

Effect of the Methanol Extract of *Centella Asiatica* as a Chemotherapeutic Agent on the Occurrence and Development of Dysplastic Cells: Histological and Immunohistochemical Analysis via the p53 and tnf- α Genes

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Abstract: This research evaluated the efficacy of *Centella asiatica* methanol extract at various concentrations on the emergence and progression of dysplastic cells induced by dimethylbenz(a)anthracene (DMBA) in male Wistar rats. The study aimed to demonstrate that the methanol extract of *Centella asiatica* is a phytochemically effective and histologically and immunohistochemically proven inhibitor of oral dysplasia cell development. Fifty male Wistar rats (*Rattus norvegicus*) were divided into five groups, with four groups receiving varying doses of the treatment and one group serving as a positive control. Phytochemical screening was conducted using qualitative and quantitative methods. Histopathological examination was performed using H&E and IHC staining techniques. Qualitative screening revealed alkaloids, flavonoids, glycosides, saponins, tannins, and triterpenoid/steroid compounds in the methanol extract of *Centella asiatica*. Quantitative screening revealed the levels of total phenolics, total flavonoids, and antioxidant activity. The study demonstrated the effectiveness of the methanol extract of *Centella asiatica* at different concentrations in reducing the incidence of DMBA-induced dysplasia. The groups not administered the extract exhibited higher dysplasia grades, indicating the efficacy of administering the methanol extract of *Centella asiatica* at varying doses in reducing DMBA-induced dysplasia.

Keywords: methanol extract of *Centella asiatica*, DMBA, dysplasia.

积雪草甲醇提取物作为化疗药物对发育不良细胞发生和发展的影响：通过p53和肿瘤坏死因子- α 基因进行组织学和免疫组织化学分析

摘要：本研究评估了不同浓度的积雪草甲醇提取物对雄性威斯塔大鼠中二甲基苯并(a)蒽(二甲基苯胺)诱导的发育不良细胞的出现和进展的功效。本研究旨在证明积雪草甲醇提取物是一种具有植物化学有效性、且经组织学和免疫组织化学证实可抑制口腔发育不良细胞发展的抑制剂。将50只雄性威斯塔大鼠(褐家鼠)分成5组，其中4组接受不同剂量的治疗，1组作为阳性对照。使用定性和定量方法进行植物化学筛选。使用他和免疫组织化学染色技术进行组织病理学检查。定性筛选显示积雪草甲醇提取物中含有生物碱、黄酮类化合物、糖苷、皂苷、单宁和三萜/类固醇化合物。定量筛选揭示了总酚、总黄酮和抗氧化活性的水平。该研究证明了不同浓度的积雪草甲醇提取物在降低二甲基苯胺诱导的发育不良发生率方面的有效性。未施用提取物的组表现出更高的发育不良等级，表明施用不同剂量的积雪草甲醇提取物在

降低二甲基苯胺诱导的发育不良方面的有效性。

关键词：积雪草甲醇提取物，二甲基苯胺，发育不良。

1. Introduction

Centella asiatica is native to tropical Asia and is often found in open, moist, and fertile soils. *Centella asiatica* exhibits various pharmacological effects, such as anticancer, antibacterial, antifungal, and anti-inflammatory, and accelerates wound healing. The triterpene component of *Centella asiatica* comprises various active components, including asiatic acid, madecassoside acid, asiaticoside, and madecassoside. Asiatic acid is a cancer-fighting compound. Cancer incidence cannot be separated from the contribution of angiogenesis. *Centella asiatica*, as an inhibitor, is assumed to play a role in initiating cancer through angiogenesis [1]. In a study by Babykutty et al. [2], plant compounds from *Centella asiatica* induced apoptosis in MCF-7 cells, as shown by nuclear condensation, increased annexin staining, loss of mitochondrial membrane potential, and DNA fragmentation.

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer reported worldwide. OSCC originates from squamous cells that exhibit a characteristic flat, scale-like morphology and line the oral cavity and pharynx. The development of squamous cells into cancer cells involves several stages, including metaplasia, dysplasia, and carcinoma in situ [2].

