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Original article

Cytotoxic test of different solvents of soursop (*Annona muricata*) leaf extract against HSC-3 cell line

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ABSTRACT

Background: *Soursop (Annona muricata) leaves have been researched extensively and found to have anticancer properties. The use of soursop as an anticancer treatment is increasingly popular due to its selective cytotoxic activity by acetogenins. The polarity of the extract solvent contributes to the biological activity of the plant, namely cytotoxicity.* ***Purpose:*** *To determine the cytotoxicity of A. muricata leaf extract with ethanol, ethyl acetate and hexane fractions against human oral squamous carcinoma (HSC-3) cell lines.* ***Methods:*** *This experimental laboratory study consisted of twenty four treatment groups tested against the HSC-3 cell line. The ethanol, ethyl acetate and hexane fractions of A. muricata leaves were administered to seven different concentrations, namely 0.3 µg/mL, 3 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, 150 µg/mL and 300 µg/mL. The control group consisted of three groups: negative control, solvent control and positive control. The percentage of cell viability was calculated by absorbent enzyme-linked immunosorbent assay (ELISA) reader. The cytotoxicity of A. muricata leaf extract against HSC-3 cells was determined by cell counting kit-8 (CCK-8) assay and expressed by IC_{50} value. The results were analysed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD).* ***Results:*** *The results show that the leaf extracts of A. muricata are moderately cytotoxic to HSC-3 cells. The highest cytotoxic activity was found in the ethyl acetate extract with an IC_{50} value of 76.66 µg/mL – making it the best solvent – then hexane (IC_{50}: 84.14 µg), then ethanol (IC_{50}: 101.32 µg/mL). Statistical analysis using one-way ANOVA and Tukey's HSD is considered significant $p < 0.001$.* ***Conclusion:*** *Ethanol, ethyl acetate and hexane fractions of A. muricata leaf extract are moderately cytotoxic, with IC_{50} values in the range of 21–200 µg/mL.*

Keywords: *Annona; cytotoxicity; antineoplastic agent; squamous cell carcinoma of head and neck*

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INTRODUCTION

Cancer is a group of diseases characterised by the uncontrolled growth and spread of abnormal cells.[1] According to the World Health Organization, in 2018, cancer became the second leading cause of death in the world, causing 9.6 million people or one in six people to die. Oral cancer is one of the top ten forms of cancer-causing deaths in the world, with an estimated 657,000 new cases and more than 330,000 deaths annually.[2] Until now, the exact aetiology of oral cancer has been unknown because of its complex and multifactorial nature. Some of the influencing factors can be local, such as poor oral hygiene, irritation or trauma to restorations or dental caries. In addition, there are also external factors that can influence its occurrence, namely bad habits such as smoking, alcohol consumption, chewing betel nut, viral infections, or it can be influenced by the *host's* genetics, age, gender and immunity.[3]

The most common oral cancers are oral squamous cell carcinoma (OSCC), with a prevalence of 80%–90% of all malignancies of oral neoplasms.[4] OSCC can occur in the lip, oral cavity, nasopharynx or pharynx. The treatment depends on several factors, such as the cancer's stage of progression, its location and the general health of the patient.[5] Some of the therapies that are often used today include surgery, radiotherapy, chemotherapy, gene therapy and/or hormonal therapy, alone or in combination. Commonly

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used chemotherapy drugs include antimetabolites or those that inhibit enzymes, DNA-interactive compounds, antitubulin compounds, hormones and molecular targeting compounds. However, the side effects of chemotherapy treatment are caused by the drugs' attack on normal cells, causing hair loss; bone marrow suppression; reduced levels of haemoglobin, platelets and white blood cells; drug resistance; weak body; etc.[6,7] The number of side effects from common chemotherapy drugs has led to numerous studies on herbal ingredients and their potential for being alternative treatments to cancer. Herbal ingredients are believed to have minimal side effects and are also more economical. The anticancer properties of herbal ingredients can be in the form of plant extracts or single active compounds that have been isolated.[7]

