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Cytotoxic Activity of Volatile Compounds in Cymbopogon nardus' Essential Oils

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ABSTRACT

Abstract: *Plants produce a variety of secondary metabolites, one of which is essential oils that contain a variety of volatile compounds and are useful for humans. Cymbopogon nardus contains volatile compounds that can inhibit the proliferation of cancer cells. This research aims to explore the antiproliferation activity of C.nardus' essential oils with different concentrations against breast cancer Michigan Cancer Foundation-7 (MCF-7) cells. Antiproliferation test was carried out with total cells method using trypan blue and cells were calculated using a microscope. Gas Chromatography-Mass Spectrometer (GC-MS) was performed to identify the volatile compounds. The results showed that the Inhibitory Concentration 50 (IC_{50}) value was 359.6 ppm with an inhibition percent of 44.49% at 170 ppm. Meanwhile, inhibition percent against Vero normal cell was 29.04%, compared to Doxorubicin 35.23%. The dominant volatile compound in C. nardus' essential oil were Geraniol and Citronellol.*

Abstrak: Tanaman menghasilkan berbagai macam metabolit sekunder, salah satunya dalam bentuk minyak atsiri yang mengandung berbagai macam senyawa volatil yang berguna bagi manusia. *Cymbopogon nardus* mengandung senyawa volatile yang dapat menghambat proliferasi sel kanker. Penelitian ini bertujuan untuk mengetahui aktifitas antiproliferasi minyak atsiri *C. nardus* dengan beberapa konsentrasi terhadap sel kanker payudara *Michigan State Foundation-7* (MCF-7). Uji antiproliferasi dilakukan dengan metode total sel menggunakan *trypan blue* dan sel dihitung menggunakan mikroskop. *Gas Chromatography-Mass Spectrometer* (GC-MS) dilakukan untuk mengidentifikasi senyawa volatil. Hasil menunjukkan bahwa konsentrasi penghambatan 50 (IC_{50}) sebesar 359.6 ppm dengan persen penghambatan sebesar 44.49% pada konsentrasi 170 ppm. Sementara itu, persen ihibisi terhadap sel normal Vero sebesar 29.04%, dibandingkan dengan Doksorubicin yang sebesar 35.23%. Senyawa volatil dominan yang terdapat pada *C. nardus* adalah Geraniol dan Citronellol.

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A. INTRODUCTION

Cancer ranks as the main purpose of death and an essential barrier to growing existence expectancy in every country of the world. In line with estimates from the World Health Organization (WHO) in 2019, cancer is the primary or second leading cause of death before the age of 70 years in 112 of 183 countries. Cancer is an ailment that results from genetic or epigenetic alterations within the somatic cells and has ordinary cell increases which can be spread to other body components (Saini et al., 2020). Breast cancer in women ranks first in the most common cancers (11.7%) with a mortality rate of 6.9% (Sung et al., 2021). Breast cancer is metastatic cancer and may typically transfer to distant organs including the bone, liver, lung and brain, which particularly accounts for its incurability. Most breast cancers arise in women and the wide variety of instances is one hundred times higher in women than that in men. Breast tumors usually start from ductal hyperproliferation, after which grow to be benign tumors or even metastatic carcinomas after continuous stimulation through diverse carcinogenic factors (Sun et al., 2017).

Most cancer cells are very similar to cells of the organism from which they originated and have comparable (but not identical) DNA and RNA. This is the reason why they are not very often detected through the immune system, in particular, if it is weakened. Few attempts that are normally used for most cancer treatment therapies are surgical procedures, radiation therapy, and chemotherapy (Bruton et al., 2005). Recent procedures in the management of breast cancer are gene therapy, oncogenes inactivation, augmentation of tumors suppressor genes, cell-target suicide, chemoprotection method, virus-mediated oncolysis and immunomodulation (Sharma et al., 2010). These strategies purpose to destroy most cancer cells and manipulate the growth of tumors within the breast tissue affected by cancer as well as control tumors increase in different tissues that are not affected by most cancers.

Treatment that is carried out in the early stages of cancer is surgery combined with other treatments to reduce recurrences such as radiation therapy, chemotherapy, hormone therapy and targeted drugs. For cancers that have undergone metastatic, the treatment is carried out more with systematic therapies including chemotherapy, hormone therapy and targeted drugs. The purpose of surgery is to remove cancer and determine the stage. This surgery is called a mastectomy where only tissue is affected by cancer and a slight edge of normal tissue that omitted/removed. However, this mastectomy is not recommended for cancer with a large tissue ratio and multicentric cancer. Regular surgery followed by radiation, patients who have undergone radiation are not recommended to perform a mastectomy. Radiotherapy is the use of high-energy particles to kill cancer cells and is commonly used after surgery to destroy cancer cells remaining in the breast, walls chest or lower area of the arms. Radiotherapy is non-selective that can also kill normal cells and have no effect on sufferers who have undergone hormone therapy for 5 years. Chemotherapy uses drugs that work by attacking cancer cells. Long-term use of chemotherapy drugs causes cardiomyopathy and congestive heart failure. Whereas hormone therapy works by blocking the body's natural

hormones or lower hormone levels which can trigger the growth of some cancers. Side effects of this hormone therapy include the risk of blood clots, bone thinning and vision changes. Targeted drugs work by attacking specific molecules in cells that have the potential to become more active in cancer cells. An example of these drugs are Trastuzumab and Herceptin. Side effects of using these drugs include heartbeats, shortness of breath, fever to bleeding that is not ordinary (Mc Donald et al., 2014).

To reduce the side effects of the use of such cancer drugs, now widely used substitutes in the form of herbal medicines. Pharmacology characteristic of common herbal ingredients found in its essential oils which contains a wide range of potential secondary metabolite compounds inhibits the proliferation of cancer cells. Essential oils are hydrophobic so it can enter the cell membrane and damage the cell.

Essential oils are secondary metabolites with a key function in plant protection, consisting commonly of terpenes with a volatile nature and a various array of chemical structures (Blowman et al., 2018). Essential oils have been proven to own anticancer properties via numerous mechanisms, including cancer preventative mechanisms, as well as appearing at the established tumors cellular itself and interaction with the microenvironment (Sitarek et al., 2017). Key hallmarks of cancer embrace resisting cell death, sustained proliferative signal and evading growth suppressors. therefore, therapeutic techniques focused on inducing apoptosis and cell arrest are of clear importance. Essential oils were proven to result in both the intrinsic (or mitochondria-based) and extrinsic (or death receptor-dependent) apoptosis pathways (Amin et al., 2009).

C. nardus is known to act as an insect repellent with some studies as antiproliferation of cancer cells (Akhila, 2010). There is still few research on antiproliferation activity in *C. nardus*. Among them, it is reported that essential oils from *C. nardus* have antiproliferation potential and contain active compounds in the form of geraniol and citronellal. *C. nardus* essential oil is also reported to have cytotoxic activity against MCM-B2 breast cancer cells (Hasim et al.,2020).

To the best of our knowledge, cytotoxic potential and antiproliferative activity of *C. nardus* essential oil against MCF-7 breast cancer cells in vitro have not been investigated previously. Therefore, the present study was focused on understanding *C. nardus* essential oil as an antiproliferative substance and figuring out its volatile compound.

B. RESEARCH METHOD

Material

The ingredients used in this study were *C. nardus* leaves taken from the Biopharmaceutical Cultivation Conservation Unit Center for Biopharmaceutical Studies Tropics with latitude (S) 6°34'37.95", longitude 106°47'20.37" and altitude (M) 238, Bogor, Indonesia. Other materials were n-hexane, water, seawater, tween 80, Artemia salina Leach, distilled water, MCF-7 cell, Vero cell, trypan blue, DMEM (Dulbecco's Modified Eagle's Medium), trypsin, gentamicin, FCS (Fetal Calf Serum), DMSO (Dimethyl Sulfoxide) and doxorubicin.

Distillation of Essential Oils

Extraction of essential oil was carried out by the method of steam distillation in a single way. A total of 3000 grams of small sliced sample were put into the distiller steam and added to distilled water in a ratio of 1:5 (sample: water). Distillation was carried out for 4 hours at a temperature of 100-105°C. Distilled essential oils then accommodated and stored at 4°C for further analysis. The percent of oil yield obtained is calculated by the formula:

$$\text{Yield Percent} = \frac{Oil\ Weight}{Dry\ Sample\ Weight}\ x\ 100\%$$

Cytotoxicity Test of Essential Oils with Brine Shrimp Lethality Test (BSLT)

Artemia salina Leach shrimp eggs and aerators were put into a container and seawater was added, allowed to stand for 24 hours until it turned into larvae. A solution of essential oil extract was dissolved in seawater with tween 80. The concentrations of the extract solution made were 1000, 500, 100, 10µg/mL and 0µg/mL (control). The test plate was prepared and stamped with seawater as much as 2 mL and then marked with a marker. A total of 1 mL of seawater was put into the test plate and then added 10 shrimp larvae and 0.5 mL of test solution for concentrations of 1000, 500, 100, 10µg / mL and 0µg / mL (control) respectively. The larvae were incubated for 24 hours and the number of dead larvae was calculated. Control was carried out by the same procedure without the addition of extracts. The LC_{50} value was determined using probit analysis using SPSS 16.0.

Antiproliferation Test of Essential Oil against MCF-7 cells and Vero

Preparation of Media and Extracts. MCF-7 cells and Vero cells were grown on 850µL of DMEM growing media in microplates of 24 wells to which 10µL of antibiotics (gentamicin), 10µL of fungizon, 30µL of FCS and extracts were added. The stock solutions of the extract with 5 different concentrations based on the results of the BSLT test were made using 10% DMSO as the solvent. Variations in extract concentrations were determined based on LC_{50} values and tested for three repetitions.

Cell Planting. The suspension of MCF-7 and Vero cells was pre-liquefied and homogenized with a vortex. Cell planting was carried out on a 24-well tissue culture plate containing a growth medium with 5 different concentrations of extracts, without the addition of essential oils as a negative control and doxorubicin as a positive control. A total of 50µL cell suspensions were added to each hole. Performed as many as three repetitions. The cell suspension was grown by incubating it in a 37°C incubator (5% CO_2).

Harvesting and Counting cells. Cell harvesting was carried out when the cells in the control hole had grown optimally covering about 70% of the hole surface (confluence) or approximately after 3 days. Cell suspensions were homogenized using micropipettes by sucking and removing them. A total of 80µl of homogeneous cell suspensions were placed in a microplate that already contains 20µl of trypan blue dye, so that the cells are visible under the microscope, then homogenized. The suspension was dripped on the Neubauer hemacytometer and a count of the number of cells under a light microscope with a

magnification of 100x was carried out. The calculated cells were those that are in the middle box of the counting room. All cells were counted, both living and dead cells

The formula used to calculate the percentage of activity cell growth and inhibition are as follows :

$$\% \text{ growth activity} = \frac{Average\ number\ of\ treatment\ cells}{Average\ number\ of\ negative\ control\ cells}\ x\ 100\%$$

$$\% \text{ of inhibitory activity} = 100\% - (\% \text{ growth activity})$$

GC-MS Essential Oil Analysis

Analysis of essential oils volatile compounds using the GC-MS tool Agilent Technologies with selective mass detectors. A total of 20µL of oil plant essentials were dissolved into 2000µL of n-hexane solvent (1:100 v/v). Sample separated using HP INNOWAX capillary columns (30 m x 0.25 mm, 0.25µm film thickness) with helium gas as a carrier (0.6 mL/min). Temperature the initial for the analysis was 60°C and raised to 150°C in increments by 2°C/min for about 1 minute, then increased to 210°C with a rate of increase of 20°C/min and kept constant for 10 minutes. The ratio of the separation rate was 250:1. The temperature at injection was 250°C by 1µL of essential oils for injection.

Statistical Analysis

Cytotoxicity data of LC_{50} BSLT were obtained using SPSS probit analysis 16.0, and IC_{50} inhibition activity of essential oils on cells was analyzed using regression analysis (log it). ANOVA analysis was carried out through the Duncan test at $p \leq 0.05$ to see the differences in each treatment.

C. RESULTS AND DISCUSSION

1. Essential Oils Productivity

The yield of essential oils produced was less than the previous studies. This difference was due to several factors such as the condition of the material and the distillation time. The condition of the withered material will produce a higher yield of essential oils compared to fresh ingredients because the moisture content in the material glands has been reduced and extraction is easier to do. A longer distillation time will result in more essential oil yields because volatile compounds that are difficult to evaporate will require a longer heating time to evaporate, in addition to the growing place and soil conditions also affect the yield of essential oils (Cassel & Vargas, 2013).

