

RESEARCH ARTICLE

The Effect of Nutmeg Seed (*M. fragrans*) Extracts Induces Apoptosis in Melanoma Maligna Cell's (B16-F10)

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Abstract

BACKGROUND: Nutmeg (*Myristica fragrans* H.), one of native plants of Maluku Indonesia, has long been used as traditional medicines especially to treat tumors, externally to treat skin infections. *M. fragrans* also has important biological activities as anticancer. However, antimelanoma activity of *M. fragrans* remains unknown. The aim of this study is to compare *M. fragrans* extracts as anticancer on melanoma B16-F10 cells by inducing apoptosis.

METHODS: *M. fragrans* seed was extracted with ethanol then fractionated with n-hexane, ethyl acetate, and n-butanol. B16-F10 melanoma cells were cultured and treated with various doses and tested using resazurin reduction assay. Apoptosis signalling via caspase-3 was measured by using western blot.

RESULTS: The extract and fractions of *M. fragrans* reduced viability of cells with IC₅₀ value for ethanol extract 21.83 µg/mL, ethyl acetate fraction 21.66 µg/mL, n-hexane fraction 47.53 µg/mL, and n-butanol fraction >1,000 µg/mL. The active fraction of ethyl acetate induced apoptosis via caspase-3 proteins similar with cisplatin as positive control in B16-F10 cells at 10 hours treatment.

CONCLUSION: Taken together, *M. fragrans* ethyl acetate fraction has the highest IC₅₀ than n-hexane and n-butanol fractions that significantly inhibited B16-F10 cell proliferation by inducing apoptosis via caspase-3. It provides the insight that it has the most potential activity as a chemopreventive agent for addressing melanoma skin cancer.

KEYWORDS: *M. fragrans*, apoptosis, fraction, melanoma

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Introduction

Melanoma is a type of skin cancer that starts from abnormal proliferation of melanocyte cells as a melanin producer.(1) This cancer has a high level of malignancy and is dangerous to the point of causing death.(2,3) Melanoma cancer cases worldwide reach >100.000 cases each year and as many as 66,000 are fatal.(4,5) Lifestyle and genetic factors are risk factors that contribute to the development of melanoma. (6) Exposure to ultraviolet (UV) radiation is the main

risk factor for melanoma cancer which can cause genetic changes in melanocytes and ultimately damage the DNA of skin cells, damage genes that regulate cell growth and division, resulting in the formation of malignant cells.(7)

Several melanoma therapy methods have been performed, such as surgical operations, chemotherapy, and radiotherapy.(8) Those treatment approaches had not only given side effects like skin damage, also caused a decrease in the effectiveness of radiation along with the number of cancer cells, and can even lead to drug resistance to chemotherapy.(9) Thus, it is necessary to develop

alternative medicine as a chemopreventive agent, one of which is derived from natural ingredients which have the activities to inhibit the abnormal growth of melanoma cells, and have lower side effects and are safer in their use.

Natural ingredients, especially herbal plants, is generally available in tropical countries, especially in Indonesia. Herbal compounds such as Curcumin, Demethoxycurcumin and Bisdemethoxycurcumin have cytotoxic activity against MCF7 breast cancer cells.(10) Nutmeg (*Myristica fragrans* H.) is one of the native plants of the Maluku Islands, Indonesia with high economic value with the largest production level of up to 80% to supply the world's nutmeg as spices.(11,12) As herbal medicinal product, *M. fragrans* has long been used as medicines for digestive problems, such as flatulence and diarrhea. Interestingly, *M. fragrans* can be used to treat tumors, externally to treat skin infections.(13) *M. fragrans* has important biological activities, such as anticancer (13-15) in addition also have antioxidants, anti-inflammatory, antibacterial (16), analgesics (17), and antidiabetic (18). However, its effectivity on melanoma cancer is still limited.

Taken together, detail informations about the activity of *M. fragrans* on B16-F10 melanoma cancer cells and its molecular mechanism has not been elucidated, therefore in the present study, we would like to compare *M. fragrans* extracts as anticancer on melanoma B16-F10 cells by inducing apoptosis.