OSCC prognosis is poor due to low estimated survival rates. Patients diagnosed with OSCC often experience a range of adverse effects as the disease progresses. Among these are persistent discomfort resulting from infections or tumors that invaded nerve tissue and difficulties with chewing, swallowing, and speaking. Data from the Global Cancer Observatory (GLOBOCAN) show that in 2020, approximately 19 million new cases of cancer worldwide were recorded, including oral and oropharyngeal cancer, with 377,713 new cases and 177,757 deaths. Asia became the country with the highest number of cases and deaths, with 248,360 cases and 131,610 deaths.

Dimethylbenz(a)anthracene (DMBA) is a compound that is procarcinogenic; the carcinogenic activity of these compounds occurs through biotransformation into more potent compounds to produce carcinogenesis. DMBA induction can lead to the development of toxic effects via oxidative stress and the generation of carcinogenic metabolites, as well as the creation of lesions on DNA bases and the binding of DNA to form DNA adducts during the carcinogenic stage. Carcinogenesis is mutation or loss of the tumor suppressor gene (p53). These mutations have implications in cell proliferation, apoptosis,

differentiation, and cell adhesion molecules and contribute to enhanced angiogenesis, which are relevant to invasion and metastasis [3].

Treatment methods for OSCC include surgery, radiation, and chemotherapy; however, these methods adversely affect normal and healthy cells. Therefore, natural medicine is recommended and considered safer, has fewer side effects, is easy to obtain and inexpensive, and can be used by everyone [4]. Plants containing asiatic acid show various pharmacological properties, such as antioxidant, anti-inflammatory, and neuroprotective [8–11], and can fight cancer. Thus, this study was conducted to determine how the methanol extract of *Centella asiatica* at doses of 25, 50, 200, and 400 mg/kg BW would affect the degree and progression of dysplasia induced by dimethylbenz(a)anthracene (DMBA) in Wistar rats.

2. Materials and Methods

2.1. Plants and Materials

Centella asiatica leaves were harvested from RT 031/RW 015, Salakmalang, Banjarharjo, Kalibawang, Kulon Progo, and Yogyakarta. *Centella asiatica* has single, long-stemmed leaves with a 10-15 cm length. The tips of the leaves are rounded with serrated edges, and the base is blunt. The organization of the leaf bones is characterized by oval-shaped leaf blades arranged in a finger-like pattern. The flesh of the leaves is permanent or perkamanteus. Each growing petiole generally amounts to 6 for the mother plant and 2-5 for the sapling. *Centella asiatica* must come from the same area and undergo the same treatment during growth. Methanol extracts of *Centella asiatica* leaves were obtained from the Laboratory of Phytochemistry, Faculty of Pharmacy, Universitas Sumatera Utara, Indonesia. The materials used were a 95% methanol solution, distilled water, and CMC-NA.

2.2. Ethical Approval

Ethical Approval No. 0580/KEPH-FMIPA/USU/2022 was obtained from the research ethics committee of Universitas Sumatera Utara.

2.3. Preparation of Crude Extracts

Centella asiatica leaves were dried in a drying box, ground with an electric blender, soaked in methanol (Lichrosolv, Germany) for 5 days, and stirred regularly. After 5 days, filtration was performed using filter paper to collect the filtrate. The filtrate was then macerated using a rotary evaporator (Heidolph VV2000,

Germany) and placed in an oven (Memmert, Germany).

2.4. Phytochemical Analysis and Antioxidant Activity of *Centella Asiatica*

2.4.1. Qualitative Phytochemical Screening

Phytochemical qualitative screening is a process used to identify and detect various classes of secondary metabolites or phytochemicals in plant extracts. In this study, qualitative phytochemical screening was conducted using a standard method.