Indonesia has biodiversity with the potential to be used as medicinal plants or herbal ingredients. One of which is the soursop plant (*Annona muricata*), which is generally very easy to find throughout the island of Indonesia because they can grow any place, especially in areas that are watery. In addition to being consumed, this plant can also be used as a medicinal plant because it has anticancer, anti-tumour, antiviral, anti-inflammatory, antidepressant, antidiabetic, antihypertensive and antibacterial properties.[8] Chemical compounds that can be found in *A. muricata* include acetogenins, alkaloids, flavonoids, phenolic compounds and other compounds. However, the secondary metabolite acetogenins have the most dominant anticancer effect and have been reported ethnobotanically regarding their possible selective cytotoxic activity against cancer cells through adenosine triphosphate (ATP) intake.[9] Flavonoids were found to induce apoptosis more strongly than the clinically proven anti-tumour agent Camptothecin.[10,11] A previous study showed the cytotoxic activity of *A. muricata* leaf extract with ethanol, ethyl acetate and hexane fractions against cervical cancer (HeLa) cell lines at concentrations of 1.5 µg/mL, 3.125 µg/mL, 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL and 200 µg/mL.[12] The leaves of the *A. muricata* have been investigated extensively for their diverse pharmacological applications and were found to be superior for their anti-inflammatory and anticancer properties. Soursop as an anticancer treatment is becoming increasingly popular, so it has been reported ethnobotanically regarding its possible selective cytotoxic activity.

Bioactivity is considered selective because there are several extracts that have been shown to be more toxic to cancer cells than to normal cells. This selective activity is reported to induce healing with minimal side effects.[13] However, most of the studies that have been previously carried out did not use the bioactive isolates responsible for the activity but were based on crude plant extracts.[14] Meanwhile, the polarity of the extract solvent used contributes to the biological activity of the plant, namely its cytotoxicity. The secondary metabolites contained tend to be more soluble in solvents of the same polarity.[15]

Therefore, we hypothesise that the secondary metabolites contained in the ethanol, ethyl acetate and hexane fraction of *A. muricata* leaf extract may have cytotoxic effects on the human oral squamous carcinoma (HSC-3) cell line. This study will explore the cytotoxic activity of *A. muricata* in three solvents with different polarities, namely ethanol (polar), ethyl acetate (semi-polar) and hexane (nonpolar), against oral cancer cells *in vitro*.

MATERIALS AND METHODS

The materials used in this study were soursop (*A. muricata*) leaves obtained from Manoko solvents, such as ethanol, ethyl acetate and hexane; supporting materials such as phosphate buffer saline (PBS); cell counting kit-8 (CCK-8) reagent; dulbecco's modified eagle medium-fetal bovine serum (DMEM-FBS) 10% as media for the cells and as a negative control; dimethyl sulfoxide (DMSO) 1% as solvent control, to dissolve each fraction of extract and to see whether the solvent contributes to the cytotoxic activity; DMSO 10% as a positive control to obtain the desired effect of the treatment, which is the cytotoxic effect, and as an antibiotic (penicillin-streptomycin) and antimycotic (amphotericin B) 1%. The tools used for the analysis included a blender, macerator, beaker glass, rotary evaporator, separatory funnel, Erlenmeyer, 96-well plate, tissue culture flask, CO_2 incubator, 450 nm microplate reader, micropipette, biosafety cabinet and microscope.

Soursop leaves originated in Bandung, West Java, Indonesia and were then extracted using maceration with 96% ethanol and continued to be fractionated using three solvents of different polarity: ethanol (polar), ethyl acetate (semi-polar) and hexane (nonpolar) using the liquid–liquid extraction method. Maceration and fractionation were performed at Natural Material Organic Chemistry Laboratory, Bandung Institute of Technology, West Java, Indonesia. Afterwards, the three extracts of A. muricata were diluted into seven different concentrations: 0.3 µg/mL, 3 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, 150 µg/mL and 300 µg/mL.

HSC-3 cells stored in liquid nitrogen were placed in a water bath and heated to 37°C until dissolved. They were then diluted with culture medium with ten times the amount of cells. Then, they were centrifuged to obtain a cell palette, resuspended in medium, and the number of cells present were counted. The growing cells were then seeded using a T-flask, incubated for 24 hours, and the cells were ready approximately one week after seeding.