Methods

***M. fragrans* Seed Extraction and Fractionation**

M. fragrans seed samples were obtained from Sarjo Village, Pasangkayu Regency, West Sulawesi Province and were identified at the Celebense Herbarium at Tadulako University, Palu, Central Sulawesi. Dry *M. fragrans* seed (4.1 kg) were extracted by maceration method using ethanol redistillation solvent for 8x24 hours. Filtrate was then concentrated with a rotary evaporator (Cat. No. R100, Buchi, Flawil, Switzerland) at a temperature of 45°C. Concentrated ethanol extract was fractionated for 24 hours sequentially with redistillation solvents of n-hexane, ethyl acetate, and n-butanol. Then the fractions of n-hexane, ethyl acetate, and n-butanol were concentrated to evaporate the solvent using a rotary evaporator at a temperature of 45°C.

B16-F10 Melanoma Cell

B16-F10 cell was acquired from American Type Culture Collection (ATCC® CRL-6475™, Manassas, Virginia,

USA). The medium used was Dulbecco's Modified Eagle's Medium (DMEM high glucose) (Cat. No. 11965118, Gibco, New York, USA) added with 10% Fetal Bovine Serum (FBS) (Cat. No. 10082147, Gibco) and 1% Penicillin-streptomycin (Cat. No. 15140112, Gibco). Cell incubation was conducted at 37°C in a 5% CO₂ incubator (Cat. No. 8000DH, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Antiproliferation Assay

B16-F10 melanoma cells (1.7x10⁴ cells/well) were cultured in 96-well microplates (Cat. No. 701001, Nest, Jiangsu, China) for 24 hours. Then, the cells were treated with *M. fragrans* ethanol extract, n-hexane fraction, ethyl acetate fraction and n-butanol fraction (variations in concentrations of 1,000; 500; 250; 125; 62.50; 31.25; 15.63; 7.81 µg/mL), cisplatin (Cat. No. C2210000, EDQM, Starsbourg, France) 42.97 µg/mL as a positive control, DMSO (Cat. No. 102952, Merck Millipore, Massachusetts, USA) 2% as a solvent control, DMEM as a media control, and DMEM with untreated cells, incubated for 48 hours. After that, Presto blue reagent (Cat. No. A13261, Thermo Fisher Scientific) was added to each well, incubated for 2 hours, then the absorbance was measured at 570 nm and reference at 600 nm by using multimode reader (Cat. No. M200 Pro, Tecan, Männedorf, Switzerland), and their cell viability and IC₅₀ values were calculated.

Western Blot

B16-F10 melanoma cells (1.0x10⁵ cells/well) were cultured in a 24-well microplate (Cat. No. 702001, Nest) for 24 hours. Then, the cells were treated using an active fraction of ethyl acetate of *M. fragrans* with a concentration variation of 125; 62.50; 31.25; 15.63 µg/mL, and cisplatin 42.97 µg/mL as positive control, incubated for 10 hours. The cells were harvested and processed *in vitro*. Lysis buffer was added as much as 100 µL/well. Lysis buffer contains (1:1) RIPA buffer (Cat. No. 89900, Thermo Fisher Scientific) : sample buffer (Cat. No. 84788, Thermo Fisher Scientific), protein inhibitor (Cat. No. S8820, Sigma Aldrich, Wisconsin, USA), and DTT (Cat. No. V3151, Sigma Aldrich). Samples were heated at 96°C for 5 minutes and deep freeze for 2-3 minutes was carried out. Ten µL lysate protein was separated with SDS-PAGE (Cat. No. A25977, Thermo Fisher Scientific) for 120 minutes. The gel produced from SDS-PAGE was further transferred to the 0.45 µm pore size of nitrocellulose membrane (Cat. No. 10600002, GE Healthcare, Illinois, USA) for 30 minutes by using blotting equipments (Cat. No. B1000, Thermo Fisher Scientific). The membrane was washed with Phosphate Buffer Saline Tween-20 0.1%

