The Effect of Methanol Extract of *Centella asiatica* leaves at different doses on Dimethylbenz (a) Anthracene (DMBA)-Induced Oral Epithelial Dysplasia in Wistar Rats

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Abstract

Oral epithelial dysplasia referred to as "potentially malignant lesions" is a condition that describes architectural and cytological changes that increase the risk of cell malignancy. Consumption of chemopreventive agents can prevent cancer because it has the ability to inhibit the early phase of carcinogenesis.

This study aimed to determine the effect of methanol extract of Centella asiatica leaves at different doses on dysplasia induced by dimethylbenz (a) anthracene (DMBA). This in-vivo study consist of 28 male Wistar rats that were divided into four groups. Each group was given different doses of methanol extract of Centella asiatica leaves once a day starting on day 1 until day 61. On day 29, each group was induced by 0,5% DMBA thrice a week for four weeks. On the last day (day 61), the rats were sacrificed and a histopathological examination was done by using H&E staining. The WHO 2017 classification was used to grade the degree of dysplasia cell. The data were analyzed using Kruskal-Wallis test. Histopathological examination results showed moderate dysplasia in group 1, mild dysplasia in group 2, and no dysplasia in group 3 and 4.

The statistical result showed that there was a significant difference in the degree of dysplasia in all groups. It can be concluded that the administration of methanol extract of Centella asiatica leaves at higher doses will inhibit the carcinogenesis caused by DMBA. Specifically, methanol extract of Centella asiatica leaves has the potential to be utilized as a chemopreventive agent.

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Introduction

Oral squamous cell carcinoma (OSCC) is an oral cancer originating from squamous epithelial tissue which accounts for more than 90% of oral cancer cases and categorized as head and neck cancer (HNC). OSCC is the 11th most common malignancy around the world.[1] In 2020, lip and oral cavity cancer accounted for 377.713 cases and 177.757 mortality worldwide. The estimated number of lip and oral cavity cancer shall increase by 2040.[2,3] In South-East Asia and some regions of the Pacific, oral cavity cancer is the most common malignancy occurred. The key factor that causes oral cavity cancer are low socioeconomic status, self-negligence, and lack of awareness, whereas, the contributing factors consist of the consumption of tobacco and betel quid.[3]

Oral cavity cancer has a poor prognosis, with a 5-year survival rate, but the survival rates can increase if oral cavity cancer is diagnosed in the early stages.[4] The survival rate also varies due to anatomical location, grade of the oral cavity cancer, age, and comorbidity.[3] The prognosis worsens as the cancer becomes more advanced and the site of the cancer is less accessible.[4]

Dimethylbenz (a) anthracene (DMBA) is one of polycyclic aromatic hydrocarbons (PAHs) and is the most potent carcinogenic agent that is known to cause tumors in rats. DMBA are known to be mutagenic, teratogenic, carcinogenic, and immunosuppressive.[5] Carcinogenesis caused by DMBA that is given locally in oral cavity is estimated to involve multiple genes, one of the genes is p53. The p53 expression will decrease and the expression of cyclin D1 will increase and

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resulting in an increase in cell proliferation.[6]

The most common methods used for treating cancer are radiotherapy, immunotherapy, and chemotherapy, however, these methods adversely will affect the normal and healthy cells. Therefore, preventive treatments against cancer were developed, either avoiding a high-fat-containing diet, avoiding excessive smoking and drinking alcohol, or consuming chemopreventive agents.[7]

Chemopreventive agents are the usage of natural substances to reverse, suppress, or prevent the early phase of carcinogenesis or to inhibit the progression of premalignant cells on becoming invasive. Particularly, chemoprevention is used to reduces cancer development in order to avoid a chemotherapy approach.[8]

Plants containing antioxidants can be used as chemopreventive agents. Antioxidants are substances that are able to prevent the process of oxidation related to free radicals. Free radicals are highly reactive electrons that are able to interact with DNA resulting in cell damage and cancer. Antioxidants play a role in inducing apoptosis, immune-enhancing effect, cell cycle arrest, gene expression, and suppressing proliferation of cancer cells.[9]

