ORIGINAL ARTICLE

Sudamala (*Artemisia vulgaris L.*) as Herb Therapy for Oral Squamous Cell Carcinoma

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ABSTRACT

Introduction: 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, while oral anti-carcinogenic active substances have not been found. The purpose of this study was to explain the effect of per oral administration of n-hexana : ethyl acetate (3:7) fraction of *Artemisia vulgaris L.* on the reduction of oncogenesis in transformed oral mucosa cells in mice due to benzopyrene induction. **Materials and Methods:** The experimental laboratory was performed on this study. We used 30 male Mus musculus mice and were divided into 5 groups. Group 1, control, received benzopyrene solvent Oleum Olivarum for 4 weeks, continued with 0.5 % CMC- Na fraction solvent. Group 2 received benzopyrene, followed with 0.5 % CMC-Na. Group 3 also received benzopyrene, followed with 50 mg/kgbw fraction of Sudamala (*Artemisia vulgaris* L.). Group 4 received benzopyrene, followed with 100 mg/kgbw fraction. Group 5 received benzopyrene, followed with 200 mg/kgbw fraction of Sudamala (*Artemisia vulgaris* L.). Tunnel assay staining and immunohistochemistry were undertaken for Ras, P53, PCNA and C-myc. Data were analyzed using Manova test. **Results:** There is an increase of apoptosis and P53 wild type expression and the reduction of the expression of P53 mutant, Ras, PCNA and C-myc to squamous cells of mice oral cavity which transformed caused by benzopyrene induced due to *Artemisia vulgaris L*. **Conclusions:** The fractions of n-hexana : ethyl acetate (3:7) is capable in killing cells which transformed without damaging the healthy ones.

Keywords: Oral cancer, Sudamala, Benzopyrene , Cancer cell

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INTRODUCTION

Oral cancer is one of the most common malignant cases in the world. In the USA, malignancy in oral cavity and oropharynx are found in 3% of approximately one million malignant cases detected in a year (1). The incidence of oral cancer in Indonesia is high and it holds the sixth rank of all most-frequent cancer cases worldwide, and it is also increasing annually. Oral Squamous Cell Carcinoma (OSCC) originating from oral mucosal epithelium is the type of cancer mostly found in oral cavity, comprising about 90% (2). The primary risk factor of oral malignancy include the history or habit of tobacco consumption, alcohol, and cigarette smoking. Benzopyrene (B(a)P) is abundant is cigarette smoke, vehicle combustion, smoke from organic materials burning process, and smoked or roasted foods. B(a)P may cause gene mutation, resulting in the transformation of normal cell to cancer cell (3).

Several findings of medicinal plants that have pharmacological effects on cancer has lead the authors to explore the bioactive materials of those plants. The reason behind this exploration was that the use of medicinal plants for cancer treatment is common in the community due to the consideration of their safery, cost-saving, and availability. The plant Sudamala (*Artemisia vulgaris* L.) is commonly used by the community as anti-tumor in digestive organs, including in oral cavity. However, studies on active agent playing a role as an anti-cancer in oral cavity have never been conducted.
Mostly found in species from the genus Artemisia, the one that grows in Indonesia is the species *Artemisia vulgaris L.*, which is called Sudamala, and can be found growing wildly in fields, forests, and prefers to grow in humid

and humus-rich soil. The use of Sudamala is common in the community, as it is empirically efficacious as an anti-inflammatory, analgesic, and anti-cancer in digestive tract and breasts (3). Studies on *Artemisia vulgaris L.* as anti-cancer remains rare. However, based on ethnopharmacological and chemotaxonomy, it could be proved that *Artemisia vulgaris L.* is an anti-cancer. Ethnopharmacology is a theoretical approach using empirical indication on the use of medicinal plants as drugs, while chemotaxonomy is an effort to find other plants from family that contains similar substance that has been proved to be active. Artemisinin is an active agent isolated from *Artemisia annua L.* that has effect as anti-cancer (4). Artesunate, the derivate of Artemisinin from *Artemisia annua L.*, has also been proved to inhibit the growth of colon cancer cells (5). The extract of *Artemisia argyi L.* leaves, which contains terpenoid and flavonoid, is abe to inhibit cervical carcinoma and have cytotoxic effect in He La cell culture (6). Several natural compounds have been proved to be able to inhibit interaction between carcinogene benzopyrene-7,8-diol-9,10-oxide with DNA through various mechanisms. Those compounds have polyphenol group or belong to flavonoid and terpenoid class that is commonly found in variou types of plant (7).