2.4.1.1. Alkaloid Content Detection

This technique entails employing Dragendorff's, Bouchardat's, and Meyer's reagents in the extract, followed by the observation of characteristic color changes that signify the presence of alkaloids. Test solution of 2 mL was evaporated on a porcelain dish until residue was obtained. The residue was then dissolved in 5 mL of 2N HCl. The resulting solution was divided into 3 test tubes. The first test tube was filled with diluted acid. The second test tube was supplemented with 3 drops of Dragendorff's reagent, and the third test tube was supplemented with 3 drops of Mayer's reagent. With Dragendorff's reagent, the presence of alkaloids is indicated by the formation of an orange or orange-red precipitate. Bouchardat's reagent forms reddish-brown precipitates, whereas Meyer's reagent forms white, yellow, or yellowish-white precipitates.

2.4.1.2. Flavonoid Detection

The plant extract is mixed with magnesium powder; then, concentrated hydrochloric acid (HCl) is added, followed by the addition of amyl alcohol. First, add 10 g of the powdered sample to 100 mL of hot water and heat it for 5 min. Filter the solution and collect 5 mL of the filtrate. Add 0.1-g magnesium powder and 1 mL of concentrated hydrochloric acid to the filtrate. Then, add 2-mL amyl alcohol to the mixture and shake. The synthesis of flavonoids with magnesium and hydrochloric acid yields a yellow or orange coloration, which is indicative of the presence of flavonoids.

2.4.1.3. Glycoside Detection

Molisch's test detects glycosides. This involves adding a few drops of Molisch's reagent, followed by the addition of concentrated sulfuric acid (H₂SO₄). The formation of a purple ring at the interface between the two layers indicates glycosides.

2.4.1.4. Tannin Detection

In this method, 1 mL of the extract is added to a test tube, followed by the addition of 0.5 mL of 5% ferric chloride (FeCl₃), and the product of the reaction displays a blue-black or greenish-black hue, which is indicative of the presence of tannins.

2.4.1.5. Saponin Detection

The determination of saponins in the extracts was made through shaking with hot water, resulting in the formation of a stable, creamy foam. To test for saponins, 10 drops of the solution were added to a test tube, followed by the addition of 5 drops of hot water. After cooling the solution, it was vigorously shaken for 10 s, and foam was formed. Persistent foam formation for 10 min, unaffected by the addition of 1 drop of 2N HCl, indicates saponin content.

2.4.1.6. Triterpene Detection

The Liebermann-Burchard test is a chemical test used to detect steroids and triterpenoids. The 2-mL test solution is evaporated in an evaporation dish. The residue was dissolved in 0.5 mL of chloroform, followed by the addition of 0.5 mL of anhydrous acetic acid and 2 mL of concentrated sulfuric acid through the wall of the tube. The presence of triterpene is indicated by a violet color, and steroid is indicated by a green color.

2.4.2. Quantitative Phytochemical Screening

In quantitative phytochemical screening, total phenolic compounds were determined using the Follin-Ciocalteu method, total flavonoid content was determined using the aluminum chloride colorimetric assay method, and antioxidant activity was assessed using the immersion DPPH free radical method.

2.4.2.1. Follin-Ciocalteu Method

Gallic acid (50 mg) was dissolved in methanol to make a 100 mL solution with a concentration of 500 ppm. Then, 2.5 mL aliquots of this solution were taken for concentrations of 500, 250, 125, 62.5, 31.25, and 15.625 ppm, each diluted to 5 mL with methanol. From each concentration, 0.1 mL was pipetted and mixed with 7.9 mL of distilled water, 0.5 mL of Folin-Ciocalteu reagent, and 1.5 mL of 20% sodium carbonate solution, followed by incubation for 90 min. The color change in the tube to blue signifies the development of a molybdenum blue complex, which occurs as a result of the redox reaction of phenol with phosphomolybdic acid in an alkaline environment. The absorbance of the gallic acid standard solution at each concentration, along with the maximum wavelength (775 nm) at a concentration of 500 ppm, was measured against the blank reagent using UV-Vis spectrophotometry (400-800 nm). This calibration was used to determine gallic acid concentration.