Centella asiatica is a domestic plant in India, South-East Asia, and part of South America. *Centella asiatica* contains polyphenols, flavonoids, tannins, vitamin C, triterpenoids (madecassic acid and Asiatic acid), and triterpene glycosides (centellasaponin, asiaticoside, sceffoleoside, and madecassoside) which act as antioxidants, therefore, *Centella asiatica* can be utilized as chemopreventive agent.[9]

In a prior study conducted by Arora R D *et al*. in 2018, *Centella asiatica* extract at a dose of 100 mg/kg bw showed the highest antioxidant activity.[10] Thus, this present study was conducted to see the effect of methanol extract of *Centella asiatica* at doses 25 mg/kg bw, 50 mg/kg bw, 200 mg/kg bw, and 400 mg/kg bw would affect the degree of dysplasia induced by dimethylbenz (a) anthracene (DMBA) in wistar rats.

Materials and methods

The methods used in this study was approved by the Animal Research Ethics Committee, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara. (No. 0865/KEPH-FMIPA/2021)

Study Design and Groups

The study was an *in-vivo* experiment with a post-test-only control group design. Animals used in this study were eight weeks old of 28 male Wistar rats (*Rattus novergicus*) with an average body weight of 200 to 300 grams. The Wistar rats used in this study must be in healthy condition and has never received any treatment before. The Wistar rats were obtained from CV. FOCUS Medical Indonesia and were housed at CV. FOCUS Medical Indonesia. Before any treatment, the Wistar rats were acclimatized for one week as an adaptation. The Wistar rats were then divided into four groups:

1. Group 1: methanol extract of *Centella asiatica* (25 mg/kg bw once a day until day 61) + 0,5% DMBA (started on day 29 for 28 days).
2. Group 2: methanol extract of *Centella asiatica* (50 mg/kg bw once a day until day 61) + 0,5% DMBA (started on day 29 for 28 days).
3. Group 3: methanol extract of *Centella asiatica* (200 mg/kg bw once a day until day 61) + 0,5% DMBA (started on day 29 for 28 days).
4. Group 4: methanol extract of *Centella asiatica* (400 mg/kg bw once a day until day 61) + 0,5% DMBA (started on day 29 for 28 days).

The study was terminated on day 61. The rats were euthanized with chloroform for histopathological examination.

Plant Materials and Extract Preparation

Methanol extract of *Centella asiatica* leaves were made at the Laboratory of Phytochemistry, Faculty of Pharmacy, Universitas Sumatera Utara, Indonesia. *Centella asiatica* leaves were obtained from RT 031/RW 015, Salakmalang, Banjarharjo, Kalibawang, Kulon Progo, Yogyakarta. The maceration extraction method was used to extract *Centella asiatica* leaves. *Centella asiatica* leaves were dried in a drying box then the dried leaves were grinded with an electric blender and soaked in methanol (Lichrosolv, Germany) for 5 days and stirred regularly. After 5 days, filtration was done using filter paper to collect the filtrate. The filtrate then macerated using rotary evaporator (Heidolph vv 2000, Germany) then placed into the oven (Memmert, Germany).[11,12]

Treatment of Methanol Extract of Centella asiatica Leaves

The methanol extracts of *Centella asiatica* leaves were administered for 61 consecutive

days. Each group received different doses of the methanol extract of *Centella asiatica* leaves orally by force feeding with syringe at 8 a.m every morning (Onemed, Indonesia).