One of the agents that most frequently undergo mutation in carcinogenetic process is Ras oncogene. Ras protein is able to sitmulate kinase protein that will activate C-myc transcription factor and has a role in cell proliferation (8). The mechanism of p53 in ceasing cell division cycle if there is DNA defect is by activating P21 that inhibits all Cyclin Dependent Kinase (CDK) and the activity of Proliferating Cell Nuclear Antigen (PCNA). The increase of wild type P53 has a role to trigger cancer cell apoptosis through the activation of caspase 3 (9). The presence of benzopyrene in the cell is able to cause control disregulation of Ras, P53, PCNA and C-myc (10). This study has an objective to prove the reduction of oncogenesis based on the expression changes of wild P53, mutant P53, Ras, PCNA, C-myc and apoptosis in oral mucosa cell in mice undergoing transformation due to benzopyrene induction after per oral administration of n-hexana : ethyl acetate (3:7) fraction from A*rtemisia vulgaris L.* The result of this study can be used as a basis for developing therapy in appropriate dose through the exploration of active agent, the terpenoid from Sudamala (*Artemisia vulgaris* L.), in cancer eradication, particularly in oral mucosa.

MATERIALS AND METHODS

Samples

Material for this study was the herb *Artemisia vulgaris* L., which was obtained and determined at Plant Conservation Center, Purwodadi Botanical Garden, Pasuruan. The herb was taken from plants aged about 24 months, with height of about 50 cm, and taken from a place of 800 m above sea level. The herb was taken from the shoot of the leaf to the sheet, cleaned from other plants and dirt, washed, and air-dried without direct sunlight. Dried materials were fined with grinding machine and sieved with powder siever. Obtained powder was kept within tightly closed container. It was in the form of powder, so that the solvent would be easier to contact the active agent to provide a more perfect extraction.

Preparation of Artemisia vulgaris L extract

The extract from the herb *Artemisia vulgaris L.* was produced by maceration, i.e., immersing the powder of *Artemisia vulgaris L.* powder within n-hexana solvent for 2 x 24 hours within closed container, kept in room temperature with frequent stirring (11). N-hexana extract that has been identified as containing terpenoid was fractionated using vacuum column chromatography. Moving phase used was n-hexana : ethyl acetate with increasing polarity.

Preparation of oral cancer in rats induced by benzopyrene

This study was obtained ethical approval from the Ethical Clearance of Ethic Committee for Research in Faculty of Dental Medicine, Universitas Airlangga with registered number 106/HRECC.FODM/X/2021. The fraction of n-hexana : ethyl acetate (3:7,v/v) (12), from *Artemisia vulgaris L.* was dissolved in 0.5% Carboxyl Methyl Cellulosa (CMC)-Na (500 mg CMC-Na within 100 ml sterile distilled water). Dose fraction of 50 mg/kgbw/d was made by taking 1.25 mg fraction, dose fraction of 100 mg/kgbw/d was made by taking 2.5 mg fraction, and dose fraction 200 mg/kgbw/d was made by taking 5 mg fraction, and each was subsequently dissolved within 0.25 ml CMC-Na 0.5% for mice with bodyweight of 25 grams. Dose administration was adjusted to body weight. Mice with body weight of 20 grams were given fraction as much as 0.2 cc. Per oral fraction was given once a day for 8 weeks according to mice body weight using mice sonde (13).

The making of benzopyrene solution was carried out by taking 10 mg benzopyrene and dissolved within 10 ml Olium olivarum or 10 mg/kgbw. Each mice was induced as much as 0.02 ml in right buccal mucosal oral cavity. Male Swiss Webster (Balb/c) strain mice (Mus musculus), aged 2.5 months, with bodyweight of 20-30 grams were divided into 5 groups. Negative control group: Ten mice were induced with 0.02 ml Oleum olivarum in buccal mucosa at the lower right of oral cavity twice a week for 4 weeks, and then were given with 0.5% CMC-Na per oral of 0.1 ml/10 gr bw once a day for 8 weeks. Positive control group: Ten mice were induced with benzopyrene 10 mg/kgbw 0.02 ml in buccal mucosa at the lower right of oral cavity twice a week for 4 weeks, and then were given with 0.5% CMC-Na per oral of 0.1 ml/10 gr bw once a day for 8 weeks. Dose 50 group: Ten mice were induced with benzopyrene 10 mg/kgbw 0.02 ml in buccal mucosa at the lower right of oral