Phytochemical screening of *A. muricata* leaf extract with ethanol, ethyl acetate and hexane was performed at the Natural Materials Organic Chemistry Laboratory, Bandung Institute of Technology to obtain the active compound using the method ascribed to J.B. Harborne.[16] Identification of alkaloid compounds was carried out by mixing 0.1 mg of extract with 10 mL of chloroform and a few drops of ammonia. To separate and acidify the chloroform fraction, a

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few drops of concentrated H_2SO_4 were added, then divided into three tubes, and Dragendorff's, Mayer's and Wagner's reagents were added. If there was a red (Dragendorff), yellowish white (Mayer) or brown (Wagner) precipitate, it indicated the presence of alkaloids.

To identify flavonoid compounds, samples were given 0.1 mg magnesium powder, 0.4 mL amyl alcohol (a mixture of 37% HCl and 95% ethanol in a ratio of 1:1) and 4 mL of alcohol, which was then shaken. The presence of flavonoids was indicated by the formation of a red, yellow or orange precipitate on the amyl alcohol layer. For phenolic compounds, 1 g of sample was extracted using 20 mL of 70% ethanol; 1 mL of the extract was then given two drops of $FeCl_3$ 5% solution. If it produced a green or blue–green colour, it contained phenolic compounds.

Saponin compounds were identified using the foam test in hot water. If the foam did not disappear when one drop of 2 N HCl was added, and it was stable for 10 minutes, then there were saponins. To identify tannin compounds, 1 g of extract was mixed with 10 mL of distilled water and brought to a boil. Then, after the filtrate had cooled, 5 mL of $FeCl_3$ was added. If a deep blue colour formed, this indicated the presence of tannins.

The HSC-3 cell line obtained from the Japan Health Science Research Resources Bank was isolated from a 64-year-old man in Japan. The main tumour from the HSC-3 cell culture was on the tongue with lymph node metastases, belonging to the category of moderately differentiated OSCC.[17] The method used to test cytotoxicity was CCK-8 assay using CCK-8 reagent. In this method, water-soluble tetrazolium salts (WST-8) dye is reduced using dehydrogenase in cells to produce formazan or orange colour, which is soluble in water through nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) produced by cellular activity.[18] The amount of formazan formed depends on dehydrogenase activity.[19] The amount of formazan produced indicated the viability of the cells. This could be seen from the optical density (OD) of formazan by using a 450 nm microplate reader.

First, the estimated number of cancer cells were calculated at 20.000 cells/well and the number inserted into the 96-well plate. Then, the concentration of the diluted extract with the culture medium was adjusted so that it reached concentrations of 0.3 µg/mL, 3 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, 150 µg/mL and 300 µg/mL. After that, it was incubated at 37°C in a 5% CO_2 incubator for 24 hours. Then, the treatment medium was rinsed with 90 µL of PBS, and 10 µL of CCK-8 was added to each well and incubated for 1 hour. This was performed three times for each group at each concentration. OD results were determined using a microplate enzyme-linked immunosorbent assay (ELISA) reader with a wavelength of 450 nm.

Cytotoxicity was measured by examining the reduction in cell viability compared with the control group using IC_{50} value. The IC_{50} value was determined by plotting a graph of cell viability versus concentration. The higher the IC_{50} value, the less cytotoxic the extract was because high concentrations were needed to inhibit cells by 50%. Absorbance data could be obtained from each well using a microplate reader or spectrophotometer (Biochrom Anthos Zenyth, USA). This data was converted to cell inhibition rate using the formula below. The percentage of inhibition was calculated using the formula:[12]

$$\%\ Inhibition = 1 - \left(\frac{absorbance\ of\ group\ with\ extract}{absorbance\ of\ control\ group}\right) x\ 100\%$$

The results were analysed using the Shapiro–Wilk data normality test. Statistical significance of the data was calculated using one-way analysis of variance (ANOVA) in addition to Tukey's honestly significant difference (HSD) post hoc test to determine the level of significance. The results were declared significant if $p < 0.05$. This analysis was carried out using SPSS 28.0 (SPSS Inc., Chicago, IL, USA) software.