(PBST) (Cat. No. 18912014, Gibco), incubated using a 0.25% BSA blocking solution (Cat. No. 1.12018, Sigma Aldrich) for 30 minutes. Membrane immunoblotting was carried out using primary antibodies of caspase-3 (Cat. No. #14220, Cell Signaling, Massachusetts, USA) with a dilution of 1:300, then it was incubated overnight at 4°C. The membrane was washed 3x with 0.1% PBST, incubated using appropriate secondary antibodies (anti-rabbit (Cat. No. C90501-02, Li-Cor, Nebraska, USA) with a 1:10,000 dilution. Proteins in membrane was detected with LI-COR Odyssey (CLx Imaging System, Li-Cor, Nebraska, USA). The thickness of the protein band was analyzed using ImageJ (NIH) software from National Institutes of Health (Bethesda, Maryland, USA). Stripping on the membranes was carried out and then it was incubated using Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (Cat. No. AF5718-SP, RnD System, Minnesota, USA) as an internal control.

Results

M. fragrans Fraction and Extract Yield

The concentrated ethanol extract obtained from the maceration process was 2.30 kg from 4.1 kg dry *M. fragrans* seeds with a yield value of 56.10%. The amount obtained from the fraction of n-hexane was 521.40 g, ethyl acetate at 602.50 g and n-butanol at 401.80 g. The highest yield was obtained using ethyl acetate extraction solvent with a yield value of 26.20%.

M. fragrans Extract and Fraction Cytotoxicity in B16-F10 Cell

The results showed after 48 hours of treatment, there was a morphological change that showed cell death in ethanol extract, ethyl acetate fraction and n-hexane fraction, but no morphological change in the n-butanol fraction (Figure 1B). Significant inhibition was showed at concentration of 1,000; 500; 250; 125; 62.50 and 31.25 µg/mL for ethanol extract and ethyl acetate fraction, followed by n-hexane fraction at concentration of 1,000; 500; 250; 125 and 62.50 µg/mL (Figure 1). Percentage of cell inhibition was calculated from liner regression equation by comparing with the 2% DMSO as a solvent control. The ethyl acetate fraction of *M. fragrans* significantly inhibited B16-F10 cell proliferation with IC₅₀ value of 21.66 µg/mL, followed by ethanol extract 21.83 µg/mL and n-hexane fraction with IC₅₀ value of 47.53 µg/mL. n-butanol fraction did not show cytotoxicity against B16-F10 cell with IC₅₀>1,000 µg/mL.

Figure 2 shows the results from the resazurin reduction assay at the one time points (48 hours) post-treatment with the different concentration where there was a significant decrease in cell viability for ethanol extract and ethyl acetate fraction (rows A and C down to <20%), and fraction n-hexane (row D down to <40%) compared to cisplatin as positive control (60-80% cell viability), whereas n-butanol did not show a decrease in cell viability. The absolute IC₅₀ value analysis used GraphPad Prism 8 software. The results showed that the ethyl acetate fraction (18.05 µg/mL) had the highest IC₅₀ value, followed by ethanol extract (27.14 µg/mL) and n-hexane fraction (52.67 µg/mL). Ethanol extract and ethyl acetate fraction were also tested for their cytotoxic activity against MCF7 (Supplementary Figure 1).

Induction of Apoptosis by Active Fraction of *M. fragrans* Ethyl Acetate

The cytotoxic assay results showed potent inhibitory activity of cell proliferation by ethyl acetate fraction of *M. fragrans* against B16-F10 cells. Therefore, the caspase-inducing activity of the fraction was examined in the B16-F10 cells using western blot. The cells were treated using an active fraction of ethyl acetate of *M. fragrans* with a concentration variation of 125; 62.50; 31.25; 15.63 µg/mL, and cisplatin 42.97 µg/mL as a positive control. The specific marker for apoptosis used caspase-3 with GAPDH as an internal control.

As shown in Figure 3, expression protein levels of caspase-3 were similar with cisplatin as a positive control in B16-F10 cells at 10 hours, including caspase 3 fragments with 37 kDa and cleaved caspase 3 with 19 kDa. These results suggested that the B16-F10 melanoma cancer cell proliferation inhibition by ethyl acetate fraction of *M. fragrans* induced apoptosis via caspase-3 proteins.