cavity twice a week for 4 weeks, and then were given with dose fraction of 50 mg/kg bw 0.1 ml/10 gr bw per oral once a day for 8 weeks. Dose 100 group: Ten mice were induced with benzopyrene 10 mg/kgbw 0.02 ml in buccal mucosa at the lower right of oral cavity twice a week for 4 weeks, and then were given with dose fraction of 100 mg/kg bw 0.1 ml/10 gr bw per oral once a day for 8 weeks. Dose 200 group: Ten mice were induced with benzopyrene 10 mg/kgbw 0.02 ml in buccal mucosa at the lower right of oral cavity twice a week for 4 weeks, and then were given with dose fraction of 200 mg/kg bw 0.1 ml/10 gr bw per oral once a day for 8 weeks.

Measurement of apoptotic cells and Ras, wild type P53, mutant P53, PCNA, and C-myc expression

At the end of week 12, all mice were sacrificed and oral mucosal tissue was taken as biopsy specimen and subjected to tissue staining using amplified streptavidin biotin immunohistochemistry. The examination was continued using light microscope in 400 times magnification. Subsequently, we counted the oral squamous cells that were apoptotic and expressed Ras, wild type P53, mutant P53, PCNA, and C-myc per 100 cells. Cancer cells that were positively Ras, wild type P53, mutant P53, PCNA and C-myc showed blackish brown color between negative cancer cells that were bluish or greenish. Each one preparation was observed in 4 visual fields clockwisely at 3, 6, 9, 12 o'clock. Each one visual field was observed and counted in two sites according to the direction of 6 and 12 o'clock using grateculae (counting chamber) and counter (14). The results were subsequently averaged.

Statistical analysis

Data were tabulated and analyzed using Manova test and followed by Bonferroni test with Wilk's Lambda ($\alpha = 0.05$).

RESULTS

The examinations carried out of this study were analyzed for the expression of Ras protein, wild P53, mutant P53, PCNA, C-myc, and apoptosis on 30 male Swiss Webster (Balb/c) strain mice (Mus musculus). The data in all groups has been presented in Table and Figures (1,2,3,4).

Table 1 shows that the expression of Ras protein, wild P53, mutant P53, PCNA, C-myc and apoptosis in all groups altogether were different, as proved statistically using multivariate test with Wilk's Lambda ($\alpha = 0.05$).

It is apparent in Figure 1 that the expression of Ras protein, mutant P53, PCNA, and C-myc decreased along with the dose increase, and wild P53 and apoptotic increase along with the increase of the dose.

Figures 2, 3 and 4 show an increase in apoptosis from transforming cells and a decrease in mutant P53 and PCNA after administration of Sudamala (*Artemisia vulgaris*).

Table: Mean and standard deviation of the counting of cells expressing Ras protein, wild P53, mutant P53, PCNA, C-myc and apoptosis

Expression	± SD					p-value
	Negative Control	Positive Control	Dose 50	Dose 100	Dose 200	
Ras	1.66 ± 0.7	44.78 ± 2.5	33.51 ± 2.0	24.75 ± 2.4	10.45 ± 1.9	0.00*
Wild P53	5.62 ± 1.5	1.36 ± 0.8	14.5 ± 2.3	24.23 ± 2.3	52.9 ± 1.6	
Mutant P53	0.0 ± 0.0	45.2 ± 1.4	23.65 ± 1.5	15.2 ± 1.3	6.075 ± 1.1	
PCNA	5.875 ± 2.9	53.95 ± 2.4	44.28 ± 2.4	34.05 ± 2.5	8.35 ± 1.9	
C-myc	5.45 ± 2.5	44.71 ± 1.7	32.087 ± 2.5	23.075 ± 2.0	6.25 ± 1.6	
Apoptosis	6.77 ± 1.9	1.125 ± 0.7	14.125 ± 2.6	21.825 ± 2.3	49.712 ± 2.8	

$\bar{x}$: mean; SD: standard deviation; *: there was a significant difference between groups (p = 0.00).