2.4.2.2. Aluminum Chloride Colorimetric Assay

50 mg of quercetin was weighed and dissolved in methanol to obtain a 50 mL solution with a concentration of 100 ppm. Then, 5 mL of this solution was pipetted into a 50 mL flask and diluted with methanol to the marked line to maintain a

concentration of 100 ppm. Subsequently, aliquots of 2.5 mL were taken from this solution for concentrations ranging from 100 to 6.25 ppm, each brought up to 5 mL with methanol. From each concentration, 2 mL of the solution was pipetted and mixed with 0.1 mL of 10% aluminum chloride (AlCl_3), 0.1 mL of sodium acetate (CH_3COONa), and 2.8 mL of distilled water, followed by 40-min incubation. The absorbance of the quercetin standard solution was measured at each concentration, and the maximum wavelength (432 nm) was observed at 100 ppm against the blank reagent using UV-Vis spectrophotometry (400-800 nm). This calibration process was used to determine quercetin concentration.

2.4.2.3. Immersion DPPH Free Radical Method

The antioxidant activity of the test sample was evaluated based on its capacity to inhibit the oxidation of DPPH (1,1-diphenyl-2-picryl-hydroxyl), a free radical, in a methanol solution. This inhibition resulted in a reduction in the purple of DPPH. The IC_{50} value, representing the concentration of the test sample required to reduce free radicals by 50%, served as a parameter to quantify the antioxidant activity of the test sample. 20 mg of DPPH was measured and placed into a 100-ml volumetric flask. It was then dissolved in methanol and further diluted with methanol up to the indicated mark on the flask, resulting in a 0.5-mM DPPH solution (200-ppm concentration).

A homogenized DPPH solution with a concentration of 40 ppm was prepared, and its absorbance was recorded using a UV-visible spectrophotometer over a wavelength range of 400-800 nm. Subsequently, in each flask, 5 mL of a 0.5-mM DPPH solution (200-ppm concentration) was added, followed by the addition of methanol to reach the marked line. The flasks were then left to stand for 60 min in a dark environment, after which absorbance was measured using a visible spectrophotometer at a wavelength of 516 nm.

The antioxidant ability was assessed by measuring the reduction in the absorbance of the DPPH solution, indicated by a decrease in its purple color, following the addition of the test solution. The change in the absorbance of the DPPH solution before and after the addition of the test solution was calculated as the percentage attenuation. The IC_{50} value denotes the concentration of the test sample (in $\mu\text{g/mL}$) required to achieve 50% DPPH inhibition, indicating its ability to reduce or inhibit the oxidation process by 50%. A 0% value indicates no antioxidant activity, whereas a 100% value indicates complete inhibition. Further testing involves diluting the test solution to determine the concentration threshold of the activity.

The obtained results were then fitted into a regression equation, with the extract concentration (in $\mu\text{g/mL}$) plotted on the X-axis and the percentage of DPPH silencing (antioxidant activity) on the Y-axis.

Based on the IC_{50} value, a compound's antioxidant potency is classified as potent antioxidant ($\text{IC}_{50} < 50$ ppm), strong antioxidant ($50 \leq \text{IC}_{50} < 100$ ppm), moderate antioxidant ($100 \leq \text{IC}_{50} < 150$ ppm), and weak antioxidant ($\text{IC}_{50} \geq 150$ ppm).

2.5. Study Design and Groups

The study was an in vivo experiment with a post-test-only control group design. The animals used in this study were eight weeks old and consisted of 50 male Wistar rats (*Rattus norvegicus*) with an average body weight of 200–300 g. The Wistar rats used in this study were in a healthy condition and never received any treatment. Prior to any treatment, the Wistar rats were acclimatized for one week as an adaptation. Then, they were divided into five groups:

1. Group 1: 0.5% DMBA (three times a week for 28 days)
2. Group 2: 0.5% DMBA (three times a week for 28 days) + methanol extract of *Centella asiatica* (25 mg/kg BW once a day from Day 29 to Day 61).
3. Group 3: 0.5% DMBA (three times a week for 28 days) + methanol extract of *Centella asiatica* (50 mg/kg BW once a day from Day 29 to Day 61).
4. Group 4: 0.5% DMBA (three times a week for 28 days) + methanol extract of *Centella asiatica* (200 mg/kg BW once a day from Day 29 to Day 61).
5. Group 5: 0.5% DMBA (three times a week for 28 days) + methanol extract of *Centella asiatica* (400 mg/kg BW once a day from Day 29 to Day 61).