RESULTS

The results of the phytochemical screening can be seen in Table 1. From the results obtained, OD was converted into the number of cells through the equation obtained through the normal curve. In Figure 1, it can be seen that as the

Table 1. Phytochemical screening results of ethanol, ethyl acetate and hexane fractions of A. muricata leaf extract using J.B. Harborne's method

Fractions of *A. muricata* leaf extract	Chemical compound	Result
Ethanol	Saponin	+
	Tannin	+
Ethyl Acetate	Alkaloid	+
	Flavonoid	+
Hexane	Steroid	+
	Triterpenoid	+

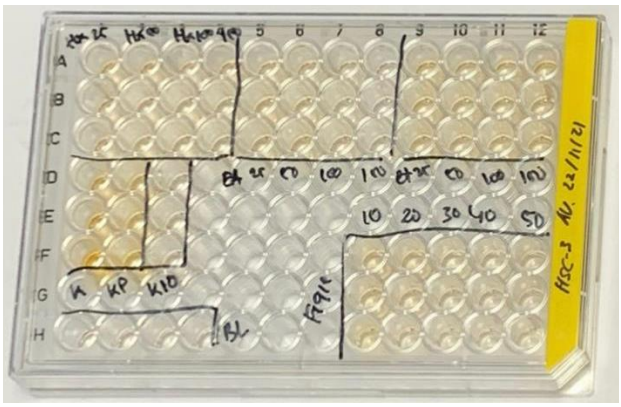

Figure 1. Colour change that occurred after the human oral squamous carcinoma (HSC-3) cells were treated with various concentrations of A. muricata leaf extract and were given cell-counting kit (CCK-8) reagent.

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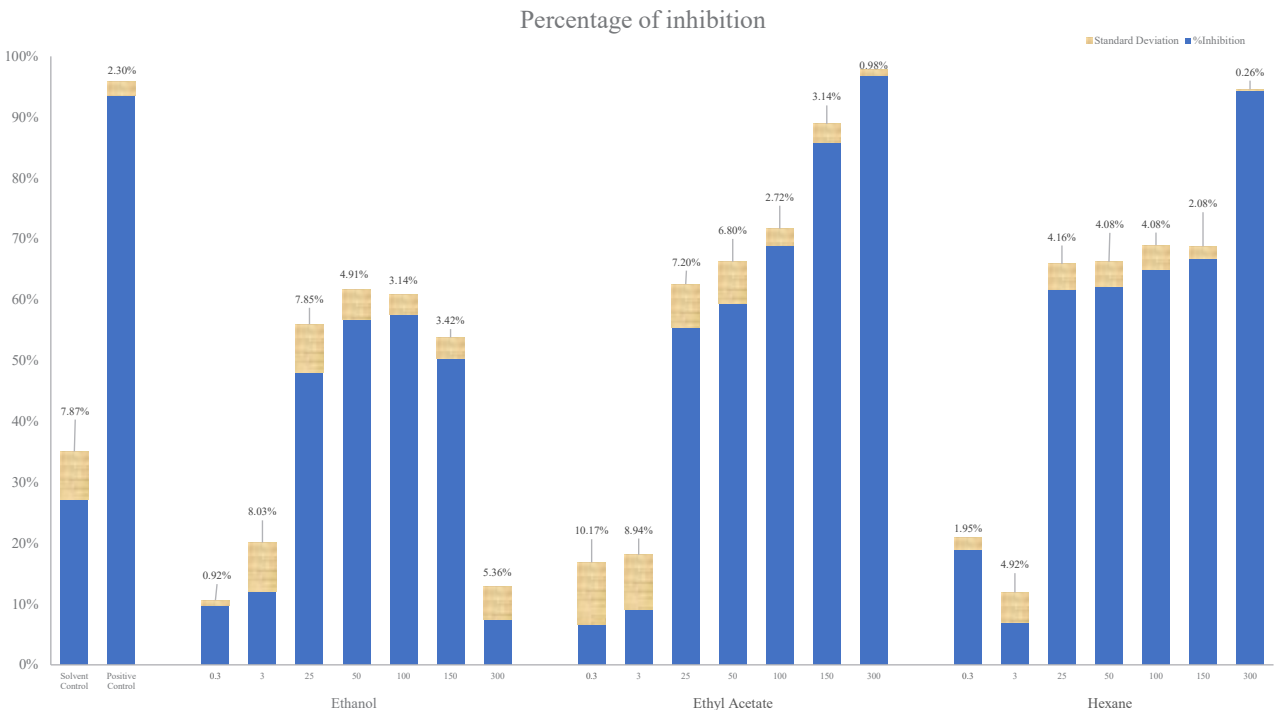