Dimethylbenz (a) anthracene (DMBA) Induction to Wistar Rats

Before the induction of DMBA to the rats, the rats were anesthetized using ketamine hydrochloride intraperitoneally at a dose of 10 mg/kg bw. Then the buccal mucosa of the rats were scratched using a 27G syringe (Onemed, Indonesia) containing 100 μg of 5% DMBA (Sigma-Aldrich corporation D3254, USA) and corn oil (Mazola, Indonesia) as solvent. The induction of the DMBA was done thrice a week for four weeks. The frequency of the DMBA induction in this study was based on a prior study conducted by Maulina T, et al in 2019, that found the application of 100 μg of 0,5% DMBA on the buccal mucosa of Sprague-dawley rats thrice a week for four weeks was effective to induced oral epithelial dysplasia in each rat.[12]

Euthanasia Procedure

On the last day of the study (day 61), each rat was euthanized. The euthanasia procedure was done by chloroform inhalation. Chloroform is commonly used as an agent for euthanizing experimental animals, mainly rodents.[13]

Histopathological Examination

After the rats were euthanized, samples were immediately excised from the rat's buccal mucosa. Then each sample was placed into a small container and was fixated in 10% formalin buffer. Then the samples were dehydrated by using alcohol starting from 70% alcohol to 95% alcohol. After dehydration, the samples were put into toluene for 30 minutes, followed by fabricating paraffin blocks and the paraffin blocks were cut by using a microtome with 5-10 μm of thickness. The samples were then placed on the object glass for H&E staining.[12] The histopathological examination was done at the Laboratory of Anatomical Pathology, Faculty of Medicine, Universitas Sumatera Utara, Indonesia.

Oral Epithelial Grading

Architectural changes and cytological changes are the criteria used for diagnosing oral epithelial dysplasia. The classification used to grade the degree of oral epithelial dysplasia is the WHO 2017 grading. The WHO 2017 grading was commonly used by pathologists and consists of three classifications, mild dysplasia, moderate dysplasia, and severe dysplasia.[13]

Statistical Analysis

The result of this study was analyzed using IBM SPSS version 21. The statistical analysis result using Kruskal-Wallis test was considered significant if the p-value was below 0,05.

Results

This study used 28 male Wistar rats, divided into four groups, one rat was found dead in every group. Therefore, 24 rats survived until the end of the study. Each rat was treated based on its group, and the degree of the oral epithelial dysplasia was classified based on the WHO 2017 classification. The Kruskal-Wallis test was used to determine whether there was a significant difference in all groups. The result of the Kruskal-Wallis test can be seen in table 1.

Group	N	Mean Rank	P Value
Group 1	6	16,83	0,059*
Group 2	6	14,25	
Group 3	6	10,42	
Group 4	6	8,50	

Table 1. Kruskal-Wallis test result. **Kruskal-Wallis test;* p < 0,05; significant.

Table 1 showed that there was a significant difference in the grade of dysplasia in all groups. Group 1 with the highest mean rank, showed moderate dysplasia, the result in group 2 showed mild dysplasia, but the result in group 3, and group 4 showed that no dysplasia occurred.

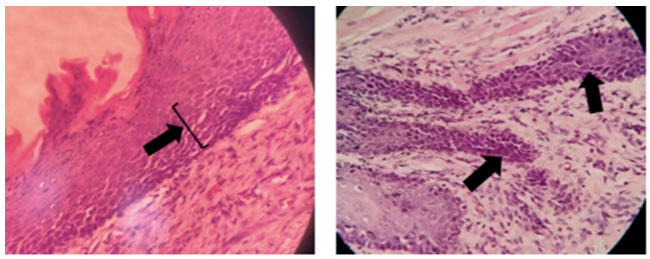

Figure 1. The histopathological examination results from group 1 and group 2.

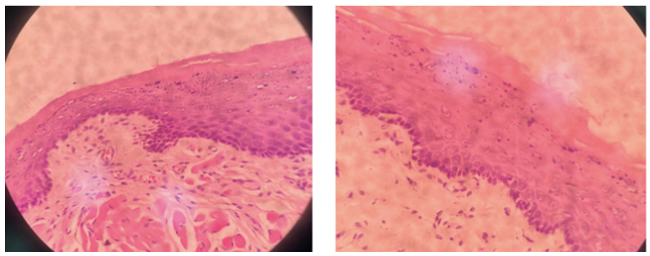

Figure 2. The histopathological examination results from group 3 and group 4.