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

2.6. Dimethylbenz(a)anthracene (DMBA) Induction in Wistar Rats

The rats were administered ketamine hydrochloride intraperitoneally at a dose of 10 mg/kg BW via the buccal mucosa for anesthesia. The buccal mucosa of each rat in the treatment group was then scratched for a distance of 1 cm using a 27G syringe filled with 100 μg of 5% DMBA and corn oil serving as a solvent. The induction of 100 μg of 0.5% DMBA into the buccal mucosa of Sprague-Dawley rats three times a week for 28 days was found to be successful in inducing oral epithelial dysplasia in each rat according to Maulina et al. [1].

2.7. Treatment of Methanol Extract of *Centella Asiatica* Leaves

After induction of DMBA three times a week for 28 days, each rat group 2-5 needs to be given a methanol extract of *Centella asiatica* for the next 33 consecutive days. The extract was given by force-feeding with a syringe every morning after breakfast.

2.8. Euthanasia Procedure

On Day 61 of the experiment, each rat was

euthanized by inhaling chloroform.

2.9. Histopathological and Immunohistochemistry: Protein Expression p53 and TNF α Examination

The buccal mucosa was immediately excised after the rats were euthanized. The samples were then placed into small containers and fixed in a 10% formalin buffer. The samples were then dehydrated using alcohol ranging in strength from 70% to 95%. Following dehydration, the samples were immersed in toluene for 30 min, made into paraffin blocks, and then cut into pieces using a microtome ranging in thickness from 5 to 10 μ m. The samples were then placed on the object glass for H&E and IHC staining.

The object glass for H&E staining was placed into a water bath set at 50°C. The glass was then secured with paraffin tape after being coated with albumin and glycerin. Staining commences with deparaffinization through the use of xylol I (2 minutes) and xylol II (2 minutes), followed by hydration through the use of alcohol, specifically 95% (2x2 minutes), 90% (2x2 minutes), 80% (2x2 minutes), and 70% (2x2 minutes). Following staining with hematoxylin and eosin for five minutes each, the sample was dehydrated using a series of alcohol solutions, from 70% to 95%. Subsequently, the sample was mounted on a glass object and examined under a microscope with a magnification of 400x.

For the IHC examination, the following deparaffinization process should be employed: utilize xylol I for 10 minutes, followed by xylol II for 5 minutes, and then alcohol (70%-95%). Subsequently, the slide should be rinsed with PBS solution (pH 7.4) and Tris EDTA. To block non-specific antibody binding, a 1% BSA solution should be applied, and the slide should be washed with PBS (three times for 5 minutes each). Finally, the slide should be dried. Polyclonal antibody droplets (P53 and TNF α) were then incubated for 24 h at 4°C and washed with PBS (3x5 minutes). Drip with DAB (20-40 minutes) and counterstain with Meyer hematoxylin. Perform mounting. The Laboratory of Histology, Faculty of Medicine, Universitas Sumatera Utara, Indonesia, performed the histopathological analysis.

2.10. Statistical Analysis

The result of this study was analyzed using IBM

SPSS Version 21. The statistical analysis results using the Kruskal-Wallis test were considered significant if the p-value was below 0.05.

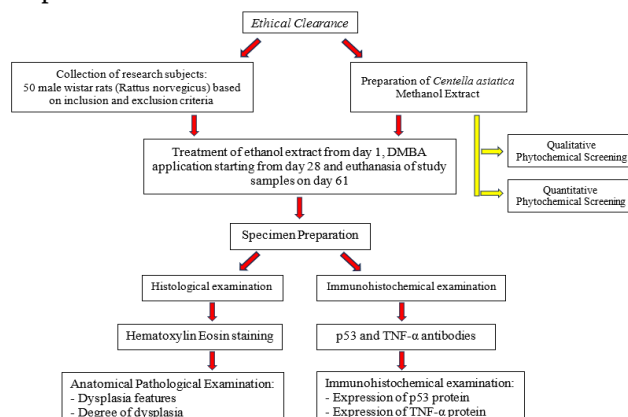


Fig. 1 Research methodology (The authors)

3. Results and Discussion

3.1. Phytochemical Screening of *Centella Asiatica*

The results of the qualitative phytochemical screening test of the methanol extract of *Centella asiatica*, which was extracted using the maceration method, showed that the extract contained alkaloid, flavonoid, glycoside, saponin, tannin, and triterpenoid/steroid compounds (Table 1).

