Effects of *Centella asiatica* Leaves Extract on Dimethyl Benz(A) Anthracene (DMBA) Induced Oral Epithelial Dysplasia in Rats

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Abstract

Oral epithelial dysplasia is the architectural and cytologic changes of the oral cavity with an increased risk to squamous cell carcinoma. Nowadays, herbal extract from plants with bioactive substance are being developed as chemopreventive agent to prevent the development of more invasive lesions. Centella asiatica is one of widely available herbal plant in Indonesia which shown pharmacological effect as anticancer. This study aimed to determine the effects of Centella asiatica leaves extract on dysplasia induced by dimethyl benz(a) anthrance (DMBA) with different frequency. In this in-vivo study, 14 male wistar rats (Ratt us novergicus) were divided into two groups. Both groups were given 2% methanolic extract of Centella asiatica leaves orally by force feeding once a day starting at day 1 until day 61. At day 29, the induction of dysplasia was done by scratching the buccal mucosa of each rat using a syringe containing 0.5% DMBA three times a week (Group 1) and twice a week (Group 2) for four weeks. The rats were then sacrificed and histopathological analysis was done using hematoxylin-eosin staining. The WHO 2017 classification was used to assess the degree of dysplasia. The data were analyzed using Mann-Whitney test. Histopathological examination results showed no dysplasia in group 2 and mild dysplasia in group 1. Statistical test result showed that there was no significant difference in the degree of dysplasia in group 1 and 2 (p=0.16). It can be concluded that administration of methanolic extract of Centella asiatica leaves in samples with more frequent exposure of DMBA will result in comparable degree of dysplasia to group with less frequent exposure. In other words, methanolic extract of Centella asiatica leaves has the potential to be developed as chemopreventive agent.

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Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer in the world. OSCC can arises in, buccal mucosa, floor of the mouth, tongue and other sites in the oral cavity.[1,2] This type of cancer occurs in more than 500,000 cases annually and has the highest death rate globally.[1] Based on the data from International Agency for Research on Cancer in 2014, the overall incidence of oral cancer in Indonesia was 1.63%, with 319 cases was diagnosed over the last fifteen years at Dharmais Indonesia National Cancer Hospital (DNCH).[3]

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The development of malignancy is a complex process. The development starts from hyperplasia to dysplasia, which then progresses into carcinoma in situ, and finally OSCC.[4] Dysplasia is defined as abnormal cell growth towards the development of oral cancer. Dysplasia occurs from irreversible cell injury that results in DNA changes that ultimately have an impact on abnormal cell growth and the subsequent changes on the cell size, shape, and color.[5,6]

Carcinogenic agents can cause tissue structures to become abnormal, resulting in changes in the tissue homeostasis. This continuous and persistent abnormalities will later induce carcinogenesis.[7] 7,12-dimethylbenz[a] Anthracene (DMBA) is a polycyclic aromatic hydrocarbon (PAH) that has been associated with tumor development in rats.[8] The DMBA dose and frequency of exposure affect the growth of squamous cell carcinoma (SSC) in mice. The

findings were consistent with a study conducted by Muchsin D in 2016 on the effect of DMBA frequency exposure, which demonstrated variances in the level of dysplasia in mice exposed to DMBA at different frequencies.[9]

Cancer can have a detrimental impact on patients, and the survival rate has been poor in the past few decades. As a result, anticancer treatments should be developed.[10] To prevent the development of more aggressive lesions, herbal extracts from plants with bioactive substances are being explored as chemopreventive agents.[11,12] *Centella asiatica* is a medicinal plant that has been used for a long time. This plant has long been utilized as an ingredient of traditional medicine in Indonesia.[13,14]

Amino acids (alanine, serine, aspartate, and glutamate), phenols (kaemferol and quercetin), and triterpenoids (asiaticoside, centelloside, madecassoside, madecasic acid, and brahmoside) are among the active components of *Centella asiatica*.[13,15] Asiaticoside, madecassoside, asiatic acid, and madecassic acid are the major triterpenoids found in Centella asiatica.[16] *Centella asiatica* has anticancer, antibacterial, antifungal, anti-inflammatory, antioxidant, and wound-healing properties and accelerates wound healing.[13,15]

A prior study published by Singh D et al. in 2012 discovered that a 200 µg/ml of *Centella asiatica* methanol extract was the optimum concentration for producing an antioxidant effect on the *Centella asiatica* plant.[17] Arora R et al. in 2018 conducted a study on the effective dose of *Centella asiatica* extract, comparing three fractions: The fractions of *Centella asiatica* extract enriched for (CAE-EF) and depleted/freed of (CAE-FF) triterpenes contents were compared with methanolic extract (CAE) at the same dose. Three fraction at a dose of 100mg/kg body demonstrated the highest antioxidant activity in vitro.[18]

There were numerous studies of the chemopreventive agent as a treatment of premalignant lesions and oral malignancies at various doses, but there are still few studies on the effect of *Centella asiatica* as a chemopreventive agent. The purpose of this study was to determine the effect of daily administration of *Centella asiatica* methanolic extract on the incidence of dysplasia induced by DMBA exposure at different frequencies.