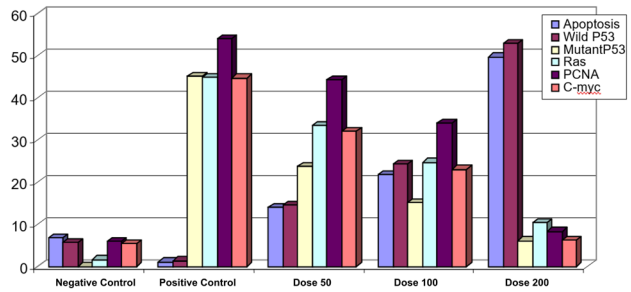

Fig. 1: Mean of expression of Ras protein, wild P53, mutant P53, PCNA, C-myc and apoptotic cell coount in five treatment groups.

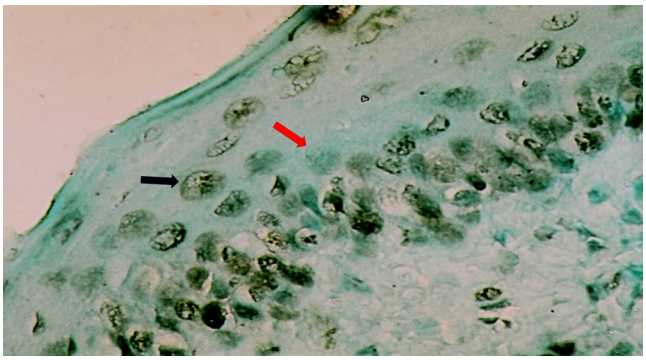

Fig. 2: TUNEL assay staining in mice oral mucosa in dose fraction of 200 mg/kgbw, magnification 400 x. Black arrow = positive reaction (brown); red arrow = negative reaction.

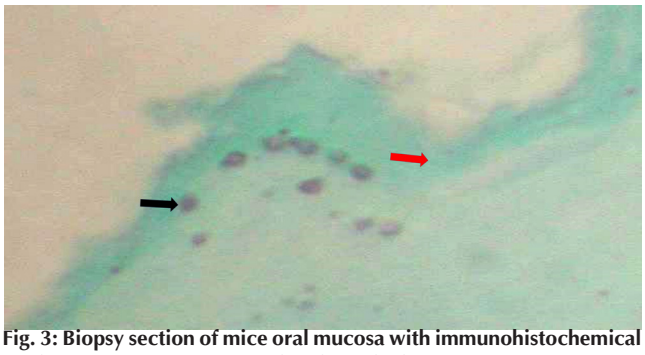

Fig. 3: Biopsy section of mice oral mucosa with immunohistochemical swab using anti-mice monoclonal antibody mutant P53 in groups with dose fraction of 200 mg/kgbw, magnification 400 x. Black arrow = positive reaction (brown) ; red arrow = negative reaction.

Fig. 4: Biopsy section of mice oral mucosa with immunohistochemical swab using anti-mice monoclonal antibody PCNA in groups with dose fraction of 200 mg/kgbw, magnification 400 x. Black arrow = positive reaction (brown) ; red arrow = negative reaction.

DISCUSSION

Studies on traditional medicine, particularly those with materials from medicinal herbs, is still undergoing and showing an increasing number recently. Nevertheless, not much of the results of those studies on medicinal plants are applied as medicine in health care. Drugs used in the community should meet the requirements of safety, efficacy, and standardization. To meet those requirements they should meet pre-clinical and clinical tests. Pre-clinical test include efficacy test based on experimental research that can be performed either in vivo or in vitro.

This was an experimental study to elaborate the effect of hexana-ethyl acetate fraction of Sudamala (*Artemisia vulgaris* L.) on the expression of Ras, wild type P53, mutant P53, PCNA, C-myc and apoptosis in oral mucosa induced by benzopyrene. This study used Balb/c mice since its genome sequence are mostly similar or homologues to those in human (15).

Cigarette smoking is the primary ethiological factor in oral cancer. Heavy cigarette smokers have a risk to oral cancer six times higher to those who never smoke (2). Benzopyrene was used in this study as chemical agent that caused cellular transformation to malignancy (transform cell), since it is produced from tobacco burning, particularly cigarette, and complete carcinogenic agent, both as tumor initiator or promotor (16). Benzopyrene (B(a)P), as one of the cause of Oral Squamous Cell Carcinoma (OSCC) may form B(a)P–7.8-diol-9.10-oxide, a strong and reactive carcinogenic mutagen. The diol oxide is highly reactive and may form covalent binding with guanin base DNA that may result in cancer (17). DNA damage due to benzopyrene may cause transversion mutation through covalent binding with guanine read as timin (18). The result of test in mice by the administration of n-hexana : ethyl acetate (3:7,v/v) *Artemisia vulgaris* L. in doses of 50, 100 and 200 mg/kgbw on 6 variables reflecting proliferative and apoptotic mechanism as the basis of oncogenesis were Ras, wild P53, mutant P53, PCNA, C-myc and several apoptotic cells.