Table 1 Secondary metabolites of the methanol extract of *Centella asiatica* with qualitative methods (The authors)

Secondary metabolite	Reagen	Result
Alkaloid	Dragendroff Bouchardat Meyer	+
Flavonoid	Powder Mg+ Amil Alcohol + HCl _p	+
Glycoside	Molish+H ₂ SO ₄	+
Saponin	Hot water/mix	+
Tannin	FeCl ₃	+
Triterpene/Steroid	Lieberman-Bourchat	+

The results of the quantitative phytochemical screening test of the methanol extract of *Centella asiatica* showed that the extract contained 95,77±0,95 mg/g GAE of total phenolic, 2,35±0,01 mg/g QE of total flavonoid, and antioxidant activity with an IC₅₀ value of 113,91±0,01 (Table 2).

Table 2 Secondary metabolites and antioxidant activity of the methanol extract of *Centella asiatica* (The authors)

Solvent type	Extraction method	Total phenolic (mg/g GAE)	Total flavonoid (mg/g QE)	Antioxidant activity (IC ₅₀)
Methanol	Maceration	95,77±0,95	2,35±0,01	113,91±0,01

3.2. Nonparametric Hematoxylin and Eosin Test

Table 3 shows the effectiveness of administering the methanol extract of *Centella asiatica* at doses of 25, 50, 200, and 400 mg/kg BW on the incidence of dysplasia induced by dimethylbenz(a)anthracene (DMBA). The group that was not given the extract had a higher dysplasia grade.

Table 3 Kruskal-Wallis test results (The authors)

Group	N	Mean ± SD	P Value
Group 1 (control)	10	2.00 ± 0.000	0.000*
Group 2 (dose 25 mg/kg BW)	10	2.00 ± 0.000	
Group 3 (dose 50 mg/kg BW)	9	1.44 ± 0.527	
Group 4 (dose 200 mg/kg BW)	10	1.30 ± 0.483	

Group 5 (dose 400 mg/kg BW) 9 1.22 ± 0.441

* Kruskal-Wallis test; p < 0.05; significant

3.3. TNFa and p53 IRS Scores

Table 4 shows the effectiveness of administering the methanol extract of *Centella asiatica* at doses of 25, 50, 200, and 400 mg/kg BW on the incidence of dysplasia induced by dimethylbenz(a)anthracene (DMBA). The group that was not given the extract had a higher dysplasia grade. Pourshahidi et al. [17] studied the carcinogenic effects of the compound dimethylbenz(a)anthracene (DMBA), and it was

explained that after DMBA was applied to the cheek mucosa of hamsters for 5 and 10 weeks, dysplasia occurred at mild to moderate severity. Arora et al. [18] conducted a study on two fractions of the methanol extract (CAE). One fraction, CAE-EF, contained triterpene active ingredients, while the other, CAE-FF, did not. The results of the study showed that the methanol extract, at a dose of 100 mg/kg BW, demonstrated the most potent antioxidant activity of the two fractions tested in vitro.