Figure 2. Ethanol, ethyl acetate and hexane fractions of A. muricata extract and the average inhibition percentage of human oral squamous carcinoma (HSC-3) cells after being treated with the test materials.

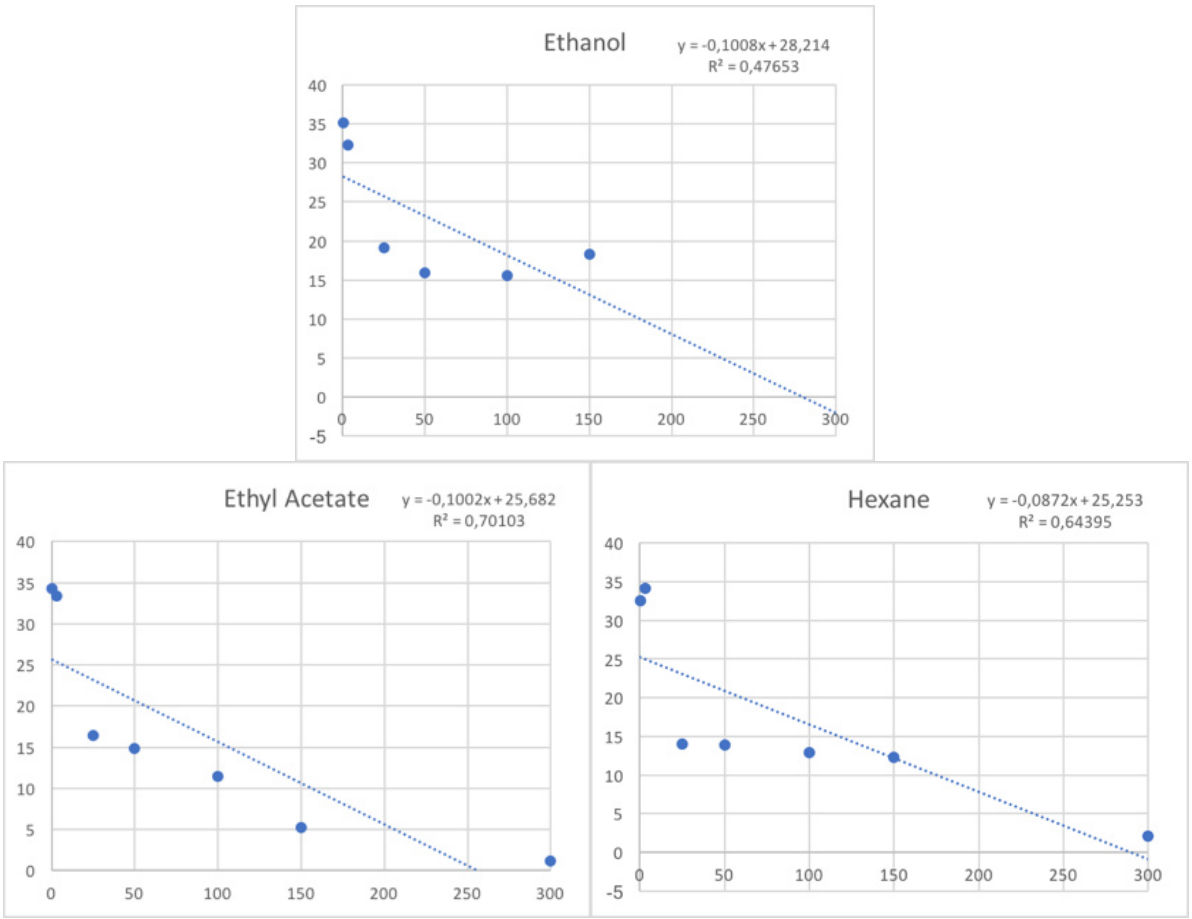

Figure 3. Linear regression formula to calculate the IC_{50} values of each fraction.