The result of examination using TUNEL assay showed the count of apoptotic cells in all treatment groups were significantly different from that in control group. Among treatment group the results were also found to be significantly different and increased along with dose increase. TUNEL assay was used to detect apoptosis in this study since this method can detect DNA fragmentation, one of the characteristics in apoptosis. The principle in TUNEL assay was to connect 3-OH end of DNA fragment with olygomer, a triphosphate nucleotide chain labelled randomly with digoxygenin. Random labeling was intended to trigger optimal binding between digoxygenin and anti-digoxygenin. The reaction was catalyzed by enzyme TDT (Terminal Deoxynucleotidyl Transferase). Digoxygenin binds antidigoxygenin peroxydase conjugate that binds DAB (Diamino Benzidine) substrate, resulting in brown color. Using TUNEL assay, apoptotic cells could be found even though morphological changes had not been occurred and it could be easily differentiated from unapoptotic cells.

The mechanism of apoptotic increase resulting from n-hexana : ethyl acetate (3:7,v/v) fraction from *Artemisia vulgaris* L. may occur through P53 wild type increase pathway that activated bax to facilitate cytochrome-c release from mitochondria and bound to Apaf 1, resulting in the activation of caspase 9 and increased the activity of caspase 3, which caused DNA damage by DNAse (19).

The result showed that n-hexana : ethyl acetate (3:7,v/v) fraction of *Artemisia vulgaris* L. was able to increase apoptosis in cells that undergo transformation due to benzopyrene induction. This confirmed previous studies that the administration of eupatilin isolated from *Artemisia asiatica Nakai* was able to increase apoptosis and the expression of Caspase 3 in leukemic cells (20). This proved that genus Artemisia provides similar effects, although the species is different. This may result from the fact that in one genus Artemisia contain active

compound with similar potentials.

The result of immunohistochemical test showed that cell count expressing mutant P53 in all treatment groups were different significantly to those of control group. PCNA expression in all treatment groups were significantly different from that in control group except in group receiving dose fraction of 200 mg/kgbw, that was not different from control group. In mutant P53 expression and PCNA among treatment groups the result was also significantly different and reduced along with the dose increase. The expression of wild type P53 was found to increase significantly along with the dose increase. Indirect immunohistochemical examination was conducted using secondary antibody that bound primary antibody that had been bound to the antigen. Subsequently, the secondary antobody was labeled to facilitate microscope observation. Using Hematoxilin or Methyl green counterstain, positive cell stained brown and normal cells stained according to the counterstain, counted in percentage and compared between groups.

Mutagen stimulus in oral epithelium, such as carcinogenic material benzopyrene in continuous smokers in a long period, results in P53 gene mutation, so that the protein that it signals becomes inactive. Binding between benzopyrene that changed into 7,8-diol-9,10-oxide and DNA damaged DNA structure. If DNA damage occurred in tumor suppressor gene P53, an unstable P53 mutant may be formed. The administration of n-hexana : ethyl acetate (3:7,v/v) fraction from *Artemisia vulgaris* L. could reduce mutant P53 and increase wild type P53. The latter may have a role in DNA repair mechanism by ceasing cell cycle from G1 to S phase and increased GADD 45 (Growth Arrest and DNA Damage), a protein that has a role in DNA repair process. Wild type P53 may increase P21 that inhibits PCNA and all CDKs. This prevents complex binding between PCNA-CDK and cyclin that subsequently prevens pRb phosphorilation through the binding with E2F transcription factor, resulting in the deactivation of E2F. In consequence, cell cycle halts in G1 phase and allows DNA repair, and if the repair is successful, the cell cycle will be continued to S phase. If the repair process fails, P53 will activate bax to stimulate mitochondria to release cytochrome-c to cytosol along with Apoptosis Protease Activating Factor-1 (Apaf-1) and pro caspase 9 to form caspase 9, and then activates caspace 3 that results in cell apoptosis, which is presenting as DNA fragmentation by DNAse (19).