Table 4 TNFa and p53 IRS score test results (The authors)

Group	TNF-α			p53		
	Mild, n (%)	Moderate, n (%)	Severe, n (%)	Mild, n (%)	Moderate, n (%)	Severe, n (%)
1	2 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
2	2 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
3	2 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
4	2 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
5	2 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total	10 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Phytochemical screening was conducted to determine the presence of various bioactive compounds in the sample. The screening results were tabulated to provide a comprehensive overview of the phytochemicals detected, including alkaloids, flavonoids, glycosides, saponins, tannins, and triterpenes. Each compound was assessed for its qualitative presence or absence, which served as a preliminary assessment of the sample's potential pharmacological properties. Alkaloids hold significant importance in inhibiting the development of cancer by regulating signaling pathways related to cell proliferation, cell cycle progression, and the metastasis of cancer cells. They are key components in various anti-cancer drugs that are used clinically. Furthermore, the review discusses the potential future applications of alkaloid nanoformulations in the treatment of lung cancer [5]. Flavonoids, with their antioxidant and anti-inflammatory properties, may mitigate oxidative stress and inflammation associated with dysplasia, contributing to anti-cancer effects [6]. Some glycosides possess anticancer properties, potentially inhibiting dysplastic cell progression to invasive cancer [7]. Saponins have immunomodulatory properties that enhance the immune response against dysplastic cells, aiding in their recognition and elimination [8]. Tannins exhibit antioxidant and antimicrobial activities, potentially reducing oxidative stress and preventing infections that worsen dysplasia, while also possessing anticancer properties [9]. Triterpenes, known for their anti-inflammatory and anticancer effects, may modulate dysplasia progression by reducing inflammation and inhibiting abnormal cell growth [10].

Quantitative analysis of phytochemicals in the methanol extract of *Centella asiatica* revealed total phenolic and flavonoid compounds alongside antioxidant properties. This investigation employed the

Folin-Ciocalteu method to measure the total phenolic content, a technique renowned for its specificity and sensitivity to phenol compounds. Upon reaction with a solution containing phenolic compounds and sodium carbonate, the Folin-Ciocalteu reagent produces a dark blue complex, whose absorbance is subsequently evaluated. Phenolic compounds, ubiquitous in various plant-based foods and beverages, constitute an essential component of the human diet, with polyphenols being identified as pivotal antioxidants in tea extracts [11]. Among the constituents of herbal plants, flavonoids are another class of phenolic compounds renowned for their antioxidant effects. Flavonoid compounds are categorized into various types exhibiting distinct polarity, which is determined by the quantity and arrangement of the hydroxyl groups. These differences in polarity influence the solubility of flavonoids in different solvents [12]. The antioxidant activity of the sample was determined using the DPPH method, which was chosen for its simplicity, speed, and sensitivity. DPPH, a stable free radical, undergoes a color change from purple to yellow upon reacting with compounds exhibiting antioxidant activity, thus allowing for absorbance assessment. The IC50 value, denoting the concentration of antioxidant compounds inhibiting 50% of free radicals, indicates antioxidant efficacy, with lower values indicating superior efficacy [11].

In this study, it can be seen that the methanol extract of *Centella asiatica* was effective at doses of 25, 50, 200, and 400 mg/kg BW on the incidence of dysplasia induced by dimethylbenz(a)anthracene (DMBA). Based on research by Wu et al. [19], asiatic acid can inhibit lung cancer cell proliferation, cause cell death, and induce apoptosis in lung cancer cells through a decrease in mitochondrial membrane potential. Asiatic acid is known to primarily induce apoptosis by interfering with the mitochondrial membrane potential,

which subsequently stimulates the release of cytochrome c and triggers the caspase signaling pathway and PARP activation, ultimately resulting in apoptotic cell death. Mitochondria and ROS play central roles in cell death processes, including apoptosis.

Centella asiatica functions as an antioxidant by reducing hydroperoxides, deactivating free radicals, and chelating metal ions. Antioxidants possessed by *Centella asiatica* have different functions, including collecting reactive oxygen, inhibiting free radicals, inhibiting p-coumaric acid, and metal chelation. Plant active components can serve as preventive agents or as ingredients to mitigate the adverse effects of cancer treatment and radiation therapy. Triterpenoids are a group of aromatic polyhydroxy compounds that can be found in plants and have pharmacological effects. Asiaticoside, a constituent of triterpenoids, exhibits pharmacological properties as an anticancer agent. Asiaticoside, as an anticancer agent, serves as a mechanism for cancer prevention through its antioxidant properties and its ability to reduce excessive gene expression. Asiaticoside can also reduce mitochondrial membranes, thereby increasing the occurrence of apoptosis in cells. Asiaticoside was found to effectively impede the proliferation of colorectal tumors and hepatocellular carcinoma by inhibiting the growth pathways of cancer cells. Asiaticoside has the capability to bring about a reduction in gene expression, which serves as a crucial regulator of the cell cycle and is also one of the targets of genetic alterations in cancer. In this way, cancer cell viability is inhibited [13].