Discussion

This research focuses on the effect of *M. fragrans* extract and fraction on B16-F10 melanoma cancer cells and its molecular mechanisms through induction of apoptosis. We also had observed that nutmeg extract and fractions reduced significantly cell viability in others cells cancer like MCF7 (Supplementary Figure 1). Our results showed that the ethanol extract, ethyl acetate fraction and n-hexane fraction suppresses the growth of B16-F10 melanoma cancer cells that seen from cell morphology changes when compare with controls (Figure 1). The morphological changes of cancer cells correlated with cellular proliferation.(19)

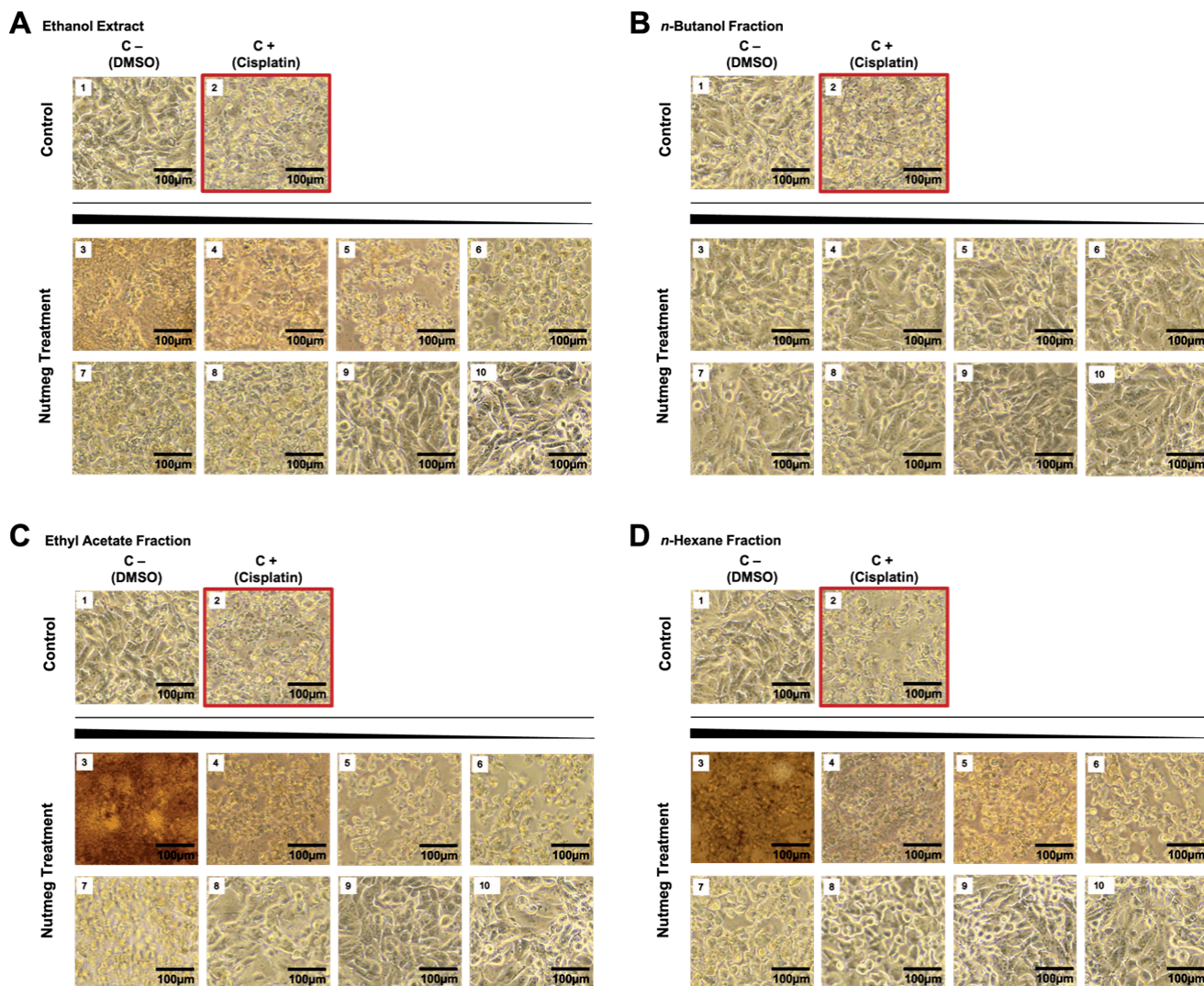


Figure 1. Morphological changes of B16-F10 cells in *M. fragrans* ethanol extract and fraction after antiproliferation assay for 48 hours incubation. 1: solvent control; 2: positive control; 3: concentration of 1,000 µg/mL; 4: concentration of 500 µg/mL; 5: concentration of 250 µg/mL; 6: concentration of 125 µg/mL; 7: concentration of 62.50 µg/mL; 8: concentration of 31.25 µg/mL; 9: concentration of 15.63 µg/mL; 10: concentration of 7.81 µg/mL.

The solvent of each extract has a different polarity so the secondary metabolites contained in the extract appropriate with the solvent polarity.