Table 1. Distilled Essential Oils Yield

Plant	Yield Experiment (%)	Yield Reference (%)	Reference
C. nardus	0.4	0.79	Cassel and Vargas 2013

2. Cytotoxicity Test of Essential Oils with BSLT

BSLT is an initial pharmacological screening method that is easy and relatively inexpensive and does not require certain specialization in its implementation (Ghosh et al. 2015). BSLT can be used as a simple bioassay to examine the toxicity level of a compound by determining the value of LC_{50}. The test was carried out using *Artemia salina* in the napoli (larval) phase. The parameter measured was the mortality rate of shrimp larvae due to the administration of test solutions, in this study it was essential oil. The cytotoxicity of essential oils was determined in the LC_{50}. The results of the BSLT test showed that *C. nardus* had toxic potential because the LC_{50} calculation results were obtained at 98.5 ppm. Meyer et al. (1982) classify the toxicity level of a compound that is said to have potential bioactivity if the LC_{50} is less than 1000 ppm so that the essential oils of *C. nardus* had the potential to be toxic and had the potential to be antiproliferation compounds for cancer cells.

3. Antiproliferation Test of Essential Oil against MCF-7 cells and Vero

The antiproliferation activity of *C. nardus* essential oil was tested against MCF-7 cancer cells. Using the total cell method according to Priosoeryanto et al. (1995) with the principle of negative control in the plate containing cancer cells and growth media is considered to proliferate as much as 100% so that the total treatment cells (which were given essential oil) and positive control (doxorubicin) will be compared with the total cells in the negative control and obtained a percent of proliferation and percent inhibition. Cells were incubated in the incubator for 3 to 4 days because it takes 3-4 days for the cells to grow and proliferate optimally so that cell counting can be done on day 3 or 4. If less than 3 days the cell has not grown optimally and for more than 4 days it is feared that the cell will experience death due to lack of growth nutrients. The calculation of total cells was carried out with a hemacytometer under a microscope with a magnification of 100 times. The cells were dripped with trypan blue to clarify the appearance of the cells under the microscope. The results showed that the proliferation of cancer cells decreased as the concentration of the administered extract increased. As shown in Figure 1, the administration of 170 ppm of *C. nardus* essential oil can inhibit the proliferation of cancer cells by 44.5% with an IC_{50} value of 359.6 ppm. Koba et al. (2009) report that citronella essential oil at a concentration of 150 ppm can inhibit the growth of HaCaT cells by 23% with IC_{50} of 450 ppm.

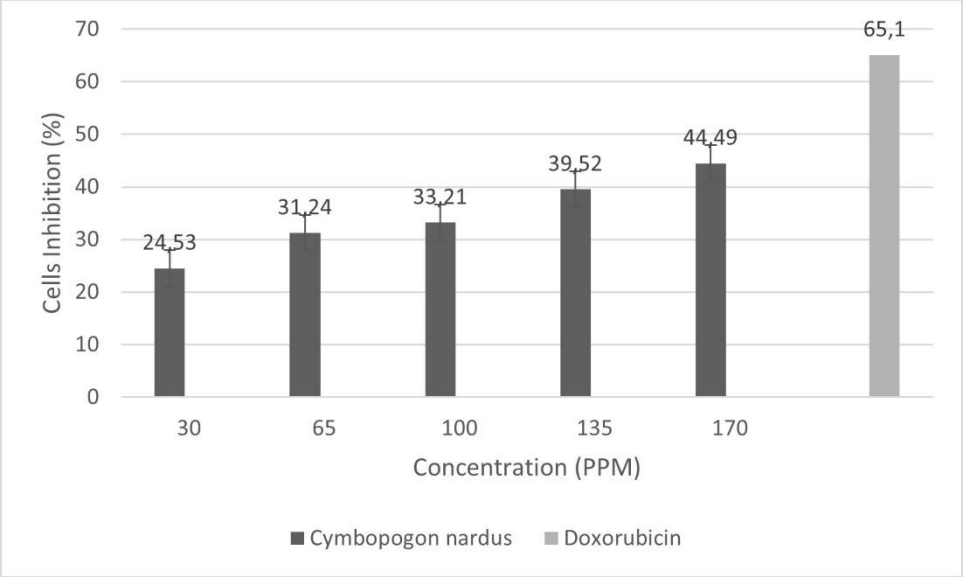

Figure 1. Effect of essential oils and doxorubicin administration on inhibition MCF-7 cells.

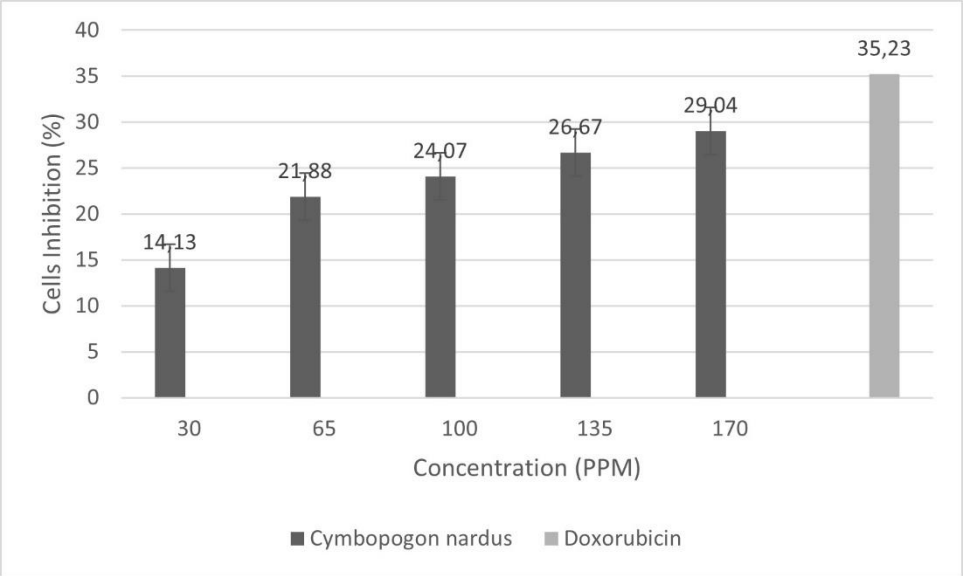

Figure 2. Effect of essential oils and doxorubicin administration on inhibition Vero normal cells.

Figure 2, the administration of *C. nardus* essential oils to Vero normal cells also shows a noticeable difference compared to doxorubicin. Testing of Vero normal cells was also carried out to determine the effect of essential oils administration on normal cell growth. *C. nardus* essential oils gave an inhibitory result of 29.0% at a concentration of 170 ppm for Vero normal cells while doxorubicin inhibited the growth of Vero normal cells by 35.23%.

As a comparison, doxorubicin (a drug used for cancer therapy) was used against MCF-7 cells and Vero cells. Figures 1 and 2 show that doxorubicin concentrations of 100 ppm inhibited MCF-7 cells by 65.1% but also damage Vero normal cell proliferation by 35.2%, this is in contrast to inhibition by *C. nardus* at the same concentration which only inhibited Vero cell proliferation by 29% and indicated

that the essential oil of *C. nardus* was safer against normal cells compared to doxorubicin.

4. GC-MS Essential Oil Analysis

The results of GC-MS obtained a total of 20 volatile compounds contained in the essential oil of *C. nardus*. It consists of 13 secondary metabolite compounds and 7 hydrocarbon compounds. The dominant volatile compounds found in *C. nardus* include geraniol (35.2%), citronellol (14%) and citronella (10.6%). The types of volatile compounds found in the essential oil of *C. nardus* will be presented in Table 2.

Table 2. Volatile Compounds Found In Essential Oils

No	Compound	Faction	Chemistry Structure	Molecule Weight (g mol^{-1})	Retention Time (min)	Peak Area (%)
1	Benzene	Hydrocarbon	C_6H_6	78	3.83	3.62
2	Acetic acid	Carboxylic acid	CH_3COOH	60	4.23	18.46
3	**Limonene**	Monoterpene	$C_{10}H_{16}$	136	7.10	0.56
4	**Cis-ocimene**	Monoterpene	$C_{10}H_{16}$	136	8.24	0.46
5	**Citronella**	Monoterpene	$C_{10}H_{18}O$	154	18.96	10.59
6	**Trans-caryophyllene**	Sesquiterpene	$C_{15}H_{24}$	204	24.66	2.16
7	Citronellyl propionate	Ester	$C_{13}H_{24}O_2$	212	28.70	0.96
8	**Z-Citral**	Monoterpene	$C_{10}H_{16}O$	152	29.45	1.73
9	**Cis-citral**	Monoterpene	$C_{10}H_{16}O$	152	32.19	2.29
10	**Naphtalene**	Monoterpene	$C_{10}H_8$	128	33.26	0.52
11	**Gamma-muurolene**	Sesquiterpene	$C_{15}H_{24}$	204	33.28	0.46
12	**Alpha-amorphene**	Sesquiterpene	$C_{15}H_{24}$	204	33.31	1.45
13	**Nerol**	Monoterpene	$C_{10}H_{18}O$	154	33.77	2.62
14	**Citronellol**	Monoterpene	$C_{10}H_{20}O$	156	34.44	14.05
15	**Geraniol**	Monoterpene	$C_{10}H_{18}O$	154	38.63	35.21
16	Methyleugenol	Eter	$C_{11}H_{14}O_2$	178	46.64	1.00
17	**Germacrene**	Sesquiterpene	$C_{15}H_{24}$	204	47.36	0.41
18	2-butanone	Ketone	C_4H_8O	72	48.46	0.23

19	Anisylaceton	Ketone	$C_{10}H_{12}O_2$	164	49.60	2.05
20	Phenol	Alcohol	C_6H_6O	94	51.97	0.59

The bolded are secondary metabolite volatile compounds

The results of GC-MS show that the majority of volatile compounds in *C. nardus* were from the terpenoid group, monoterpenes (45.00%) or sesquiterpenes (20.00%). Other volatile compounds were derived from the group of hydrocarbons (5.00 %), carboxylic acids (5.00%), esters (5.00%), ethers (5.00%), ketones (10.00%) and alcohols (5.00%).

The dominant volatile compounds in *C. nardus* essential oil that were alleged to have potential to inhibit the proliferation of cancer cells were geraniol, citronellol, citronella, cis-citral, trans-caryophyllene, limonene, naphthalene and germacrene. Such compounds were alleged to inhibit the proliferation of cancer cells by certain inhibitory mechanisms.

Geraniol belongs to the monoterpene class of acyclic alcohols and is commonly contained in drugs for cancer therapy such as 5-fluorouracil and docetaxel (Carnesecchi et al.2002). Geraniol can suppress the growth of MCF-7 cells by reducing protein levels of cyclin D1, cyclin-dependent kinase 4 (CDK-4), cyclin E and cyclin A and increasing P27 levels so that cell cycle containment occurs in phase G1 and cell proliferation stops (Duncan et al. 2004). Citronellol compound according to Zhuang et al. (2009) has anticancer potential but the inhibition mechanism is not yet known. Antiproliferation test found that the essential oil of *C. nardus* had the potential to inhibit the growth of MCF-7 cancer cells but also inhibits the growth of normal Vero cells, in other word is also toxic to Vero cells. This is due to the presence of citronella volatile compound, a monoterpenoid compound $C_{10}H_{18}O$. As reported by Stone et al. (2013), citronella is toxic in Vero cells because with 0.74.10-2% it can inhibit 50% of cell proliferation and has a Selectivity Index (SI) of 1.6 whereas if a compound has an SI below 3 it can be said that the compound is non-selective (Prayong et al. 2008). *C. Nardus* essential oils also contain cis-citral (neral) which is alleged to have antiproliferation potential for cancer cells. Zeng et al. (2015) reported that citral compounds can inhibit the proliferation of 4TI cancer cells (rat breast cancer) in vivo but the mechanism of inhibition is not yet known with certainty. Trans Caryophyllene is also a compound found in *C. nardus* that is alleged to be able to inhibit the proliferation of cancer cells. According to Dahham et al (2015) trans-caryophyllene can inhibit the proliferation of bowel cancer cells HCT-116 and HT-29 in-vitro by inducing the process of apoptosis of the mitochondrial pathway.

Limonene is alleged to inhibit cancer cell proliferation by activating the apoptotic pathway through the activation of caspase 3 and 9 and increasing the cleaved PARP protein expression. These mechanisms help to establish Limonene as a powerful pro-apoptotic agent to serve as an important therapeutic target (Araújo-

Filho et al., 2020). Naphthalene, according to (Luo et al., 2020) had in-vitro cytotoxic activity by arresting the cell cycle in the S phase and inducing apoptosis in MDA-MB-231 cells. The percentage of MDA-MB-231 cells in the G1 phase decrease significantly from 74.39% to 39.14% and 55.31%. Whereas, the cells in the S phase increased from 19.84% to 36.99% and 21.86%. According to (Cao et al., 2018) germacrene had anti-cancer activity toward T24 cells. The amount of T24 cells was reduced drastically. Whereas the shape of the cells gradually changed from wedge-shaped and polygonal into a round then the cells soon fell off, atrophied and disintegrated. Cells then were observed to be dead after the administration of germacrene. The inhibitory mechanism of germacrene needs to be explored further.