p53 is a tumor suppressor gene that is frequently mutated in malignancies. The gene product, a transcriptional factor, regulates the expression of genes involved in cell cycle arrest or apoptosis in response to genomic damage or cell stress. Tumor necrosis factor alpha (TNF- α) is the primary cytokine in the acute inflammatory response to gram-negative bacteria and other microbes and can also induce cell survival and apoptosis. In our study, there was no significant difference in the average levels of TNF- α and p53 between the control and treatment groups. In this study, the moderate and severe grades of dysplasia were highly correlated with p53 staining found in the suprabasal layer of epithelium; therefore, p53 staining had a higher specificity and positive predictive value (PPV) for malignant transformation than for the assessment of dysplasia but lower sensitivity. According to these TNF- α , a cell's individual properties determine whether it will differentiate, proliferate, or undergo apoptosis. Therefore, it is postulated that the administration of the methanol extract of *Centella asiatica* cannot prevent the increase of TNF- α for this period of study. The extended administration of the methanol extract of *Centella asiatica* could lead to a higher expression of TNF- α

and p53 [14, 15].

In this study, there was a significant difference between the groups that were given the treatment and those that were not given the methanol extract of *Centella asiatica*. The outcome demonstrates that the methanol extract of *Centella asiatica* can exert an impact on the occurrence of dysplasia as this plant is commonly utilized in medicinal contexts as an anticancer agent, owing to its constituent active ingredients. From the quantitative and qualitative phytochemical analyses, it can be observed that secondary metabolite components can induce differences in the degree of dysplasia between samples treated with *Centella asiatica* extract and those not. Phenolic compounds have anticancer properties as they can inhibit the growth of cancer cells and promote apoptosis in these cells. The primary components of this plant are triterpenoids, which include asiaticoside, madecassoside, and asiatic acid. Asiaticoside possesses anti-inflammatory and antioxidant properties. In this study, the results of the average degree of dysplasia in each group showed that there was a decrease in the degree of dysplasia in the 200- and 400-mg/kg BW dose groups. This suggests that the dose plays a role in influencing the incidence of dysplasia induced by dimethylbenz(a)anthracene (DMBA). Based on the study by Velu et al. [20], the extract administration at a dose of 100 mg/kg BW can inhibit the overall occurrence of dysplasia in the buccal mucosa of DMBA-induced rats. In this research, the methanol extract of *Centella asiatica*, when administered in lower doses, was found to be effective in decreasing the level of dysplasia in Wistar rat (*Rattus norvegicus*) test subjects [16].

4. Conclusion

In summary, the results from the dysplasia grading were analyzed using the Kruskal-Wallis test followed by the Mann-Whitney test. The findings revealed the potency of delivering methanol extracts of *Centella asiatica* at concentrations of 25, 50, 200, and 400 mg/kg BW in reducing the frequency of dysplasia triggered by dimethylbenz(a)anthracene (DMBA). The group that was not given the extract had a higher dysplasia grade. This study found that the methanol extract from *Centella asiatica* leaves can effectively influence the incidence of dysplasia because of its bioactive content, which acts as an anticancer. Secondary metabolites from *Centella asiatica* leaves have been shown to influence significant differences in the degree of dysplasia between groups given the extract and those not. Ingredients such as phenolics, asiaticoside, madecassoside, and asiatic acid can prevent cancer cell growth, support cell apoptosis, and act as anti-inflammatory and antioxidant agents. Future research can extend the administration time of the methanol extract from *Centella asiatica* leaves to achieve more effective results in increasing the

expression of *tnf-α* and p53.

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