(20) Solvent polarity from polar to non-polar: ethanol > ethyl acetate > n-hexane. Ethyl acetate is a semi-polar solvent that can collect secondary metabolites, both polar and non-polar. From the extraction results, the ethyl acetate fraction has the highest yield value, this indicates that many compounds are collected to the ethyl acetate fraction which have an effect on inhibiting cell proliferation.

The effect of ethyl acetate fraction of *M. fragrans* seeds showed the highest cytotoxic activity, followed by ethanol extract and the n-hexane fraction while the n-butanol fraction did not show cytotoxic activity against B16-F10 melanoma cancer cells. Ethyl acetate fraction

could significantly inhibit B16-F10 cell proliferation with IC_{50} value of 21.66 µg/mL smaller than cisplatin as a positive control. This means that ethyl acetate fraction has higher cytotoxic activity than the cisplatin because at smaller concentrations this fraction has been able to inhibit of 50% on the growth of B16-F10 melanoma cancer cells.

Cell viability and absolute IC_{50} (or the theoretically possible effect) analysis were performed.(21) The data showed that the ethyl acetate fraction had significant decrease in cell viability to >80% with the highest absolute IC_{50} value of 18,05 µg/mL (Figure 2), it means ethyl acetate fraction has strong cytotoxic activity of B16-F10 cells. Based on the cytotoxicity classification, the IC_{50} value is less than <50 µg/mL has strong cytotoxic activity.(22)