C. CONCLUSIONS AND SUGGESTIONS

The essential oil of C. nardus has the potential to inhibit the proliferation of MCF-7 cancer cells in vitro. In inhibiting the proliferation of normal cells of Vero, the essential oil of C. nardus is better than doxorubicin. This inhibition is caused by the presence of volatile compounds contained in essential oils that are alleged to inhibit cell proliferation through several pathways such as the cell cycle arrest to trigger the apoptosis process. It is necessary to conduct an induction test of cell apoptosis, analysis of mitochondrial damage and analysis of flow cytometry to find out the exact mechanism in the process of inhibition of cancer cells by essential oils.

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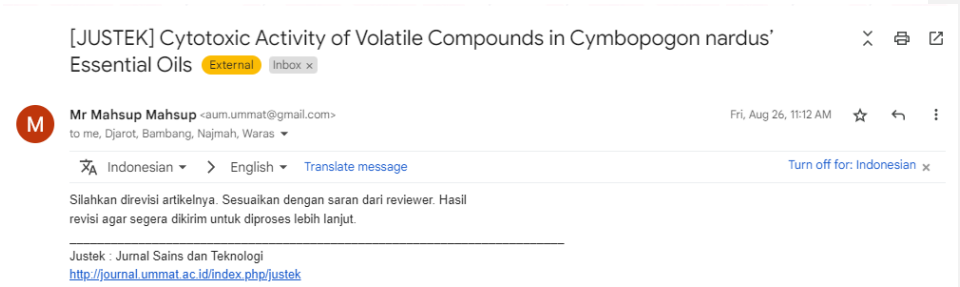

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Cytotoxic Activity of Volatile Compounds in Cymbopogon nardus' Essential Oils

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ABSTRACT

Abstract: *Plants produce a variety of secondary metabolites, one of which is essential oils that contain a variety of volatile compounds and are useful for humans. Cymbopogon nardus contains volatile compounds that can inhibit the proliferation of cancer cells. This research aims to explore the antiproliferation activity of C.nardus' essential oils with different concentrations against breast cancer Michigan Cancer Foundation-7 (MCF-7) cells. Antiproliferation test was carried out with total cells method using trypan blue and cells were calculated using a microscope. Gas Chromatography-Mass Spectrometer (GC-MS) was performed to identify the volatile compounds. The results showed that the Inhibitory Concentration 50 (IC_{50}) value was 359.6 ppm with an inhibition percent of 44.49% at 170 ppm. Meanwhile, inhibition percent against Vero normal cell was 29.04%, compared to Doxorubicin 35.23%. The dominant volatile compound in C. nardus' essential oil were Geraniol and Citronellol.*

Abstrak: Tanaman menghasilkan berbagai macam metabolit sekunder, salah satunya dalam bentuk minyak atsiri yang mengandung berbagai macam senyawa volatil yang berguna bagi manusia. *Cymbopogon nardus* mengandung senyawa volatile yang dapat menghambat proliferasi sel kanker. Penelitian ini bertujuan untuk mengetahui aktifitas antiproliferasi minyak atsiri *C. nardus* dengan beberapa konsentrasi terhadap sel kanker payudara *Michigan State Foundation-7* (MCF-7). Uji antiproliferasi dilakukan dengan metode total sel menggunakan *trypan blue* dan sel dihitung menggunakan mikroskop. *Gas Chromatography-Mass Spectrometer* (GC-MS) dilakukan untuk mengidentifikasi senyawa volatil. Hasil menunjukkan bahwa konsentrasi penghambatan 50 (IC_{50}) sebesar 359.6 ppm dengan persen penghambatan sebesar 44.49% pada konsentrasi 170 ppm. Sementara itu, persen ihibisi terhadap sel normal Vero sebesar 29.04%, dibandingkan dengan Doksorubicin yang sebesar 35.23%. Senyawa volatil dominan yang terdapat pada *C. nardus* adalah Geraniol dan Citronellol.

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A. INTRODUCTION

Cancer ranks as the main purpose of death and an essential barrier to growing existence expectancy in every country of the world. In line with estimates from the World Health Organization (WHO) in 2019, cancer is the primary or second leading cause of death before the age of 70 years in 112 of 183 countries. Cancer is an ailment that results from genetic or epigenetic alterations within the somatic cells and has ordinary cell increases which can be spread to other body components (Saini et al., 2020). Breast cancer in women ranks first in the most common cancers (11.7%) with a mortality rate of 6.9% (Sung et al., 2021). Breast cancer is metastatic cancer and may typically transfer to distant organs including the bone, liver, lung and brain, which particularly accounts for its incurability. Most breast cancers arise in women and the wide variety of instances is one hundred times higher in women than that in men. Breast tumors usually start from ductal hyperproliferation, after which grow to be benign tumors or even metastatic carcinomas after continuous stimulation through diverse carcinogenic factors (Sun et al., 2017).

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Most cancer cells are very similar to cells of the organism from which they originated and have comparable (but not identical) DNA and RNA. This is the reason why they are not very often detected through the immune system, in particular, if it is weakened. Few attempts that are normally used for most cancer treatment therapies are surgical procedures, radiation therapy, and chemotherapy (Bruton et al., 2005). Recent procedures in the management of breast cancer are gene therapy, oncogenes inactivation, augmentation of tumors suppressor genes, cell-target suicide, chemoprotection method, virus-mediated oncolysis and immunomodulation (Sharma et al., 2010). These strategies purpose to destroy most cancer cells and manipulate the growth of tumors within the breast tissue affected by cancer as well as control tumors increase in different tissues that are not affected by most cancers.

Treatment that is carried out in the early stages of cancer is surgery combined with other treatments to reduce recurrences such as radiation therapy, chemotherapy, hormone therapy and targeted drugs. For cancers that have undergone metastatic, the treatment is carried out more with systematic therapies including chemotherapy, hormone therapy and targeted drugs. The purpose of surgery is to remove cancer and determine the stage. This surgery is called a mastectomy where only tissue is affected by cancer and a slight edge of normal tissue that omitted/removed. However, this mastectomy is not recommended for cancer with a large tissue ratio and multicentric cancer. Regular surgery followed by radiation, patients who have undergone radiation are not recommended to perform a mastectomy. Radiotherapy is the use of high-energy particles to kill cancer cells and is commonly used after surgery to destroy cancer cells remaining in the breast, walls chest or lower area of the arms. Radiotherapy is non-selective that can also kill normal cells and have no effect on sufferers who have undergone hormone therapy for 5 years. Chemotherapy uses drugs that work by attacking cancer cells. Long-term use of chemotherapy drugs causes cardiomyopathy and congestive heart failure. Whereas hormone therapy works by blocking the body's natural

hormones or lower hormone levels which can trigger the growth of some cancers. Side effects of this hormone therapy include the risk of blood clots, bone thinning and vision changes. Targeted drugs work by attacking specific molecules in cells that have the potential to become more active in cancer cells. An example of these drugs are Trastuzumab and Herceptin. Side effects of using these drugs include heartbeats, shortness of breath, fever to bleeding that is not ordinary (Mc Donald et al., 2014).

To reduce the side effects of the use of such cancer drugs, now widely used substitutes in the form of herbal medicines. Pharmacology characteristic of common herbal ingredients found in its essential oils which contains a wide range of potential secondary metabolite compounds inhibits the proliferation of cancer cells. Essential oils are hydrophobic so it can enter the cell membrane and damage the cell.

Essential oils are secondary metabolites with a key function in plant protection, consisting commonly of terpenes with a volatile nature and a various array of chemical structures (Blowman et al., 2018). Essential oils have been proven to own anticancer properties via numerous mechanisms, including cancer preventative mechanisms, as well as appearing at the established tumors cellular itself and interaction with the microenvironment (Sitarek et al., 2017). Key hallmarks of cancer embrace resisting cell death, sustained proliferative signal and evading growth suppressors. therefore, therapeutic techniques focused on inducing apoptosis and cell arrest are of clear importance. Essential oils were proven to result in both the intrinsic (or mitochondria-based) and extrinsic (or death receptor-dependent) apoptosis pathways (Amin et al., 2009).

C. nardus is known to act as an insect repellent with some studies as antiproliferation of cancer cells (Akhila, 2010). There is still few research on antiproliferation activity in *C. nardus*. Among them, it is reported that essential oils from *C. nardus* have antiproliferation potential and contain active compounds in the form of geraniol and citronellal. *C. nardus* essential oil is also reported to have cytotoxic activity against MCM-B2 breast cancer cells (Hasim et al.,2020).

To the best of our knowledge, cytotoxic potential and antiproliferative activity of *C. nardus* essential oil against MCF-7 breast cancer cells in vitro have not been investigated previously. Therefore, the present study was focused on understanding *C. nardus* essential oil as an antiproliferative substance and figuring out its volatile compound.

B. RESEARCH METHOD

Material

The ingredients used in this study were *C. nardus* leaves taken from the Biopharmaceutical Cultivation Conservation Unit Center for Biopharmaceutical Studies Tropics with latitude (S) 6°34'37.95", longitude 106°47'20.37" and altitude (M) 238, Bogor, Indonesia. Other materials were n-hexane, water, seawater, tween 80, Artemia salina Leach, distilled water, MCF-7 cell, Vero cell, trypan blue, DMEM (Dulbecco's Modified Eagle's Medium), trypsin, gentamicin, FCS (Fetal Calf Serum), DMSO (Dimethyl Sulfoxide) and doxorubicin.

Distillation of Essential Oils

Extraction of essential oil was carried out by the method of steam distillation in a single way. A total of 3000 grams of small sliced sample were put into the distiller steam and added to distilled water in a ratio of 1:5 (sample: water). Distillation was carried out for 4 hours at a temperature of 100-105°C. Distilled essential oils then accommodated and stored at 4°C for further analysis. The percent of oil yield obtained is calculated by the formula:

$$\text{Yield Percent} = \frac{Oil\ Weight}{Dry\ Sample\ Weight}\ x\ 100\%$$

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Sesuaikan penulisan rumus yg lain

Cytotoxicity Test of Essential Oils with Brine Shrimp Lethality Test (BSLT)

Artemia salina Leach shrimp eggs and aerators were put into a container and seawater was added, allowed to stand for 24 hours until it turned into larvae. A solution of essential oil extract was dissolved in seawater with tween 80. The concentrations of the extract solution made were 1000, 500, 100, 10µg/mL and 0µg/mL (control). The test plate was prepared and stamped with seawater as much as 2 mL and then marked with a marker. A total of 1 mL of seawater was put into the test plate and then added 10 shrimp larvae and 0.5 mL of test solution for concentrations of 1000, 500, 100, 10µg / mL and 0µg / mL (control) respectively. The larvae were incubated for 24 hours and the number of dead larvae was calculated. Control was carried out by the same procedure without the addition of extracts. The LC_{50} value was determined using probit analysis using SPSS 16.0.

Antiproliferation Test of Essential Oil against MCF-7 cells and Vero

Preparation of Media and Extracts. MCF-7 cells and Vero cells were grown on 850µL of DMEM growing media in microplates of 24 wells to which 10µL of antibiotics (gentamicin), 10µL of fungizon, 30µL of FCS and extracts were added. The stock solutions of the extract with 5 different concentrations based on the results of the BSLT test were made using 10% DMSO as the solvent. Variations in extract concentrations were determined based on LC_{50} values and tested for three repetitions.

Cell Planting. The suspension of MCF-7 and Vero cells was pre-liquefied and homogenized with a vortex. Cell planting was carried out on a 24-well tissue culture plate containing a growth medium with 5 different concentrations of extracts, without the addition of essential oils as a negative control and doxorubicin as a positive control. A total of 50µL cell suspensions were added to each hole. Performed as many as three repetitions. The cell suspension was grown by incubating it in a 37°C incubator (5% CO_2).

Harvesting and Counting cells. Cell harvesting was carried out when the cells in the control hole had grown optimally covering about 70% of the hole surface (confluence) or approximately after 3 days. Cell suspensions were homogenized using micropipettes by sucking and removing them. A total of 80µl of homogeneous cell suspensions were placed in a microplate that already contains 20µl of trypan blue dye, so that the cells are visible under the microscope, then homogenized. The suspension was dripped on the Neubauer hemacytometer and a count of the number of cells under a light microscope with a

magnification of 100x was carried out. The calculated cells were those that are in the middle box of the counting room. All cells were counted, both living and dead cells

The formula used to calculate the percentage of activity cell growth and inhibition are as follows :

$$\% \text{ growth activity} = \frac{Average\ number\ of\ treatment\ cells}{Average\ number\ of\ negative\ control\ cells}\ x\ 100\%$$

$$\% \text{ of inhibitory activity} = 100\% - (\% \text{ growth activity})$$

GC-MS Essential Oil Analysis

Analysis of essential oils volatile compounds using the GC-MS tool Agilent Technologies with selective mass detectors. A total of 20µL of oil plant essentials were dissolved into 2000µL of n-hexane solvent (1:100 v/v). Sample separated using HP INNOWAX capillary columns (30 m x 0.25 mm, 0.25µm film thickness) with helium gas as a carrier (0.6 mL/min). Temperature the initial for the analysis was 60°C and raised to 150°C in increments by 2°C/min for about 1 minute, then increased to 210°C with a rate of increase of 20°C/min and kept constant for 10 minutes. The ratio of the separation rate was 250:1. The temperature at injection was 250°C by 1µL of essential oils for injection.