Discussion

This study is aimed to determine the effect of methanol extract of *Centella asiatica* leaves at different doses on dysplasia induced by dimethylbenz (a) anthracene (DMBA). Based on a prior study by Maulia T, et al in 2019, the induction of DMBA was done thrice a week for four weeks has effectively induced dysplasia in rats.[12] The carcinogenesis caused by DMBA involved several genes and proteins, namely p53, p21, p16, pRb, and CDKN2A were changed in cell proliferation. The cyclin D1 had increased expression and p27 was downregulated. These changes cause cell cycle disruption leading to uncontrolled proliferation.[6]

The induction of dimethylbenz (a) anthracene (DMBA) will cause oxidative stress leading to the increase of reactive oxygen species (ROS). ROS are involved in the carcinogenesis process. Excessive ROS will cause oxidative damage to DNA in its structure and function causing cancer initiation.[14]

Thus *Centella asiatica* is known to possess anticancer and antioxidant activity, there are no data available regarding its chemopreventive effect at doses of 25 mg/kg bw, 50 mg/kg bw, 200 mg/kg bw, and 400 mg/kg bw against oral cancer. Sakonsinsiri C et al in 2018, reported that *Asiatic acid* possesses promising anti-cancer activity on human cholangiocarcinoma cells by inhibition of proliferation and induction of apoptosis.[15] Jain S, et al 2016 reported that methanol extract of *Centella asiatica* showed induction of apoptosis on MCF-7 cells in vitro.[16]

In this study, oral administration of methanol extract of *Centella asiatica* at doses of 200 mg/kg bw and 400 mg/kg bw completely prevented the dysplasia development in the buccal mucosa of the rats. Meanwhile, the methanol extract of *Centella asiatica* at doses of 25 mg/kg bw and 50 mg/kg bw showed moderate dysplasia and mild dysplasia. This probably happened because the antioxidant activity of *Centella asiatica* at lower doses were unable to compensate for the free radicals caused by oxidative stress by DMBA.[14] The imbalance between antioxidants and free radicals will rapidly increase the normal cell division process, resulting in abnormal cell growth that leads to the formation of cancer.[17,18]

The bioactive compounds in *Centella asiatica* are *Asiatic acid*, *asiaticoside*, *madecassic acid, glycosides,* and *madecassoside*. The other bioactive compounds are brahmoside, centelloside, carotenoids, tannins, vitamin c, and minerals.[9,19] The main compounds of *Centella asiatica* that are responsible for anti-cancer activity are *asiatic acid* and *asiaticoside*.[19,20,21] Wu T, et al in 2017, reported that *Asiatic acid* has the ability to inhibit cell proliferation in lung cancer cells and cause cell death in vivo and in vitro. Asiatic acid also induces apoptosis in lung cancer cells through interference with the mitochondrial membrane potential leading to apoptotic cell death.[17] Anti-cancer activity caused by *asiaticoside* was reported by Zhou X, et al in 2020. *Asiaticoside* decreases the expression of cyclin D1 resulting in cell cycle arrest at the G0/G1 phase, promoting cell apoptosis, and suppressing the viability of the cells.[21] The overexpression of cyclin D1 will shorten the cell cycle in the G1 phase, resulting in uncontrolled cell proliferation and causing DNA lesions. Uncontrolled cell proliferation in the G1 phase will cause dysplasia to transform into malignancy.[21,22]

The present study concludes that methanol extract of *Centella asiatica* at doses of 200 mg/kg bw and 400 mg/kg bw has the potential to be develope as a chemopreventive agent. Methanol extract of *Centella asiatica* at doses of 200 mg/kg bw and 400 mg/kg bw might have prevented the DMBA-induced dysplasia formation by inhibition of abnormal cell proliferation, cell cycle arrest at the G0/G1 phase, and promoting cell apoptosis.

Conclusions

From this study, we can conclude that there was a significant difference in the degree of dysplasia induced by dimethylbenz (a) anthracene (DMBA) in each treatment group. Methanol extract of *Centella asiatica* at doses of 200 mg/kg bw and 400 mg/kg bw completely prevented dysplasia development in DMBA-treated rats.

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Declaration of Interest

The authors declare that there is no conflict of interest related to this study.

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