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concentrations increase, the resulting colour is lighter in the well. The darker the resulting colour, the greater the number of surviving cells.

The results of the mean ± standard deviation of the number of surviving cells can be seen in Table 2. After obtaining the number of surviving cells from each group, the percentage of inhibition was calculated using the formula. The results of the calculation of the percentage of inhibition can be seen in Figure 2. The ethyl acetate and hexane fractions' percentages of inhibition escalate as the concentration increases. However, in the ethanol fraction, the highest percentage of inhibition was at a concentration of 100 µg/mL and decreased at a concentration of 150 µg/mL.

The IC_{50} value can be found to determine at what concentration the extract causes 50% cell death. The higher the IC_{50} value, the less toxic the extract. From the calculation of the number of surviving cells, IC_{50} values were also calculated for the ethanol, ethyl acetate and hexane fraction extracts using the regression formula (Figure 3), and as can be seen in Table 3, the ethyl acetate fraction extract had the lowest IC_{50} value. However, the IC_{50} values of the hexane and ethanol fractions show results that do not differ much from the ethyl acetate fraction. Therefore, the three fractions can be categorised as moderately cytotoxic according to the U.S. National Cancer Institute because they are in the range of 21–200 µg/mL.[20]

Table 2. Viable human oral squamous carcinoma (HSC-3) cells after being treated with various concentrations of each fraction as well as the negative control, solvent control and positive control for 24 hours

Test material: *A. muricata* leaf extracts and controls	Mean ± Standard deviation (cells)
Negative control	36.751 ± 4.317
Solvent control (DMSO 1%)	26.754 ± 2.893
Positive control (DMSO 10%)	2.365 ± 846
Ethanol 0.3 µg/mL	35.114 ± 3.353
Ethanol 3 µg/mL	32.310 ± 2.951
Ethanol 25 µg/mL	19.066 ± 2.886
Ethanol 50 µg/mL	15.900 ± 1.802
Ethanol 100 µg/mL	15.566 ± 1.154
Ethanol 150 µg/mL	18.233 ± 1.258
Ethanol 300 µg/mL	34.013 ± 1.968
Ethyl acetate 0.3 µg/mL	34.299 ± 3.737
Ethyl acetate 3 µg/mL	33.389 ± 3.286
Ethyl acetate 25 µg/mL	16.400 ± 2.645
Ethyl acetate 50 µg/mL	14.900 ± 2.499
Ethyl acetate 100 µg/mL	11.400 ± 1.000
Ethyl acetate 150 µg/mL	5.233 ± 1.154
Ethyl acetate 300 µg/mL	1.181 ± 360
Hexane 0.3 µg/mL	35.749 ± 2.421
Hexane 3 µg /mL	34.183 ± 1.809
Hexane 25 µg /mL	14.066 ± 1.527
Hexane 50 µg /mL	13.900 ± 1.500
Hexane 100 µg /mL	12.900 ± 1.500
Hexane 150 µg /mL	12.233 ± 763
Hexane 300 µg /mL	2.087 ± 95

Table 3. IC_{50} value of A. muricata leaf extracts

A. muricata leaf extract	IC_{50} value (µg/mL)
Ethanol	101.32
Ethyl acetate	76.66
Hexane	84.14

DISCUSSION

The chemical compounds in the *A. muricata* are known to show bioactivity, such as antioxidant, antimicrobial, anti-inflammatory and cytotoxic properties, against cancer cells.[8] A previous study by Qorina et al. shows that ethanol, ethyl acetate and hexane fractions of *A. muricata* extract have high cytotoxic activity against HeLa cells and have an IC_{50} value of under 20 µg/mL.[12] The results of the phytochemical tests that have been carried out in the study show that saponin and tannin compounds are present in the ethanol fraction, alkaloid and flavonoid compounds are in the ethyl acetate fraction and triterpenoid and steroid compounds are in the hexane fraction. This may have been caused by the different polarities of the solvents used.[21]