Statistical Analysis

Cytotoxicity data of LC_{50} BSLT were obtained using SPSS probit analysis 16.0, and IC_{50} inhibition activity of essential oils on cells was analyzed using regression analysis (log it). ANOVA analysis was carried out through the Duncan test at $p \leq 0.05$ to see the differences in each treatment.

C. RESULTS AND DISCUSSION

1. Essential Oils Productivity

The yield of essential oils produced was less than the previous studies. This difference was due to several factors such as the condition of the material and the distillation time. The condition of the withered material will produce a higher yield of essential oils compared to fresh ingredients because the moisture content in the material glands has been reduced and extraction is easier to do. A longer distillation time will result in more essential oil yields because volatile compounds that are difficult to evaporate will require a longer heating time to evaporate, in addition to the growing place and soil conditions also affect the yield of essential oils (Cassel & Vargas, 2013).

Table 1. Distilled Essential Oils Yield

Plant	Yield Experiment (%)	Yield Reference (%)	Reference
C. nardus	0.4	0.79	Cassel and Vargas 2013

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2. Cytotoxicity Test of Essential Oils with BSLT

BSLT is an initial pharmacological screening method that is easy and relatively inexpensive and does not require certain specialization in its implementation (Ghosh et al. 2015). BSLT can be used as a simple bioassay to examine the toxicity level of a compound by determining the value of LC_{50}. The test was carried out using *Artemia salina* in the napoli (larval) phase. The parameter measured was the mortality rate of shrimp larvae due to the administration of test solutions, in this study it was essential oil. The cytotoxicity of essential oils was determined in the LC_{50}. The results of the BSLT test showed that *C. nardus* had toxic potential because the LC_{50} calculation results were obtained at 98.5 ppm. Meyer et al. (1982) classify the toxicity level of a compound that is said to have potential bioactivity if the LC_{50} is less than 1000 ppm so that the essential oils of *C. nardus* had the potential to be toxic and had the potential to be antiproliferation compounds for cancer cells.

3. Antiproliferation Test of Essential Oil against MCF-7 cells and Vero

The antiproliferation activity of *C. nardus* essential oil was tested against MCF-7 cancer cells. Using the total cell method according to Priosoeryanto et al. (1995) with the principle of negative control in the plate containing cancer cells and growth media is considered to proliferate as much as 100% so that the total treatment cells (which were given essential oil) and positive control (doxorubicin) will be compared with the total cells in the negative control and obtained a percent of proliferation and percent inhibition. Cells were incubated in the incubator for 3 to 4 days because it takes 3-4 days for the cells to grow and proliferate optimally so that cell counting can be done on day 3 or 4. If less than 3 days the cell has not grown optimally and for more than 4 days it is feared that the cell will experience death due to lack of growth nutrients. The calculation of total cells was carried out with a hemacytometer under a microscope with a magnification of 100 times. The cells were dripped with trypan blue to clarify the appearance of the cells under the microscope. The results showed that the proliferation of cancer cells decreased as the concentration of the administered extract increased. As shown in Figure 1, the administration of 170 ppm of *C. nardus* essential oil can inhibit the proliferation of cancer cells by 44.5% with an IC_{50} value of 359.6 ppm. Koba et al. (2009) report that citronella essential oil at a concentration of 150 ppm can inhibit the growth of HaCaT cells by 23% with IC_{50} of 450 ppm.

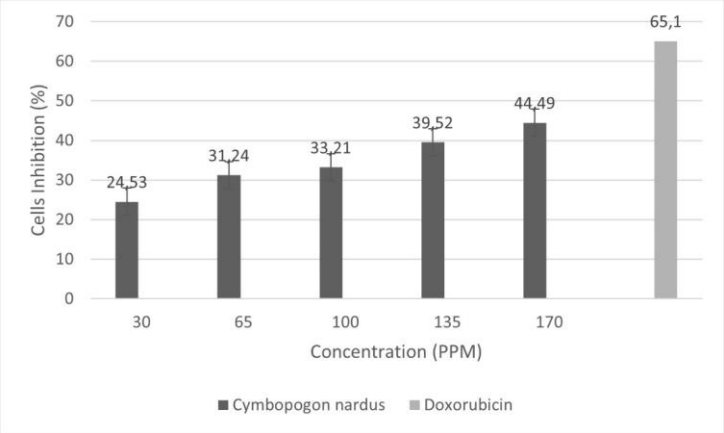

Figure 1. Effect of essential oils and doxorubicin administration on inhibition MCF-7 cells.

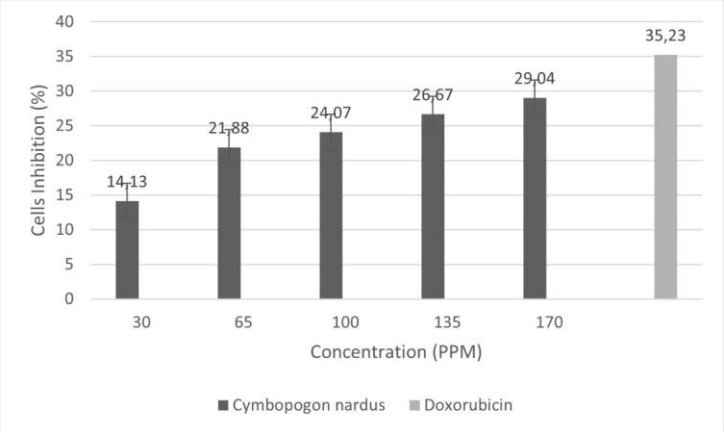

Figure 2. Effect of essential oils and doxorubicin administration on inhibition Vero normal cells.

Figure 2, the administration of *C. nardus* essential oils to Vero normal cells also shows a noticeable difference compared to doxorubicin. Testing of Vero normal cells was also carried out to determine the effect of essential oils administration on normal cell growth. *C. nardus* essential oils gave an inhibitory result of 29.0% at a concentration of 170 ppm for Vero normal cells while doxorubicin inhibited the growth of Vero normal cells by 35.23%.

As a comparison, doxorubicin (a drug used for cancer therapy) was used against MCF-7 cells and Vero cells. Figure 1 and figure 2 show that doxorubicin concentrations of 100 ppm inhibited MCF-7 cells by 65.1% but also damage Vero normal cell proliferation by 35.2%, this is in contrast to inhibition by *C. nardus* at the same concentration which only inhibited Vero cell proliferation by 29% and

indicated that the essential oil of *C. nardus* was safer against normal cells compared to doxorubicin.

4. GC-MS Essential Oil Analysis

The results of GC-MS obtained a total of 20 volatile compounds contained in the essential oil of *C. nardus*. It consists of 13 secondary metabolite compounds and 7 hydrocarbon compounds. The dominant volatile compounds found in *C. nardus* include geraniol (35.2%), citronellol (14%) and citronella (10.6%). The types of volatile compounds found in the essential oil of *C. nardus* will be presented in Table 2.

Table 2. Volatile Compounds Found In Essential Oils

No	Compound	Faction	Chemistry Structure	Molecule Weight (g mol^{-1})	Retention Time (min)	Peak Area (%)
1	Benzene	Hydrocarbon	C_6H_6	78	3.83	3.62
2	Acetic acid	Carboxylic acid	CH_3COOH	60	4.23	18.46
3	**Limonene**	Monoterpene	$C_{10}H_{16}$	136	7.10	0.56
4	**Cis-ocimene**	Monoterpene	$C_{10}H_{16}$	136	8.24	0.46
5	**Citronella**	Monoterpene	$C_{10}H_{18}O$	154	18.96	10.59
6	**Trans-caryophyllene**	Sesquiterpene	$C_{15}H_{24}$	204	24.66	2.16
7	Citronellyl propionate	Ester	$C_{13}H_{24}O_2$	212	28.70	0.96
8	**Z-Citral**	Monoterpene	$C_{10}H_{16}O$	152	29.45	1.73
9	**Cis-citral**	Monoterpene	$C_{10}H_{16}O$	152	32.19	2.29
10	**Naphtalene**	Monoterpene	$C_{10}H_8$	128	33.26	0.52
11	**Gamma-muurolene**	Sesquiterpene	$C_{15}H_{24}$	204	33.28	0.46
12	**Alpha-amorphene**	Sesquiterpene	$C_{15}H_{24}$	204	33.31	1.45
13	**Nerol**	Monoterpene	$C_{10}H_{18}O$	154	33.77	2.62
14	**Citronellol**	Monoterpene	$C_{10}H_{20}O$	156	34.44	14.05
15	**Geraniol**	Monoterpene	$C_{10}H_{18}O$	154	38.63	35.21
16	Methyleugenol	Eter	$C_{11}H_{14}O_2$	178	46.64	1.00
17	**Germacrene**	Sesquiterpene	$C_{15}H_{24}$	204	47.36	0.41
18	2-butanone	Ketone	C_4H_8O	72	48.46	0.23

19	Anisylaceton	Ketone	$C_{10}H_{12}O_2$	164	49.60	2.05
20	Phenol	Alcohol	C_6H_6O	94	51.97	0.59

The bolded are secondary metabolite volatile compounds

The results of GC-MS show that the majority of volatile compounds in *C. nardus* were from the terpenoid group, monoterpenes (45.00%) or sesquiterpenes (20.00%). Other volatile compounds were derived from the group of hydrocarbons (5.00 %), carboxylic acids (5.00%), esters (5.00%), ethers (5.00%), ketones (10.00%) and alcohols (5.00%).

The dominant volatile compounds in *C. nardus* essential oil that were alleged to have potential to inhibit the proliferation of cancer cells were geraniol, citronellol, citronella, cis-citral, trans-caryophyllene, limonene, naphthalene and germacrene. Such compounds were alleged to inhibit the proliferation of cancer cells by certain inhibitory mechanisms.

Geraniol belongs to the monoterpene class of acyclic alcohols and is commonly contained in drugs for cancer therapy such as 5-fluorouracil and docetaxel (Carnesecchi et al.2002). Geraniol can suppress the growth of MCF-7 cells by reducing protein levels of cyclin D1, cyclin-dependent kinase 4 (CDK-4), cyclin E and cyclin A and increasing P27 levels so that cell cycle containment occurs in phase G1 and cell proliferation stops (Duncan et al. 2004). Citronellol compound according to Zhuang et al. (2009) has anticancer potential but the inhibition mechanism is not yet known. Antiproliferation test found that the essential oil of *C. nardus* had the potential to inhibit the growth of MCF-7 cancer cells but also inhibits the growth of normal Vero cells, in other word is also toxic to Vero cells. This is due to the presence of citronella volatile compound, a monoterpenoid compound $C_{10}H_{18}O$. As reported by Stone et al. (2013), citronella is toxic in Vero cells because with 0.74.10-2% it can inhibit 50% of cell proliferation and has a Selectivity Index (SI) of 1.6 whereas if a compound has an SI below 3 it can be said that the compound is non-selective (Prayong et al. 2008). *C. Nardus* essential oils also contain cis-citral (neral) which is alleged to have antiproliferation potential for cancer cells. Zeng et al. (2015) reported that citral compounds can inhibit the proliferation of 4TI cancer cells (rat breast cancer) in vivo but the mechanism of inhibition is not yet known with certainty. Trans Caryophyllene is also a compound found in *C. nardus* that is alleged to be able to inhibit the proliferation of cancer cells. According to Dahham et al (2015) trans-caryophyllene can inhibit the proliferation of bowel cancer cells HCT-116 and HT-29 in-vitro by inducing the process of apoptosis of the mitochondrial pathway.

Limonene is alleged to inhibit cancer cell proliferation by activating the apoptotic pathway through the activation of caspase 3 and 9 and increasing the cleaved PARP protein expression. These mechanisms help to establish Limonene as a powerful pro-apoptotic agent to serve as an important therapeutic target (Araújo-

Filho et al., 2020). Naphthalene, according to (Luo et al., 2020) had in-vitro cytotoxic activity by arresting the cell cycle in the S phase and inducing apoptosis in MDA-MB-231 cells. The percentage of MDA-MB-231 cells in the G1 phase decrease significantly from 74.39% to 39.14% and 55.31%. Whereas, the cells in the S phase increased from 19.84% to 36.99% and 21.86%. According to (Cao et al., 2018) germacrene had anti-cancer activity toward T24 cells. The amount of T24 cells was reduced drastically. Whereas the shape of the cells gradually changed from wedge-shaped and polygonal into a round then the cells soon fell off, atrophied and disintegrated. Cells then were observed to be dead after the administration of germacrene. The inhibitory mechanism of germacrene needs to be explored further.