Materials and methods

Ethical Clearance

The methods used in this study was approved by the Animal Research Ethics Committee, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara (No.0125/KEPH-FMIPA/2021)

Study Design and groups

The study conducted was an *in-vivo* experiment with posttest only control group design. Animals used in this study were 14 male wistar rats (Rattus novergicus) of eight weeks old with an average body weight of 200-300 grams. The rats should be in a healthy condition characterized by active movement, clean fur, clear eyes and had never received any treatment before. The rats were obtained from and housed at CV. Focus Medical Indonesia animal house. The rats were acclimatized for 1 weeks before any treatment to assure good adaptation. The rats were then divided into 2 groups: Group 1 was given 2% methanolic extract of *Centella asiatica* leaves once a day and 0.5% DMBA three times a week, while group 2 was given 2% methanolic extract of *Centella asiatica* once a day and 0.5% DMBA twice a week. *Centella asiatica* leaves methanolic extract was given at day 1 until day 61 while DMBA induction was started at day 29 for four weeks. The rats were then sacrificed on day 61 for histopathological analysis.

Plant Materials and Extract Preparation

Methanolic extract of *Centella asiatica* leaves were made at the Pharmacology Laboratory, Faculty of Pharmacy, Universitas Sumatera Utara. *Centella asiatica* leaves were collected from RT 031/RW 015, Salakmalang, Banjarharjo, Kalibawang, Kulon Progo, Yogyakarta. The extraction was done using maceration method. *Centella asiatica* leaves were initially dried in a drying box to ensure complete drying. The dried leaves were then grinded into powder with an electric blender (Philips, Dutch) and soaked in methanol (Lichrosolv, Germany) for 7 days with regular stirring. After that, filtration was done using cotton dan filter paper to collect the macerate/filtrate. The macerated samples were then concentrated using rotary evaporator (Heidolph vv 2000, Germany) and then placed into the oven (Memmert, Germany).[19,20]

***Centella asiatica* Leaves Methanolic Extract Treatment**

The methanolic extract of *Centella asiatica leaves* was administered for 61 days. Each rat received 100 mg/kg BW of 2% Centella asiatica leaves methanolic extract orally by force feeding with a syringe at 8 a.m (Onemed, Indonesia). The selected dose was based on a recent study by Arora R et al. who reported that *Centella asiatica* methanolic extract with the dose of 100 mg/kg BW had the best antioxidant effect in rats.[18]

Oral Epithelial Dysplasia Induction by DMBA

The induction was conducted in at the Focus Medical Laboratory, Medan, Indonesia. The rats were anesthetized using ketamine hydrochloride intraperitoneally with the dose of 10 mg/kg BW after 28 days of treatment with 2% *Centella asiatica* methanolic extract. The buccal mucosa of each rat in group 1 was scratched three times a week (every Monday, Wednesday, and Friday) and group 2 twice a week (every Monday and Friday) with a syringe (Onemed, Indonesia) containing 100 µg of 5% DMBA (Sigma-Aldrich corporation D3254, USA) and corn oil (Tropicana slim, Indonesia) as a solvent, with a length of 1 cm measured using a probe UNC-15 (Osung, Korea) for four weeks**.** On day 61, the rats were sacrificed by cervical dislocation. The frequency of DMBA used in this study was based on a previous study about curcumin extract as a chemo preventive agent in mice study models by Maulina T et al. in 2019, which found that application of 100 µg of 0.5% DMBA to the buccal mucosa of Sprague-Dawley mice three times a week for four weeks was effective in inducing dysplasia.[21] A study by Muchsin D in 2016 on the frequency effect of DMBA discovered that DMBA exposure twice a week could cause dysplasia.[9]