The results of the cytotoxicity evaluation using the CCK-8 assay show that the ethyl acetate and hexane fraction extracts have a cytotoxic effect on HSC-3 cells that increases with the concentration of the extracts. However, the ethanol fraction extract had the greatest cytotoxic effect at a concentration of 100 g/mL, which started decreasing at a concentration of 150 µg/mL. At the concentration of 300 µg/mL, the number of viable cells in the ethanol fraction approached the negative control. From these results, it can be seen that the hexane fraction extract contains steroid and triterpenoid compounds, which can produce almost the same cytotoxic activity as the ethyl acetate fraction. In the ethanol fraction extract, there are saponin compounds that are known to be involved in DNA replication, prevent cancer cell proliferation pathways and inhibit cancer cell proliferation by stopping the cell cycle in the G1/S and G2/M phases.[13] Meanwhile, the ethyl acetate fraction extract contains alkaloids and flavonoids.

In previous studies on the cytotoxicity of *A. muricata*, ethanol, ethyl acetate and hexane fractions against HeLa cell lines proved that the three fractions were strongly cytotoxic, with IC_{50} in the ethanol fraction of 5.91 µg/mL, in the ethyl acetate fraction of 7.56 µg/mL and in the hexane fraction of 8.39 µg/mL.[12] The three values of IC_{50} in this study are close to the IC_{50} value of the positive control, namely Cisplatin (IC_{50} value: 1.78 µg/mL). Cisplatin is one of the well-known chemotherapy drugs and has been used in the treatment of various cancers, one of which is head and neck cancer. The mode of action of this drug is related to its ability to cross-link with purine bases in DNA, interfere with DNA repair mechanisms, cause DNA damage and induce cancer cell apoptosis. However, for the treatment of head and neck cancer, Cisplatin is not an

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effective drug, so it must be combined with various other drugs, such as Doxorubicin, Methotrexate, Vinblastine and others. In addition, Cisplatin can cause various side effects, such as kidney problems, decreased immunity to infection, bleeding and others.[22]

In addition, previous studies also showed an increase in the percentage of inhibition in the three fractions along with an increase in the concentration of *A. muricata*.[12] However, the results of the ethanol fraction in this study were different. The percentage of inhibition of the ethanol fraction of *A. muricata* at a concentration of 100 µg/mL was 57.64% and then decreased with increasing concentration. The difference in results may have been caused by the different cell lines or the extraction method used; the research conducted in 2020 used the vacuum dry method. In addition, according to several other previous studies, *A. muricata* was cytotoxic to the HepG2 liver cancer cell line, the ethyl acetate fraction was cytotoxic against leukaemia cancer cell lines U937 and lung cancer A549 and the hexane fraction was cytotoxic against HeLa cell lines and pancreatic cancer Capan-1.[14] In conclusion, there was cytotoxic activity of *A. muricata* leaf extract in the ethanol, ethyl acetate and hexane fractions against HSC-3 cell lines. Extracts with the ethyl acetate solvent had the highest cytotoxic activity, making it the best solvent.

However, this study required the use of 1% DMSO due to the limitations associated with the dissolution of the extract. The study was also performed twice and in stages, adding three sets of extract concentrations to obtain the IC_{50} values. In addition to the secondary metabolite content in the extracts, there are other factors that can affect the efficacy of *A. muricata* ethanol, ethyl acetate and hexane fractions, such as biological variations of the plants, the plants' habitats and the leaves used. Environmental factors, such as humidity, temperature, types of nutrients from the soil and solar radiation, also play an important role in physiological functions, anatomical forms and plant life cycles.[23] Further research is needed on the cytotoxic ability of *A. muricata* leaf extract against HSC-3 cells in order to learn the optimal dose, long-term safety and side effects on normal cells. Quantitative research is needed to phytochemically test and determine the levels of active compounds contained in the extract.

This research shows that three extracts of *A. muricata* have a moderate cytotoxicity against HSC-3 cancer cells, with the greatest cytotoxicity in the ethyl acetate extract (IC_{50} = 76.66 µg/mL). Thus, extracts of *A. muricata* need to be researched further to find the most effective concentration with the potential to be developed as a novel alternative therapy for oral cancer due to its antineoplastic agents.

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