C. CONCLUSIONS AND SUGGESTIONS

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The essential oil of C. nardus has the potential to inhibit the proliferation of MCF-7 cancer cells in vitro. In inhibiting the proliferation of normal cells of Vero, the essential oil of C. nardus is better than doxorubicin. This inhibition is caused by the presence of volatile compounds contained in essential oils that are alleged to inhibit cell proliferation through several pathways such as the cell cycle arrest to trigger the apoptosis process. It is necessary to conduct an induction test of cell apoptosis, analysis of mitochondrial damage and analysis of flow cytometry to find out the exact mechanism in the process of inhibition of cancer cells by essential oils.

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Cytotoxic Activity of Volatile Compounds in Cymbopogon nardus' Essential Oils

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ABSTRACT

Abstract: *Plants produce a variety of secondary metabolites, one of which is essential oils that contain a variety of volatile compounds and are useful for humans. Cymbopogon nardus contains volatile compounds that can inhibit the proliferation of cancer cells. This research aims to explore the antiproliferation activity of C.nardus' essential oils with different concentrations against breast cancer Michigan Cancer Foundation-7 (MCF-7) cells. Antiproliferation test was carried out with total cells method using trypan blue and cells were calculated using a microscope. Gas Chromatography-Mass Spectrometer (GC-MS) was performed to identify the volatile compounds. The results showed that the Inhibitory Concentration 50 (IC_{50}) value was 359.6 ppm with an inhibition percent of 44.49% at 170 ppm. Meanwhile, inhibition percent against Vero normal cell was 29.04%, compared to Doxorubicin 35.23%. The dominant volatile compound in C. nardus' essential oil were Geraniol and Citronellol.*

Abstrak: Tanaman menghasilkan berbagai macam metabolit sekunder, salah satunya dalam bentuk minyak atsiri yang mengandung berbagai macam senyawa volatil yang berguna bagi manusia. *Cymbopogon nardus* mengandung senyawa volatile yang dapat menghambat proliferasi sel kanker. Penelitian ini bertujuan untuk mengetahui aktifitas antiproliferasi minyak atsiri *C. nardus* dengan beberapa konsentrasi terhadap sel kanker payudara *Michigan State Foundation-7* (MCF-7). Uji antiproliferasi dilakukan dengan metode total sel menggunakan *trypan blue* dan sel dihitung menggunakan mikroskop. *Gas Chromatography-Mass Spectrometer* (GC-MS) dilakukan untuk mengidentifikasi senyawa volatil. Hasil menunjukkan bahwa konsentrasi penghambatan 50 (IC_{50}) sebesar 359.6 ppm dengan persen penghambatan sebesar 44.49% pada konsentrasi 170 ppm. Sementara itu, persen ihibisi terhadap sel normal Vero sebesar 29.04%, dibandingkan dengan Doksorubicin yang sebesar 35.23%. Senyawa volatil dominan yang terdapat pada *C. nardus* adalah Geraniol dan Citronellol.

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A. INTRODUCTION

Cancer ranks as the main purpose of death and an essential barrier to growing existence expectancy in every country of the world. In line with estimates from the World Health Organization (WHO) in 2019, cancer is the primary or second leading cause of death before the age of 70 years in 112 of 183 countries. Cancer is an ailment that results from genetic or epigenetic alterations within the somatic cells and has ordinary cell increases which can be spread to other body components (Saini et al., 2020). Breast cancer in women ranks first in the most common cancers (11.7%) with a mortality rate of 6.9% (Sung et al., 2021). Breast cancer is metastatic cancer and may typically transfer to distant organs including the bone, liver, lung and brain, which particularly accounts for its incurability. Most breast cancers arise in women and the wide variety of instances is one hundred times higher in women than that in men. Breast tumors usually start from ductal hyperproliferation, after which grow to be benign tumors or even metastatic carcinomas after continuous stimulation through diverse carcinogenic factors (Sun et al., 2017).

Most cancer cells are very similar to cells of the organism from which they originated and have comparable (but not identical) DNA and RNA. This is the reason why they are not very often detected through the immune system, in particular, if it is weakened. Few attempts that are normally used for most cancer treatment therapies are surgical procedures, radiation therapy, and chemotherapy (Brunton et al., 2018). Recent procedures in the management of breast cancer are gene therapy, oncogenes inactivation, augmentation of tumors suppressor genes, cell-target suicide, chemoprotection method, virus-mediated oncolysis and immunomodulation (Sharma et al., 2010). These strategies purpose to destroy most cancer cells and manipulate the growth of tumors within the breast tissue affected by cancer as well as control tumors increase in different tissues that are not affected by most cancers.

Treatment that is carried out in the early stages of cancer is surgery combined with other treatments to reduce recurrences such as radiation therapy, chemotherapy, hormone therapy and targeted drugs. For cancers that have undergone metastatic, the treatment is carried out more with systematic therapies including chemotherapy, hormone therapy and targeted drugs. The purpose of surgery is to remove cancer and determine the stage. This surgery is called a mastectomy where only tissue is affected by cancer and a slight edge of normal tissue that omitted/removed. However, this mastectomy is not recommended for cancer with a large tissue ratio and multicentric cancer. Regular surgery followed by radiation, patients who have undergone radiation are not recommended to perform a mastectomy. Radiotherapy is the use of high-energy particles to kill cancer cells and is commonly used after surgery to destroy cancer cells remaining in the breast, walls chest or lower area of the arms. Radiotherapy is non-selective that can also kill normal cells and have no effect on sufferers who have undergone hormone therapy for 5 years. Chemotherapy uses drugs that work by attacking cancer cells. Long-term use of chemotherapy drugs causes cardiomyopathy and congestive heart failure. Whereas hormone therapy works by blocking the body's natural

hormones or lower hormone levels which can trigger the growth of some cancers. Side effects of this hormone therapy include the risk of blood clots, bone thinning and vision changes. Targeted drugs work by attacking specific molecules in cells that have the potential to become more active in cancer cells. An example of these drugs are Trastuzumab and Herceptin. Side effects of using these drugs include heartbeats, shortness of breath, fever to bleeding that is not ordinary (McDonald et al., 2004).

To reduce the side effects of the use of such cancer drugs, now widely used substitutes in the form of herbal medicines. Pharmacology characteristic of common herbal ingredients found in its essential oils which contains a wide range of potential secondary metabolite compounds inhibits the proliferation of cancer cells. Essential oils are hydrophobic so it can enter the cell membrane and damage the cell.

Essential oils are secondary metabolites with a key function in plant protection, consisting commonly of terpenes with a volatile nature and a various array of chemical structures (Blowman et al., 2018). Essential oils have been proven to own anticancer properties via numerous mechanisms, including cancer preventative mechanisms, as well as appearing at the established tumors cellular itself and interaction with the microenvironment (Sitarek et al., 2017). Key hallmarks of cancer embrace resisting cell death, sustained proliferative signal and evading growth suppressors. therefore, therapeutic techniques focused on inducing apoptosis and cell arrest are of clear importance. Essential oils were proven to result in both the intrinsic (or mitochondria-based) and extrinsic (or death receptor-dependent) apoptosis pathways (Amin et al., 2009).

C. nardus is known to act as an insect repellent with some studies as antiproliferation of cancer cells (Akhila, 2009). There is still few research on antiproliferation activity in *C. nardus*. Among them, it is reported that essential oils from *C. nardus* have antiproliferation potential and contain active compounds in the form of geraniol and citronellal. *C. nardus* essential oil is also reported to have cytotoxic activity against MCM-B2 breast cancer cells (Hasim et al., 2020).

To the best of our knowledge, cytotoxic potential and antiproliferative activity of *C. nardus* essential oil against MCF-7 breast cancer cells in vitro have not been investigated previously. Therefore, the present study was focused on understanding *C. nardus* essential oil as an antiproliferative substance and figuring out its volatile compound.

B. RESEARCH METHOD

Material

The ingredients used in this study were *C. nardus* leaves taken from the Biopharmaceutical Cultivation Conservation Unit Center for Biopharmaceutical Studies Tropics with latitude (S) 6°34'37.95", longitude 106°47'20.37" and altitude (M) 238, Bogor, Indonesia. Other materials were n-hexane, water, seawater, tween 80, Artemia salina Leach, distilled water, MCF-7 cell, Vero cell, trypan blue, DMEM (Dulbecco's Modified Eagle's Medium), trypsin, gentamicin, FCS (Fetal Calf Serum), DMSO (Dimethyl Sulfoxide) and doxorubicin.

Distillation of Essential Oils

Extraction of essential oil was carried out by the method of steam distillation in a single way. A total of 3000 grams of small sliced sample were put into the distiller steam and added to distilled water in a ratio of 1:5 (sample: water). Distillation was carried out for 4 hours at a temperature of 100-105°C. Distilled essential oils then accommodated and stored at 4°C for further analysis. The percent of oil yield obtained is calculated by the formula (1):

$$\text{Yield Percent} = \frac{Oil\ Weight}{Dry\ Sample\ Weight}\ x\ 100\% \qquad (1)$$

Cytotoxicity Test of Essential Oils with Brine Shrimp Lethality Test (BSLT)

Artemia salina Leach shrimp eggs and aerators were put into a container and seawater was added, allowed to stand for 24 hours until it turned into larvae. A solution of essential oil extract was dissolved in seawater with tween 80. The concentrations of the extract solution made were 1000, 500, 100, 10µg/mL and 0µg/mL (control). The test plate was prepared and stamped with seawater as much as 2 mL and then marked with a marker. A total of 1 mL of seawater was put into the test plate and then added 10 shrimp larvae and 0.5 mL of test solution for concentrations of 1000, 500, 100, 10µg / mL and 0µg / mL (control) respectively. The larvae were incubated for 24 hours and the number of dead larvae was calculated. Control was carried out by the same procedure without the addition of extracts. The LC_{50} value was determined using probit analysis using SPSS 16.0.

Antiproliferation Test of Essential Oil against MCF-7 cells and Vero

Preparation of Media and Extracts. MCF-7 cells and Vero cells were grown on 850µL of DMEM growing media in microplates of 24 wells to which 10µL of antibiotics (gentamicin), 10µL of fungizon, 30µL of FCS and extracts were added. The stock solutions of the extract with 5 different concentrations based on the results of the BSLT test were made using 10% DMSO as the solvent. Variations in extract concentrations were determined based on LC_{50} values and tested for three repetitions.

Cell Planting. The suspension of MCF-7 and Vero cells was pre-liquefied and homogenized with a vortex. Cell planting was carried out on a 24-well tissue culture plate containing a growth medium with 5 different concentrations of extracts, without the addition of essential oils as a negative control and doxorubicin as a positive control. A total of 50µL cell suspensions were added to each hole. Performed as many as three repetitions. The cell suspension was grown by incubating it in a 37°C incubator (5% CO_2).

Harvesting and Counting cells. Cell harvesting was carried out when the cells in the control hole had grown optimally covering about 70% of the hole surface (confluence) or approximately after 3 days. Cell suspensions were homogenized using micropipettes by sucking and removing them. A total of 80µl of homogeneous cell suspensions were placed in a microplate that already contains 20µl of trypan blue dye, so that the cells are visible under the microscope, then homogenized. The suspension was dripped on the Neubauer hemacytometer and a count of the number of cells under a light microscope with a

magnification of 100x was carried out. The calculated cells were those that are in the middle box of the counting room. All cells were counted, both living and dead cells.

The formula used to calculate the percentage of activity cell growth (2) and inhibition (3) are as follows :

$$\% \text{ growth activity} = \frac{Average\ number\ of\ treatment\ cells}{Average\ number\ of\ negative\ control\ cells}\ x\ 100\% \quad (2)$$

$$\% \text{ of inhibitory activity} = 100\% - (\% \text{ growth activity}) \quad (3)$$

GC-MS Essential Oil Analysis

Analysis of essential oils volatile compounds using the GC-MS tool Agilent Technologies with selective mass detectors. A total of 20µL of oil plant essentials were dissolved into 2000µL of n-hexane solvent (1:100 v/v). Sample separated using HP INNOWAX capillary columns (30 m x 0.25 mm, 0.25µm film thickness) with helium gas as a carrier (0.6 mL/min). Temperature the initial for the analysis was 60°C and raised to 150°C in increments by 2°C/min for about 1 minute, then increased to 210°C with a rate of increase of 20°C/min and kept constant for 10 minutes. The ratio of the separation rate was 250:1. The temperature at injection was 250°C by 1µL of essential oils for injection.

Statistical Analysis

Cytotoxicity data of LC_{50} BSLT were obtained using SPSS probit analysis 16.0, and IC_{50} inhibition activity of essential oils on cells was analyzed using regression analysis (log it). ANOVA analysis was carried out through the Duncan test at $p \leq 0.05$ to see the differences in each treatment.

C. RESULTS AND DISCUSSION

1. Essential Oils Productivity

The yield of essential oils produced was less than the previous studies (Table 1). This difference was due to several factors such as the condition of the material and the distillation time. The condition of the withered material will produce a higher yield of essential oils compared to fresh ingredients because the moisture content in the material glands has been reduced and extraction is easier to do. A longer distillation time will result in more essential oil yields because volatile compounds that are difficult to evaporate will require a longer heating time to evaporate, in addition to the growing place and soil conditions also affect the yield of essential oils (Cassel & Vargas, 2006).