The obtained result showed that n-hexana : ethylacetate (3:7,v/v) fraction is able to reduce mutant P53 expression, reduce PCNA expression and increase wild type P53 expression in cells undergoing transformation due to benzopyrene induction. This confirmed previous studies that in the administration of eupatilin isolated from *Artemisia asiatica Nakai* is able to reduce mutant P53 expression, increase wild type P53 expression adn increase P21 expression in gastric cancer in vitro (4). The administration of artesunate isolated from *Artemisia annua* L. is also proved to increase wild type P53 and P21 expression in colon cancer (5). This proved that genus Artemisia had similar effect although the species was different. This is because one genus Artemisia contains active compounds with similar potentials.

The result of immunohistochemical examination showed that cell count expressing Ras in all treatment groups was significantly different from that in control group. C-myc expression in all treatment groups was significantly different from that in control group except in group receiving dose fraction of 200 mg/kgbw, which had no difference from control group. In Ras and C-myc expression between treatment groups also revealed significant difference and decreased alongn with the dose increase.

External signals, such as growth factor ligand that binds to its receptor, may activate Ras pathway. In addition, if there is mutation in growth factor receptor, growth signal through tyrosine kinase receptor will be continuously sent even though there is no growth factor. Mutation may render ras protooncogene to become oncogene, so that the protein it signals become overactive. Ras has a role to transmit signals from receptor to the nucleus to stimulate cell proliferation and differentiation. Inactive Ras-GDP complex became active Ras-GTP. Active Ras binds Raf, a cellular kinase, that subsequently triggers a sequence of consecutive protein phosphorilation and stimulates MAP kinase pathway. MAPK pathway transmits signals to the nucleus through the activity of transcription factor C-myc. Myc signals DNA binding protein specific sequence that has a role to control proliferation and differentiation. C-myc protein is signaled by c-myc gene that has a role as transcription factor for DNA replication. Mutagene may cause c-myc protooncogene to become oncogene and continuous c-myc activation may trigger oncogenesis. MAPK pathway can synthesize and maintain cyclin D stability. Cyclin D is bound to cdk 4/6 to form D-cdk 4/6 cyclin complex that along with PCNA protein may activate cell replication process (21).

Mutation in ras oncogene was a dot mutation. The change of one nucleotida is sufficientt to change a benign protooncogene to becoma a hazardous one. Dot mutation is the one that causes increased function of a gene called function enhancer mutation, so that the oncogene effect may become predominant, meaning that the mutation needed only one of both alleles to induce the effect. Cancer may occur due to excessive expression of normal protooncogene product, which may result due to protooncogene amplification or protooncogene activation that was previously inactive (22).

In oral squamous cell carcinoma there was no

predominant Ras mutation between N-H-K Ras (23). In this study, the reduction of oncogenesis was proved by the result, which showed that n-hexana : ethyl acetate fraction (3:7,v/v) was able to reduce Ras and C-myc protein in benzopyrene-induced transformed cells. This result was in line with those from previous studies that the administration of eupatilin isolated from *Artemisia asiatica Nakai* was able to reduce H-Ras expression in epithelial cell in breast cancer. The administration of jaceosidin isolated from Artemisia argyi also reduced Ras expression in breast cancer epithelial cells (4). This proved that the genus Artemisia, despite in different species, has the same effect, so that the fraction can be developed as a medicine agent to overcome cancer in oral cavity.

The n-hexana : ethyl acetate fraction (3:7,v/v) is able to reduce Ras protein expression, likely due to the inhibition of farnesyltransferase enzyme. The administration of Artemisia tridentata was able to inhibit farnesyltransferase enzyme. The latter is needed as a catalyst to release GDP and exchange it with GTP in Ras activation. GTP group bound with Ras protein may activate the protein to perform MAPK cascade phosphorylation (23). The plants in one genus Artemisia, based on chemotaxonomical approach by finding other plant in the same genus, are suspected to have the similar type of substance that is evidently active. The limitation of this study is needed to measure other parameters in evaluating the probability of alternate mechanism pathways.

CONCLUSIONS

In conclusion, n-hexana : ethyl acetate fraction (3:7,v/v) from *Artemisia vulgaris* L. is able to reduce oncogenesis based on the reduced expression of mutant P53, Ras, and C-myc proteins that result in the reduction of PCNA protein expression, resulting in reduced proliferation and increased wild type P53 protein, triggering apoptosis ot mice oral mucosal cells, which are transformed due benzopyrene induction without damaging the healthy cells.

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