Histopathological Examination

After being sacrificed on day 61, dysplasia was assessed with haematoxyline eosin staining at Anatomical Pathology, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia. The buccal mucosa of the rats was excised and fixed in10% formalin. The tissue was cut into smaller piece for dehydration process. Dehydration was done with alcohol from 70% to 80% to 95% for 1 hour and 30 minutes. After that, each tissue placed in toluene for 30 minutes. The tissue was then infiltrated with liquefied paraffin at a temperature of 58-60℃ for 30 minutes to 6 hours in an incubator to remove the toluen from the tissue and replace it with paraffin. The paraffin block was cut by using a rotatory microtome with 4-5 µm thickness. The sectioned tissue was placed in a water bath at a temperature of 46℃ and collected on a clean object glass. The slide was labelled using non removable ink. The slides were then stained with hematoxylin-eosin stain.[22,23,24] The stained tissue slides were then observed under light microscope with magnification of 40x, 100x, and 400x times. The degree of dysplasia in this study was scored using WHO 2017 classification system, with a score of 0 for no dysplasia, 1 for mild dysplasia, 2 for moderate dysplasia, and 3 for severe dysplasia.[25]

Statistical Analysis

The data were compared using Mann-Whitney test to determine if there was a significant difference in the degree of dysplasia between the groups. The results were considered as significant if the p-value was below 0.05. Statistical analyses were performed using Statistical Package for the Social Sciences (IBM SPSS, software version 21).

Results

The study used 14 Wistar rats (*Rattus novergicus*) but on the 61 days of the study, one rat from group 1 and one rat from group 2 were found dead. Thus, 12 rats remained survive until day 61. Each rat was treated according to its respective group, then the degrre of dysplasia was assessed based on the 2017 WHO classification. The Mann-Whitney test was used to see whether or not there was a significant difference in all treatment groups. The result can be seen in table 1.

Group	N	Average ± SD	P-value
Group 1	6	1.00 ± 0.89	0.16
Group 2	6	0.33 ± 0.51	

Table 1. Mann-Whitney test results of the mean dysplasia level. *Mann-Whitney test; $p < 0.05$; significant.

Table 1 showed that there are no significant difference in the degree of dysplasia between both groups, although group 1 showed higher degree of dysplasia compared to group 2

(p=1.16). Group 1 showed mild dysplasia with the average grade of 1.00 ± 0.89 while group 2 showed no dysplasia with the average grade of 0.33 ± 0.51. Histopathological examination with hematoxylin-eosin staining showed that there was mild dysplasia in group 1 and no dysplasia in group 2. Mild dysplasia in group 1 was characterized by the precence of abnormal cells with a variation in the nuclear size (anisonucleosis) and shape (nuclear pleomorfism), increased nuclear/cytoplasmic ratio and hyperchromasia. Meanwhile in group 2, nuclear size and shape was normal, nuclear/cytoplasmic ratio was normal, and no hyperchromasia. The dysplasia occurred in both groups are shown in Figure 1.

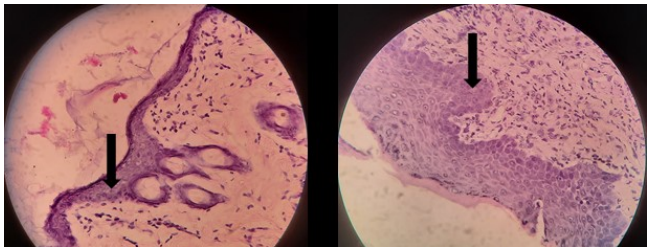

Figure 1. The dysplasia occurred in both groups are shown in figure.

Discussion

This study aimed to determine the different effect of *Centella asiatica* leaves on the incidence of dysplasia induced by dimethylbenz(a) anthracene (DMBA) two and three times a week. In previous study by Maulina T in 2019 regarding the effects of Curcumin extract as a chemopreventive agent with using DMBA to induce cancer, it was found that exposure of DMBA 0.5% three times a week for 28 days is effective for inducing moderate and severe dysplasia.[21]