Table 1. Distilled Essential Oils Yield

Plant	Yield Experiment (%)	Yield Reference (%)	Reference
C. nardus	0.4	0.79	Cassel and Vargas 2013

2. Cytotoxicity Test of Essential Oils with BSLT

BSLT is an initial pharmacological screening method that is easy and relatively inexpensive and does not require certain specialization in its implementation (Ghosh et al., 2015). BSLT can be used as a simple bioassay to examine the toxicity level of a compound by determining the value of LC_{50}. The test was carried out using *Artemia salina* in the napoli (larval) phase. The parameter measured was the mortality rate of shrimp larvae due to the administration of test solutions, in this study it was essential oil. The cytotoxicity of essential oils was determined in the LC_{50}. The results of the BSLT test showed that *C. nardus* had toxic potential because the LC_{50} calculation results were obtained at 98.5 ppm. (Meyer et al., 1982) classify the toxicity level of a compound that is said to have potential bioactivity if the LC_{50} is less than 1000 ppm so that the essential oils of *C. nardus* had the potential to be toxic and had the potential to be antiproliferation compounds for cancer cells.

3. Antiproliferation Test of Essential Oil against MCF-7 cells and Vero

The antiproliferation activity of *C. nardus* essential oil was tested against MCF-7 cancer cells. Using the total cell method according to (Priosoeryanto et al., 1995)with the principle of negative control in the plate containing cancer cells and growth media is considered to proliferate as much as 100% so that the total treatment cells (which were given essential oil) and positive control (doxorubicin) will be compared with the total cells in the negative control and obtained a percent of proliferation and percent inhibition. Cells were incubated in the incubator for 3 to 4 days because it takes 3-4 days for the cells to grow and proliferate optimally so that cell counting can be done on day 3 or 4. If less than 3 days the cell has not grown optimally and for more than 4 days it is feared that the cell will experience death due to lack of growth nutrients. The calculation of total cells was carried out with a hemacytometer under a microscope with a magnification of 100 times. The cells were dripped with trypan blue to clarify the appearance of the cells under the microscope. The results showed that the proliferation of cancer cells decreased as the concentration of the administered extract increased. As shown in Figure 1, the administration of 170 ppm of *C. nardus* essential oil can inhibit the proliferation of cancer cells by 44.5% with an IC_{50} value of 359.6 ppm. (Koba et al., 2008) report that citronella essential oil at a concentration of 150 ppm can inhibit the growth of HaCaT cells by 23% with IC_{50} of 450 ppm.

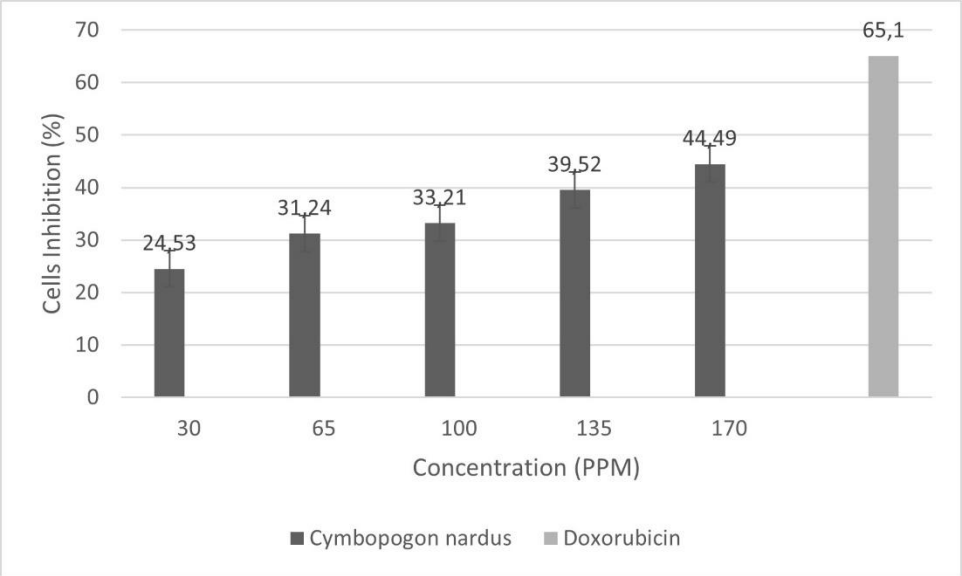

Figure 1. Effect of essential oils and doxorubicin administration on inhibition MCF-7 cells.

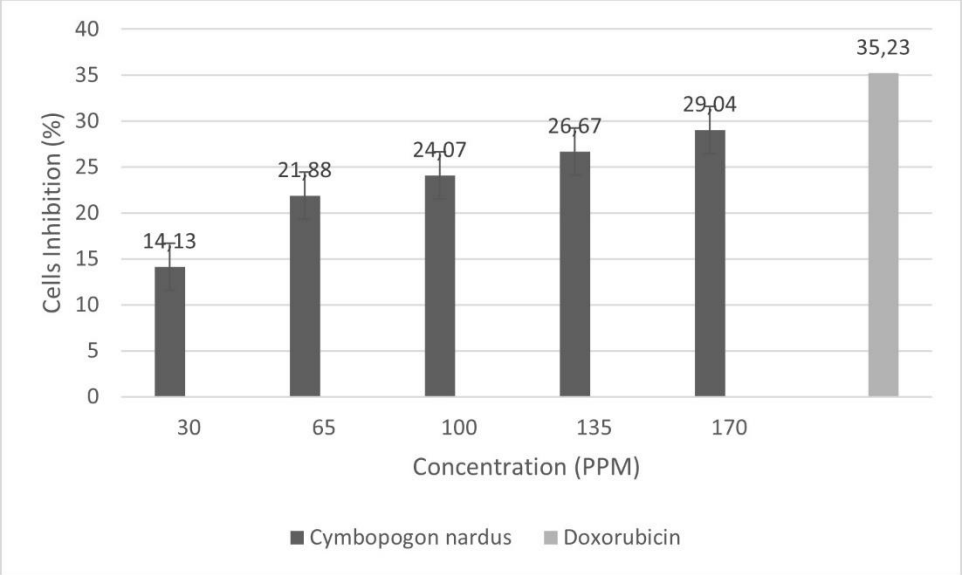

Figure 2. Effect of essential oils and doxorubicin administration on inhibition Vero normal cells.

Figure 2, the administration of *C. nardus* essential oils to Vero normal cells also shows a noticeable difference compared to doxorubicin. Testing of Vero normal cells was also carried out to determine the effect of essential oils administration on normal cell growth. *C. nardus* essential oils gave an inhibitory result of 29.0% at a concentration of 170 ppm for Vero normal cells while doxorubicin inhibited the growth of Vero normal cells by 35.23%.

As a comparison, doxorubicin (a drug used for cancer therapy) was used against MCF-7 cells and Vero cells. Figures 1 and 2 show that doxorubicin concentrations of 100 ppm inhibited MCF-7 cells by 65.1% but also damage Vero normal cell proliferation by 35.2%, this is in contrast to inhibition by *C. nardus* at the same concentration which only inhibited Vero cell proliferation by 29% and indicated that the essential oil of *C. nardus* was safer against normal cells compared to doxorubicin.

4. GC-MS Essential Oil Analysis

The results of GC-MS obtained a total of 20 volatile compounds contained in the essential oil of *C. nardus*. It consists of 13 secondary metabolite compounds and 7 hydrocarbon compounds. The dominant volatile compounds found in *C. nardus* include geraniol (35.2%), citronellol (14%) and citronella (10.6%). The types of volatile compounds found in the essential oil of *C. nardus* will be presented in Table 2.

Table 2. Volatile Compounds Found In Essential Oils

No	Compound	Faction	Chemistry Structure	Molecule Weight (g mol^{-1})	Retention Time (min)	Peak Area (%)
1	Benzene	Hydrocarbon	C_6H_6	78	3.83	3.62
2	Acetic acid	Carboxylic acid	CH_3COOH	60	4.23	18.46
3	**Limonene**	Monoterpene	$C_{10}H_{16}$	136	7.10	0.56
4	**Cis-ocimene**	Monoterpene	$C_{10}H_{16}$	136	8.24	0.46
5	**Citronella**	Monoterpene	$C_{10}H_{18}O$	154	18.96	10.59
6	**Trans-caryophyllene**	Sesquiterpene	$C_{15}H_{24}$	204	24.66	2.16
7	Citronellyl propionate	Ester	$C_{13}H_{24}O_2$	212	28.70	0.96
8	**Z-Citral**	Monoterpene	$C_{10}H_{16}O$	152	29.45	1.73
9	**Cis-citral**	Monoterpene	$C_{10}H_{16}O$	152	32.19	2.29
10	**Naphtalene**	Monoterpene	$C_{10}H_8$	128	33.26	0.52
11	**Gamma-muurolene**	Sesquiterpene	$C_{15}H_{24}$	204	33.28	0.46
12	**Alpha-amorphene**	Sesquiterpene	$C_{15}H_{24}$	204	33.31	1.45
13	**Nerol**	Monoterpene	$C_{10}H_{18}O$	154	33.77	2.62
14	**Citronellol**	Monoterpene	$C_{10}H_{20}O$	156	34.44	14.05
15	**Geraniol**	Monoterpene	$C_{10}H_{18}O$	154	38.63	35.21
16	Methyleugenol	Eter	$C_{11}H_{14}O_2$	178	46.64	1.00
17	**Germacrene**	Sesquiterpene	$C_{15}H_{24}$	204	47.36	0.41
18	2-butanone	Ketone	C_4H_8O	72	48.46	0.23
19	Anisylaceton	Ketone	$C_{10}H_{12}O_2$	164	49.60	2.05
20	Phenol	Alcohol	C_6H_6O	94	51.97	0.59

The bolded are secondary metabolite volatile compounds

The results of GC-MS show that the majority of volatile compounds in *C. nardus* were from the terpenoid group, monoterpenes (45.00%) or sesquiterpenes (20.00%). Other volatile compounds were derived from the group of hydrocarbons

(5.00 %), carboxylic acids (5.00%), esters (5.00%), ethers (5.00%), ketones (10.00%) and alcohols (5.00%).

The dominant volatile compounds in *C. nardus* essential oil that were alleged to have potential to inhibit the proliferation of cancer cells were geraniol, citronellol, citronella, cis-citral, trans-caryophyllene, limonene, naphthalene and germacrene. Such compounds were alleged to inhibit the proliferation of cancer cells by certain inhibitory mechanisms.

Geraniol belongs to the monoterpene class of acyclic alcohols and is commonly contained in drugs for cancer therapy such as 5-fluorouracil and docetaxel (Carnesecchi et al., 2002). Geraniol can suppress the growth of MCF-7 cells by reducing protein levels of cyclin D1, cyclin-dependent kinase 4 (CDK-4), cyclin E and cyclin A and increasing P27 levels so that cell cycle containment occurs in phase G1 and cell proliferation stops (Duncan et al., 2004). Citronellol compound according to (Zhuang et al., 2009) has anticancer potential but the inhibition mechanism is not yet known. Antiproliferation test found that the essential oil of *C. nardus* had the potential to inhibit the growth of MCF-7 cancer cells but also inhibits the growth of normal Vero cells, in other words is also toxic to Vero cells. This is due to the presence of citronella volatile compound, a monoterpenoid compound $C_{10}H_{18}O$. As reported by (Stone et al., 2013), citronella is toxic in Vero cells because with 0.74.10-2% it can inhibit 50% of cell proliferation and has a Selectivity Index (SI) of 1.6 whereas if a compound has an SI below 3 it can be said that the compound is non-selective (Prayong et al., 2008). *C. Nardus* essential oils also contain cis-citral (neral) which is alleged to have antiproliferation potential for cancer cells. (Zeng et al., 2015) reported that citral compounds can inhibit the proliferation of 4TI cancer cells (rat breast cancer) in vivo but the mechanism of inhibition is not yet known with certainty. Trans Caryophyllene is also a compound found in *C. nardus* that is alleged to be able to inhibit the proliferation of cancer cells. According to Dahham et al., (2015) trans-caryophyllene can inhibit the proliferation of bowel cancer cells HCT-116 and HT-29 in-vitro by inducing the process of apoptosis of the mitochondrial pathway.

Limonene is alleged to inhibit cancer cell proliferation by activating the apoptotic pathway through the activation of caspase 3 and 9 and increasing the cleaved PARP protein expression. These mechanisms help to establish Limonene as a powerful pro-apoptotic agent to serve as an important therapeutic target (Araújo-Filho et al., 2021). Naphthalene, according to Luo et al., (2021) had in-vitro cytotoxic activity by arresting the cell cycle in the S phase and inducing apoptosis in MDA-MB-231 cells. The percentage of MDA-MB-231 cells in the G1 phase decrease significantly from 74.39% to 39.14% and 55.31%. Whereas, the cells in the S phase increased from 19.84% to 36.99% and 21.86%. According to (Cao et al., 2018) germacrene had anti-cancer activity toward T24 cells. The amount of T24 cells was reduced drastically. Whereas the shape of the cells gradually changed from wedge-shaped and polygonal into a round then the cells soon fell off, atrophied and

disintegrated. Cells then were observed to be dead after the administration of germacrene. The inhibitory mechanism of germacrene needs to be explored further.