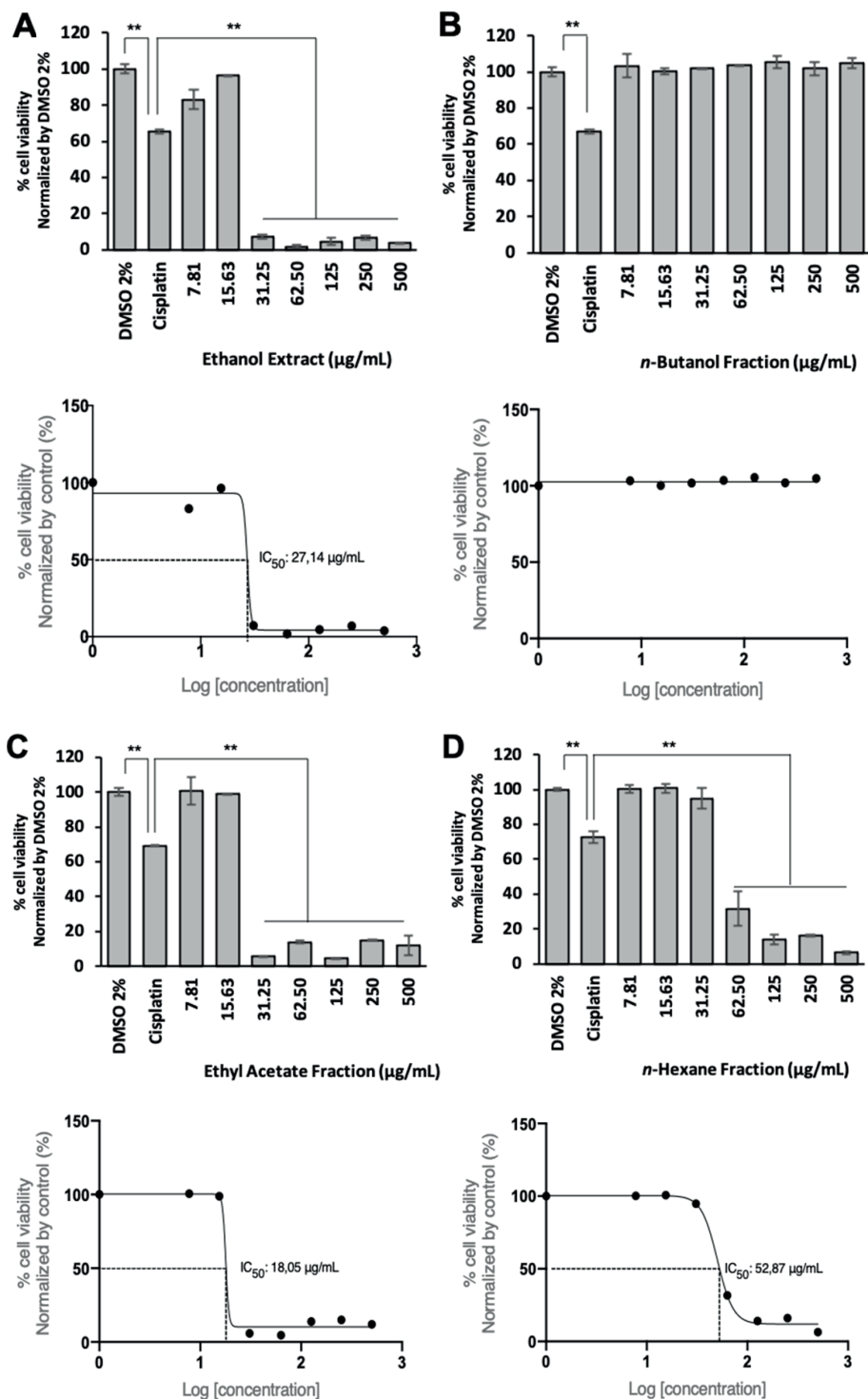


Figure 2. Data of cell viability of *M. fragrans* ethanol extract, n-butanol, ethyl acetate, n-hexane fractions were analyzed and presented as average and standard deviation. **Showed significant difference.

Previous studies reported that ethanol extract of *M. fragrans* with concentration 100 $\mu\text{g/mL}$ can inhibit the growth of 50% A-549 (Lung) cells using SRB assay.(23) However, there has not been a detailed report regarding the fraction shown to have cytotoxic activity. Our results showed that ethanol extract and ethyl acetate fraction were also tested for their cytotoxic activity against MCF7 (Supplementary Figure 1). These indicate that ethanol

extract and ethyl acetate fraction of *M. fragrans* have a promising cytotoxic activity.

Several compounds found in *M. fragrans* ethyl acetate fraction and responsible for cytotoxic activity are lignans, diarylnonanoid and phenylpropanoids.(24-26) Interestingly, we discover an ethyl acetate fraction which has cytotoxic activity. This fact provides the possibility of new active compounds that have the potential to act as

