Results of phytochemical screening to Sudamala extract with n-hexane obtained several compounds which give a positive result that the compound terpenoids, flavonoids and alkaloids. Terpenoids are chemical compounds derived from plants that contain molecules of isoprene (C5) and the carbon framework constructed by connecting 2 or more units of C5. Terpenoids cause tumor cells split into several fragments[7]. This can explain one of the terpenoids mechanisms in destroying cancer cells.

Dose of 200 mg/kg body weight (bw) proved to be more potent than the dose of 50 and 100 mg/kg bw, because it contain more of the active compound, according to the results of preliminary research. The result of the effect n-hexane : ethyl acetate (3:7, v/v) fraction of Sudamala dose of 200 mg/kg bw in healthy mice did not different significantly with control on the expression of PCNA and apoptosis.

TUNEL *assay* is used to detect apoptosis since this method could detect the fragmentation of DNA as a characteristic of apoptosis. The principle of TUNEL *assay* is connecting the edge of 3-OH from the fragment of DNA with oligomer which is the chain of nucleotide triphosphate labelled randomly with *digoxigenin.* That random labelling aims to trigger the bonding between *digoxygenin* and *antidigoxygenin* optimally. This reaction is catalyzed by the TDT enzyme (*Terminal Deoxynucleotidyl Transferase). Digoxygenin* bonds *antidigoxygenin peroxydase conjugate* bonding the substrate of DAB *(Diamino Benzidine*) into brown. By using this TUNEL *assay* method, cells undergoing apoptosis could be found even though the morphological changes have not occurred yet and could be differentiated from cells that do not undergo apoptosis[8]. The immunohistochemistry examination is used indirectly by using secondary antibody that will bind the primary antibody which have already binded to antigens. Then, the secondary antibody is labelled to ease the microscope observation. *Counterstain Hematoxilin* or *Methyl green* is used so the positive cells will be brown and the normal cells will have color which is similar to *counterstain*, it will be counted on percentage and compared among groups[2].

As anti-cancer, research on *Artemisia vulgaris L.* is not much. However, based on chemotaxonomic and etnofarmakologi approach, it can be proved that *Artemisia vulgaris L.* can be used as an anti-cancer. Etnofarmakologi is a theoretical approach which utilizes empirical indications of the using plants as medicine, while chemotaxonomic is a theoretical approach which looks for another plant of the parts containing active similar substances. Artemisinin is an active ingredient of *Artemisia annua L.* which has effect as an anti-cancer[9]. Artesunate derivating Artemisinin from *Artemisia annua L* is also proven in inhibiting the growth of colon cancer cells[10]. *Artemisia Argyi L.* leaf extract containing terpenoids and flavonoids can inhibit cervical carcinoma and has cytotoxic effects in cell culture He La[11]. Some natural compounds have been proved in inhibiting the interaction between carcinogens Benzopirene-7,8-diol-9,10-oxide with DNA through various mechanisms. Those compounds have a group of polyphenols or are included into flavonoids and terpenoids groups found in various plant species[7].

Extracts of the plant Artemisia have the ability as an anti-inflammatory through inhibition of the cyclooxygenase 2 enzyme[11]. Sudamala has anti-inflammatory effects through inhibition of the cyclooxygenase 2 enzyme[1]. Jaceosidin active ingredient of *Artemisia argyi* known to inhibit 12-o-tetradecanoylphorbol-13-acetate (TPA) which is a promoter of breast tumor through inhibition of the enzyme cyclooxygenase 2, MMP 9 and ERK 1-2[12]. Eupatilin active ingredient of *Artemisia asiatica* is known to inhibit the 12-o-tetradecanoylphorbol-13-acetate (TPA) which is a promoter of breast tumor through inhibition of the enzyme cyclooxygenase 2, NFkB and Ras[13]. Jaceosidin has the ability to inhibit greater than eupatilin against 12-o-tetradecanoylphorbol-13-acetate (TPA) which is a promoter of breast tumor[14]. Anti-cancer mechanisms of *Artemisia vulgaris L.* can through the inhibit effect against cyclooxygenase 2 enzyme which acts to catalyze the oxidation of B (a) P-7,8-diol form B (a) P-7,8-diol-9,10-oxide which is a strong and reactive mutagenic carcinogens[15].

4. Conclusions

It can be concluded that n-hexane : ethyl acetate (3:7, v/v) the fraction of Sudamala not toxic and can be used as the basis for anti-cancer drug that contains the active ingredient n-hexane : ethyl acetate (3:7, v/v) the fraction of Sudamala.

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