C. CONCLUSIONS AND SUGGESTIONS

The essential oil of *C. nardus* has the potential to inhibit the proliferation of MCF-7 cancer cells in vitro with a percent inhibition of 44.49% in 170 ppm (highest concentration). In inhibiting the proliferation of normal cells of Vero, the essential oil of *C. nardus* was better than doxorubicin with a percent inhibition of 29.04%. This inhibition was caused by the presence of volatile compounds contained in essential oils such as geraniol (35.21%), citronellol (14.05%), citronella (10.59%), cis-citral (2.29%), trans-caryophyllene (2.16%), limonene (0.56%), naphthalene (0.52%) and germacrene (0.41%) that were alleged to inhibit cell proliferation through several pathways such as the cell cycle arrest until triggering the apoptosis process. It is necessary to conduct an induction test of cell apoptosis, analysis of mitochondrial damage and analysis of flow cytometry to find out the exact mechanism in the process of inhibition of cancer cells by essential oils.

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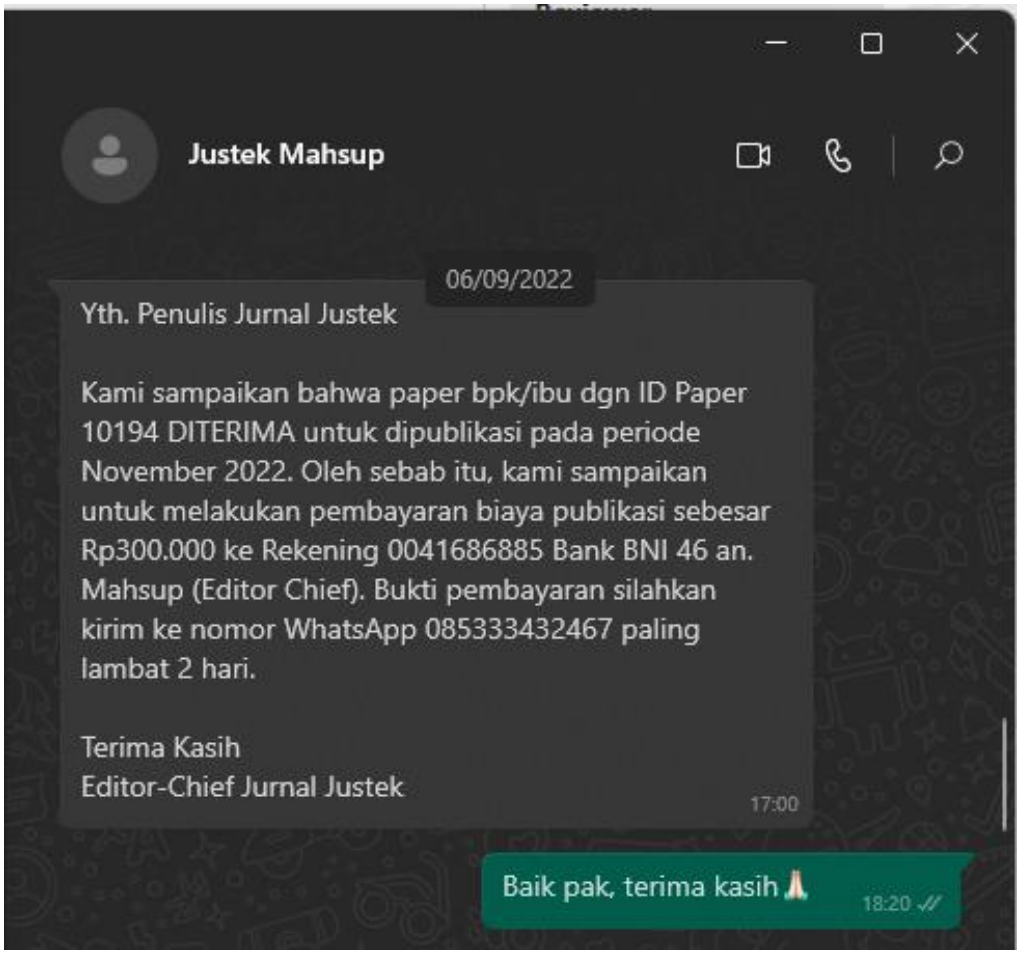

#10194 Review

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Cytotoxic Activity of Volatile Compounds in *Cymbopogon nardus*' Essential Oils

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ABSTRACT

Abstract: *Plants produce a variety of secondary metabolites, one of which is essential oils that contain a variety of volatile compounds and are useful for humans. Cymbopogon nardus contains volatile compounds that can inhibit the proliferation of cancer cells. This research aims to explore the antiproliferation activity of C.nardus' essential oils with different concentrations against breast cancer Michigan Cancer Foundation-7 (MCF-7) cells. Antiproliferation test was carried out with total cells method using trypan blue and cells were calculated using a microscope. Gas Chromatography-Mass Spectrometer (GC-MS) was performed to identify the volatile compounds. The results showed that the Inhibitory Concentration 50 (IC_{50}) value was 359.6 ppm with an inhibition percent of 44.49% at 170 ppm. Meanwhile, inhibition percent against Vero normal cell was 29.04%, compared to Doxorubicin 35.23%. The dominant volatile compound in C. nardus' essential oil were Geraniol and Citronellol.*

Abstrak Tanaman menghasilkan berbagai macam metabolit sekunder, salah satunya dalam bentuk minyak atsiri yang mengandung berbagai macam senyawa volatil yang berguna bagi manusia. *Cymbopogon nardus* mengandung senyawa volatil yang dapat menghambat proliferasi sel kanker. Penelitian ini bertujuan untuk mengetahui aktifitas antiproliferasi minyak atsiri *C. nardus* dengan beberapa konsentrasi terhadap sel kanker payudara *Michigan State Foundation-7* (MCF-7). Uji antiproliferasi dilakukan dengan metode total sel menggunakan *trypan blue* dan sel dihitung menggunakan mikroskop. *Gas Chromatography-Mass Spectrometer* (GC-MS) dilakukan untuk mengidentifikasi senyawa volatil. Hasil menunjukkan bahwa konsentrasi penghambatan 50 (IC_{50}) sebesar 359.6 ppm dengan persen penghambatan sebesar 44.49% pada konsentrasi 170 ppm. Sementara itu, persen ihibisi terhadap sel normal Vero sebesar 29.04%, dibandingkan dengan Doksorubisin yang sebesar 35.23%. Senyawa volatil dominan yang terdapat pada *C. nardus* adalah Geraniol dan Citronellol.

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A. INTRODUCTION

Cancer ranks as the main purpose of death and an essential barrier to growing existence expectancy in every country of the world. In line with estimates from the World Health Organization (WHO) in 2019, cancer is the primary or second leading cause of death before the age of 70 years in 112 of 183 countries. Cancer is an ailment that results from genetic or epigenetic alterations within the somatic cells and has ordinary cell increases which can be spread to other body components (Saini et al., 2020). Breast cancer in women ranks first in the most common cancers (11.7%) with a mortality rate of 6.9% (Sung et al., 2021). Breast cancer is metastatic cancer and may typically transfer to distant organs including the bone, liver, lung and brain, which particularly accounts for its incurability. Most breast cancers arise in women and the wide variety of instances is one hundred times higher in women than that in men. Breast tumors usually start from ductal hyperproliferation, after which grow to be benign tumors or even metastatic carcinomas after continuous stimulation through diverse carcinogenic factors (Sun et al., 2017).

Most cancer cells are very similar to cells of the organism from which they originated and have comparable (but not identical) DNA and RNA. This is the reason why they are not very often detected through the immune system, in particular, if it is weakened. Few attempts that are normally used for most cancer treatment therapies are surgical procedures, radiation therapy, and chemotherapy (Brunton et al., 2018). Recent procedures in the management of breast cancer are gene therapy, oncogenes inactivation, augmentation of tumors suppressor genes, cell-target suicide, chemoprotection method, virus-mediated oncolysis and immunomodulation (Sharma et al., 2010). These strategies purpose to destroy most cancer cells and manipulate the growth of tumors within the breast tissue affected by cancer as well as control tumors increase in different tissues that are not affected by most cancers.

Treatment that is carried out in the early stages of cancer is surgery combined with other treatments to reduce recurrences such as radiation therapy, chemotherapy, hormone therapy and targeted drugs. For cancers that have undergone metastatic, the treatment is carried out more with systematic therapies including chemotherapy, hormone therapy and targeted drugs. The purpose of surgery is to remove cancer and determine the stage. This surgery is called a mastectomy where only tissue is affected by cancer and a slight edge of normal tissue that omitted/removed. However, this mastectomy is not recommended for cancer with a large tissue ratio and multicentric cancer. Regular surgery followed by radiation, patients who have undergone radiation are not recommended to perform a mastectomy. Radiotherapy is the use of high-energy particles to kill cancer cells and is commonly used after surgery to destroy cancer cells remaining in the breast, walls chest or lower area of the arms. Radiotherapy is non-selective that can also kill normal cells and have no effect on sufferers who have

undergone hormone therapy for 5 years. Chemotherapy uses drugs that work by attacking cancer cells. Long-term use of chemotherapy drugs causes cardiomyopathy and congestive heart failure. Whereas hormone therapy works by blocking the body's natural hormones or lower hormone levels which can trigger the growth of some cancers. Side effects of this hormone therapy include the risk of blood clots, bone thinning and vision changes. Targeted drugs work by attacking specific molecules in cells that have the potential to become more active in cancer cells. An example of these drugs are Trastuzumab and Herceptin. Side effects of using these drugs include heartbeats, shortness of breath, fever to bleeding that is not ordinary (McDonald et al., 2004).

To reduce the side effects of the use of such cancer drugs, now widely used substitutes in the form of herbal medicines. Pharmacology characteristic of common herbal ingredients found in its essential oils which contains a wide range of potential secondary metabolite compounds inhibits the proliferation of cancer cells. Essential oils are hydrophobic so it can enter the cell membrane and damage the cell.

Essential oils are secondary metabolites with a key function in plant protection, consisting commonly of terpenes with a volatile nature and a various array of chemical structures (Blowman et al., 2018). Essential oils have been proven to own anticancer properties via numerous mechanisms, including cancer preventative mechanisms, as well as appearing at the established tumors cellular itself and interaction with the microenvironment (Sitarek et al., 2017). Key hallmarks of cancer embrace resisting cell death, sustained proliferative signal and evading growth suppressors. therefore, therapeutic techniques focused on inducing apoptosis and cell arrest are of clear importance. Essential oils were proven to result in both the intrinsic (or mitochondria-based) and extrinsic (or death receptor-dependent) apoptosis pathways (Amin et al., 2009).

C. nardus is known to act as an insect repellent with some studies as antiproliferation of cancer cells (Akhila, 2009). There is still few research on antiproliferation activity in *C. nardus*. Among them, it is reported that essential oils from *C. nardus* have antiproliferation potential and contain active compounds in the form of geraniol and citronellal. *C. nardus* essential oil is also reported to have cytotoxic activity against MCM-B2 breast cancer cells (Hasim et al., 2020).

To the best of our knowledge, cytotoxic potential and antiproliferative activity of *C. nardus* essential oil against MCF-7 breast cancer cells in vitro have not been investigated previously. Therefore, the present study was focused on understanding *C. nardus* essential oil as an antiproliferative substance and figuring out its volatile compound.

B. RESEARCH METHOD

Material

The ingredients used in this study were *C. nardus* leaves taken from the Biopharmaceutical Cultivation Conservation Unit Center for Biopharmaceutical Studies

Tropics with latitude (S) 6°34'37.95", longitude 106°47'20.37" and altitude (M) 238, Bogor, Indonesia. Other materials were n-hexane, water, seawater, tween 80, Artemia salina Leach, distilled water, MCF-7 cell, Vero cell, trypan blue, DMEM (Dulbecco's Modified Eagle's Medium), trypsin, gentamicin, FCS (Fetal Calf Serum), DMSO (Dimethyl Sulfoxide) and doxorubicin.