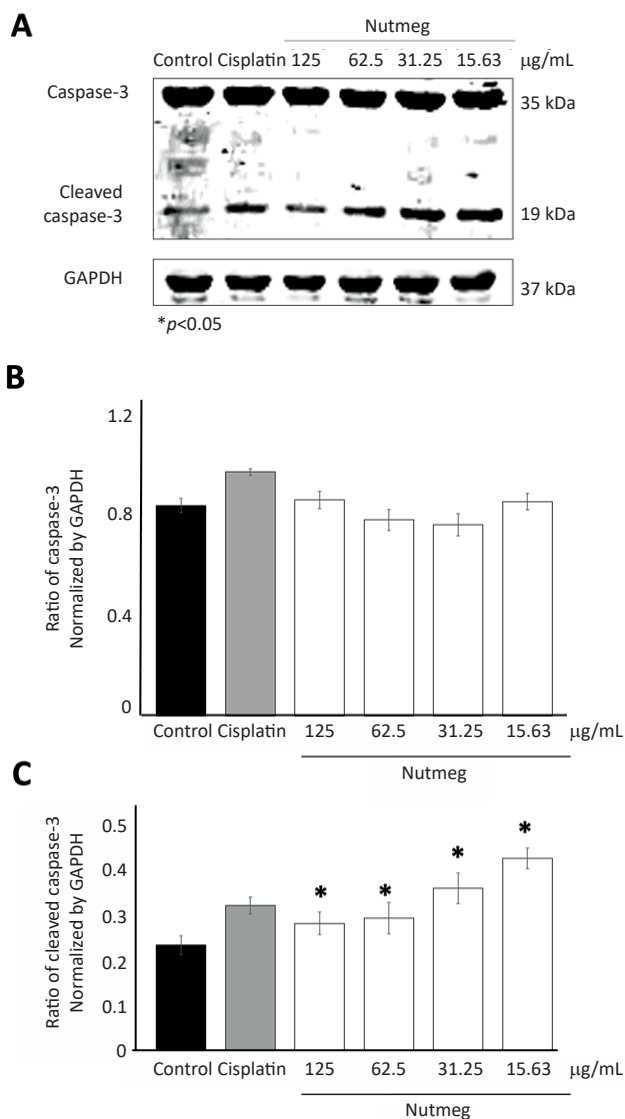


Figure 3. Active fraction of *M. fragrans* ethyl acetate potentially activate caspase-3 proteins in B16-F10 cells. A: Immunoblotting representation of caspase-3; B: Ratio total caspase-3 is normalized by GAPDH. C: Ratio cleaved caspase-3 normalized by GAPDH. Data is presented as mean average with Standard Error Minimum. **p*-value was significant with *p*-value<0.05.

anti-cancer from the ethyl acetate fraction. Also, the effect of nutmeg is widely reported in breast cancer (24,25,27) and other cancers (25,27-30) Using a skin cancer model can provide an alternative use of nutmeg fraction or its active compound as an alternative to skin cancer treatment supplements.

Further research was carried out to find out the molecular mechanism underlying the cytotoxic effect of the active fraction of nutmeg ethyl acetate on B16-F10 cells, one of which was through apoptosis induction. Apoptosis is programmed cell death that can be triggered by endogenous

or exogenous signals.(31) Apoptosis has two main signaling pathways, namely extrinsic and intrinsic apoptosis. Most anticancer effects give rise to intrinsic apoptosis pathways. (32-34) One of the apoptosis signaling pathways is caspase through the activation of caspase-3, in which activation of caspase-3 as an effector of the apoptosis process.(35) Licarin A, a lignan from *M. fragrans* cause autophagy dependent apoptosis in NCI-H23 and A549 non-small cell lung cancer cells where autophagy activation was shown by decreased in p62, increased in levels of LC3II and beclin 1, and arrest of G1. For cell death by apoptosis, it was shown by degradation in procaspase 3, increased in ROS, cleaved PARP, and MMP loss.(28)

Our study shows that *M. fragrans* ethyl acetate fraction can reduce the viability of B16-F10 cells for 10 hours of treatment by inducing B16-F10 cell death through apoptosis via caspase-3 as indicated by the results of western blot. These results are similar with cisplatin as a positive control (Figure 3). However, further research requires for investigating single *M. fragrans* active compounds that play a role in the process of inhibition of B16-F10 melanoma cells.

The drawback of this research is the limited detailed exploration of others apoptosis upstream and downstream mechanisms like involvement of beclin, PARP, and other potential mechanisms which needs to be explored and investigated.

Conclusion

M. fragrans ethyl acetate fraction has the highest IC50 than n-hexane and n-butanol fractions that significantly inhibited B16-F10 cell proliferation by inducing apoptosis via caspase-3. It provides the insight that it has the most potential activity as a chemopreventive agent for addressing melanoma skin cancer.

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Authors Contribution

S and SS performed the experiments, analyzed the data and drafted the manuscript. RL and US designed the experiments, supervised the work and wrote the manuscript. EJ, YSP, NS, AK and HG analyzed the data and drafted the manuscript.

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