Rat strain susceptibility, genetic mutations, and carcinogenic dose can influence carcinogenesis in rats.[9] In this study, the mean results of dysplasia level in each group showed that dysplasia levels were lower in group with less exposure frequency of DMBA although the finding was not statistically significant. This finding is in accordance with study conducted by Mice exposed to DMBA four times for two weeks and eight times for four weeks showed mild dysplasia. Mice exposed to DMBA with higher frequency which were 12 times during six weeks and 16 times for eight weeks histopathologically obtained squamous cell carcinoma. This study shows that DMBA exposure with more frequency can accelerate growth dysplasia and squamous cell carcinoma.[9] It is known that cancer is the result of complex interactions between genetic and environmental factors that turn normal cells into cancer cells.[10] In this study, a carcinogenic agent DMBA (7,12 dimethylbenz(a) anthracene) was used to induce dysplasia. The mechanism of cancer induced by DMBA occurs because these compounds are metabolized into active metabolites, such as epoxide diols and free radicals. The two active metabolites can bind to DNA so that DNA adducts are formed in the process of carcinogenesis. The result of DMBA induction causes oxidative stress so that lesions form on DNA bases and form DNA adduct.[26,27] Carcinogenesis is a stage of changing normal cells into cancer cells namely initiation, promotion, and progression phases. At the initiation stage, there is a change in the cell nucleus DNA that allows normal cells gradually turned into cancer cells. Cancer initiators can be obtained from repeated exposure to environment or chemicals that can cause genetic changes in DNA so that they can trigger DNA lesions. Failure in the repair mechanism of DNA lesions can cause mutations in certain genes like oncogenes and tumor suppressing cells, which is cell cycle regulators that is used in cell growth, cell division, cell differentiation, and apoptosis. DMBA not only causes oxidative stress but can induce the expression of gens that have a role as a regulator or suppressor in the cell cycle. At the initiation stage, DMBA acts as an initiator that will cause mutations in DNA. Mutations that occur in this gene encourage the transformation of cells to become abnormal, but in this process, it is still reversible.[26,28,29,30]

In this study, both groups were treated with methanolic extract of *Centella asiatica* and statistical test result showed that there was no significant difference in the degree of dysplasia between group 1, which was exposed to DMBA three times a week and group 2, which was exposed twice a week to DMBA (p>0.05). It can be concluded that in this study, the frequency of DMBA exposure did not significantly affect the dysplasia, especially in group 1 with higher exposure of DMBA which showed comparable results to group 2. This is probably due to the therapeutic potential and pronounced effect of antioxidant from *Centella asiatica extract* which

inhibit the development of DMBA-induced oral epithelial dysplasia.

In general, *Centella asiatica* has medicinal uses, one of which is anticancer.[15] This chemopreventive effect is obtained from the active ingredients in *Centella asiatica*. The main active ingredients in the *Centella asiatic* are triterpenoids consisting of asiaticoside, madecassoside, asiatic acid, and madecasic acid. Other important active ingredients are triterpenoids and saponins, which consisted of centelosid and other components such as volatile oils, flavonoids, tannins, amino acids, and carbohydrates. All the active ingredients of *Centella asiatica* had benefits as antioxidants that are beneficial for the human body in improving the immune system.[31] Antioxidants possessed by *Centella asitica* had different functions, including collecting reactive oxygen, inhibiting free radicals, inhibiting p-coumaric acid, and chelating metals.[32] Oxidative stress is basically an instability between the production of free radicals and the body's ability to neutralize their harmful effects by antioxidant.[33]

One of the active ingredients in *Centella asiatica* which has anti-inflammatory and antioxidant activity is asiaticoside.[33] Asiaticoside has been widely studied as an anticancer with different mechanisms based on cancer cell types and cancer cell lines. In a study by Zhou X, asiaticoside was shown to significantly inhibit the growth of colorectal tumors by suppressing cancer cell growth pathways. Asiaticoside can significantly reduce cyclin D1 expression and also induce cell cycle arrest in the G0/G1 phase to induce DNA repair.[34] Cyclin D1 is an important regulator of cell cycle processes. Cyclin D1 and CDKs (Cyclin Dependent-Kinase) are one of the genetic change targets in cancer. The expression of D1 cyclin in the G1 phase is constant or low, excessive D1 cyclin expression will cause the G1 phase to be shorter, so that cell proliferation becomes abnormal and causing DNA lesions. Overexpressions of cyclin D1 can cause it to become an oncogene. In various studies, it was found that this overexpression is present in dysplasia. Inducing a decrease in the expression of cyclin D1that can be used to be a potential anticancer.[35] Besides being useful as an antioxidant, several other studies on the effectiveness of the active ingredient asiaticoside as an anticancer have also been widely studied, because of these active ingredient of *Centella asiatica* with various benefits, there are differences in dysplasia level.

Conclusions

There was no significant differences in the degree of dysplasia induced by three times and twice a week exposure of dimethyl benz(a) anthracene (DMBA) when combined with daily administration of methanolic extract of *Centella asiatica*. It can be concluded that administration of methanolic extract of *Centella asiatica* leaves in samples with more frequent exposure of DMBA will result in comparable degree of dysplasia to group with less frequent exposure. In other words, methanolic extract of *Centella asiatica* leaves has the potential to be developed as chemopreventive agent.

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Declaration of Interest

The authors report no conflict of interest.

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