Distillation of Essential Oils

Extraction of essential oil was carried out by the method of steam distillation in a single way. A total of 3000 grams of small sliced sample were put into the distiller steam and added to distilled water in a ratio of 1:5 (sample: water). Distillation was carried out for 4 hours at a temperature of 100-105°C. Distilled essential oils then accommodated and stored at 4°C for further analysis. The percent of oil yield obtained is calculated by the formula (1):

$$\text{Yield Percent} = \frac{Oil\ Weight}{Dry\ Sample\ Weight}\ x\ 100\% \qquad (1)$$

Cytotoxicity Test of Essential Oils with Brine Shrimp Lethality Test (BSLT)

Artemia salina Leach shrimp eggs and aerators were put into a container and seawater was added, allowed to stand for 24 hours until it turned into larvae. A solution of essential oil extract was dissolved in seawater with tween 80. The concentrations of the extract solution made were 1000, 500, 100, 10µg/mL and 0µg/mL (control). The test plate was prepared and stamped with seawater as much as 2 mL and then marked with a marker. A total of 1 mL of seawater was put into the test plate and then added 10 shrimp larvae and 0.5 mL of test solution for concentrations of 1000, 500, 100, 10µg / mL and 0µg / mL (control) respectively. The larvae were incubated for 24 hours and the number of dead larvae was calculated. Control was carried out by the same procedure without the addition of extracts. The LC_{50} value was determined using probit analysis using SPSS 16.0.

Antiproliferation Test of Essential Oil against MCF-7 cells and Vero

Preparation of Media and Extracts. MCF-7 cells and Vero cells were grown on 850µL of DMEM growing media in microplates of 24 wells to which 10µL of antibiotics (gentamicin), 10µL of fungizon, 30µL of FCS and extracts were added. The stock solutions of the extract with 5 different concentrations based on the results of the BSLT test were made using 10% DMSO as the solvent. Variations in extract concentrations were determined based on LC_{50} values and tested for three repetitions.

Cell Planting. The suspension of MCF-7 and Vero cells was pre-liquefied and homogenized with a vortex. Cell planting was carried out on a 24-well tissue culture plate containing a growth medium with 5 different concentrations of extracts, without the addition of essential oils as a negative control and doxorubicin as a positive control. A total of 50µL cell suspensions were added to each hole. Performed as many as three repetitions. The cell suspension was grown by incubating it in a 37°C incubator (5% CO_2).

Harvesting and Counting cells. Cell harvesting was carried out when the cells in the control hole had grown optimally covering about 70% of the hole surface (confluence) or approximately after 3 days. Cell suspensions were homogenized using micropipettes by

sucking and removing them. A total of 80µl of homogeneous cell suspensions were placed in a microplate that already contains 20µl of trypan blue dye, so that the cells are visible under the microscope, then homogenized. The suspension was dripped on the Neubauer hemacytometer and a count of the number of cells under a light microscope with a magnification of 100x was carried out. The calculated cells were those that are in the middle box of the counting room. All cells were counted, both living and dead cells.

The formula used to calculate the percentage of activity cell growth (2) and inhibition (3) are as follows :

$$\% \text{ growth activity} = \frac{Average\ number\ of\ treatment\ cells}{Average\ number\ of\ negative\ control\ cells}\ x\ 100\% \qquad (2)$$

$$\% \text{ of inhibitory activity} = 100\% - (\% \text{ growth activity}) \qquad (3)$$

GC-MS Essential Oil Analysis

Analysis of essential oils volatile compounds using the GC-MS tool Agilent Technologies with selective mass detectors. A total of 20µL of oil plant essentials were dissolved into 2000µL of n-hexane solvent (1:100 v/v). Sample separated using HP INNOWAX capillary columns (30 m x 0.25 mm, 0.25µm film thickness) with helium gas as a carrier (0.6 mL/min). Temperature the initial for the analysis was 60°C and raised to 150°C in increments by 2°C/min for about 1 minute, then increased to 210°C with a rate of increase of 20°C/min and kept constant for 10 minutes. The ratio of the separation rate was 250:1. The temperature at injection was 250°C by 1µL of essential oils for injection.

Statistical Analysis

Cytotoxicity data of LC_{50} BSLT were obtained using SPSS probit analysis 16.0, and IC_{50} inhibition activity of essential oils on cells was analyzed using regression analysis (log it). ANOVA analysis was carried out through the Duncan test at $p \leq 0.05$ to see the differences in each treatment.

C. RESULTS AND DISCUSSION

1. Essential Oils Productivity

The yield of essential oils produced was less than the previous studies (Table 1). This difference was due to several factors such as the condition of the material and the distillation time. The condition of the withered material will produce a higher yield of essential oils compared to fresh ingredients because the moisture content in the material glands has been reduced and extraction is easier to do. A longer distillation time will result in more essential oil yields because volatile compounds that are difficult to evaporate will require a longer heating time to evaporate, in addition to the growing place and soil conditions also affect the yield of essential oils (Cassel & Vargas, 2006).

Table 1. Distilled Essential Oils Yield

Plant	Yield Experiment (%)	Yield Reference (%)	Reference
C. nardus	0.4	0.79	Cassel and Vargas 2013

2. Cytotoxicity Test of Essential Oils with BSLT

BSLT is an initial pharmacological screening method that is easy and relatively inexpensive and does not require certain specialization in its implementation (Ghosh et al., 2015). BSLT can be used as a simple bioassay to examine the toxicity level of a compound by determining the value of LC_{50}. The test was carried out using *Artemia salina* in the napoli (larval) phase. The parameter measured was the mortality rate of shrimp larvae due to the administration of test solutions, in this study it was essential oil. The cytotoxicity of essential oils was determined in the LC_{50}. The results of the BSLT test showed that *C. nardus* had toxic potential because the LC_{50} calculation results were obtained at 98.5 ppm. (Meyer et al., 1982) classify the toxicity level of a compound that is said to have potential bioactivity if the LC_{50} is less than 1000 ppm so that the essential oils of *C. nardus* had the potential to be toxic and had the potential to be antiproliferation compounds for cancer cells.

3. Antiproliferation Test of Essential Oil against MCF-7 cells and Vero

The antiproliferation activity of *C. nardus* essential oil was tested against MCF-7 cancer cells. Using the total cell method according to (Priosoeryanto et al., 1995)with the principle of negative control in the plate containing cancer cells and growth media is considered to proliferate as much as 100% so that the total treatment cells (which were given essential oil) and positive control (doxorubicin) will be compared with the total cells in the negative control and obtained a percent of proliferation and percent inhibition. Cells were incubated in the incubator for 3 to 4 days because it takes 3-4 days for the cells to grow and proliferate optimally so that cell counting can be done on day 3 or 4. If less than 3 days the cell has not grown optimally and for more than 4 days it is feared that the cell will experience death due to lack of growth nutrients. The calculation of total cells was carried out with a hemacytometer under a microscope with a magnification of 100 times. The cells were dripped with trypan blue to clarify the appearance of the cells under the microscope. The results showed that the proliferation of cancer cells decreased as the concentration of the administered extract increased. As shown in Figure 1, the administration of 170 ppm of *C. nardus* essential oil can inhibit the proliferation of cancer cells by 44.5% with an IC_{50} value of 359.6 ppm. (Koba et al., 2008) report that citronella essential oil at a concentration of 150 ppm can inhibit the growth of HaCaT cells by 23% with IC_{50} of 450 ppm.

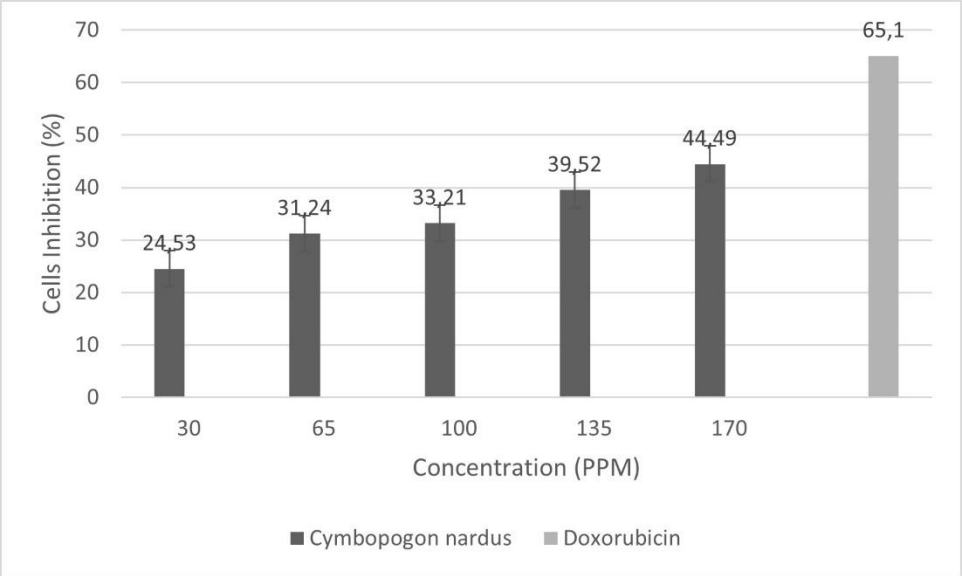

Figure 1. Effect of essential oils and doxorubicin administration on inhibition MCF-7 cells.

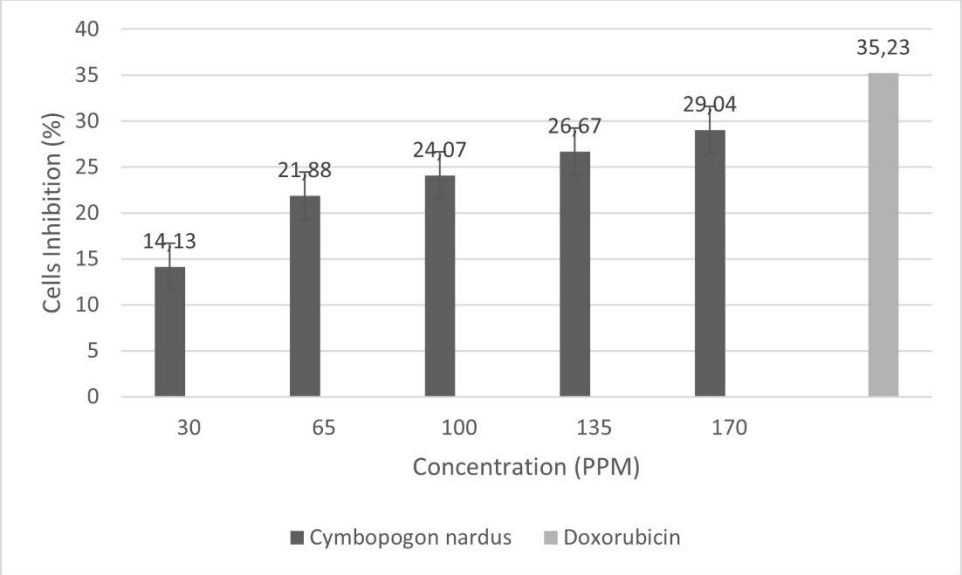

Figure 2. Effect of essential oils and doxorubicin administration on inhibition Vero normal cells.

Figure 2, the administration of *C. nardus* essential oils to Vero normal cells also shows a noticeable difference compared to doxorubicin. Testing of Vero normal cells was also carried out to determine the effect of essential oils administration on normal cell growth. *C. nardus* essential oils gave an inhibitory result of 29.0% at a concentration of 170 ppm for Vero normal cells while doxorubicin inhibited the growth of Vero normal cells by 35.23%.

As a comparison, doxorubicin (a drug used for cancer therapy) was used against MCF-7 cells and Vero cells. Figures 1 and 2 show that doxorubicin concentrations of 100 ppm inhibited MCF-7 cells by 65.1% but also damage Vero normal cell proliferation by 35.2%, this is in contrast to inhibition by *C. nardus* at the same concentration which only inhibited Vero cell proliferation by 29% and indicated that the essential oil of *C. nardus* was safer against normal cells compared to doxorubicin.

4. GC-MS Essential Oil Analysis

The results of GC-MS obtained a total of 20 volatile compounds contained in the essential oil of *C. nardus*. It consists of 13 secondary metabolite compounds and 7 hydrocarbon compounds. The dominant volatile compounds found in *C. nardus* include geraniol (35.2%), citronellol (14%) and citronella (10.6%). The types of volatile compounds found in the essential oil of *C. nardus* will be presented in Table 2.

Table 2. Volatile Compounds Found In Essential Oils

No	Compound	Faction	Chemistry Structure	Molecule Weight (g mol^{-1})	Retention Time (min)	Peak Area (%)
1	Benzene	Hydrocarbon	C_6H_6	78	3.83	3.62
2	Acetic acid	Carboxylic acid	CH_3COOH	60	4.23	18.46
3	**Limonene**	Monoterpene	$C_{10}H_{16}$	136	7.10	0.56
4	**Cis-ocimene**	Monoterpene	$C_{10}H_{16}$	136	8.24	0.46
5	**Citronella**	Monoterpene	$C_{10}H_{18}O$	154	18.96	10.59
6	**Trans-caryophyllene**	Sesquiterpene	$C_{15}H_{24}$	204	24.66	2.16
7	Citronellyl propionate	Ester	$C_{13}H_{24}O_2$	212	28.70	0.96
8	**Z-Citral**	Monoterpene	$C_{10}H_{16}O$	152	29.45	1.73
9	**Cis-citral**	Monoterpene	$C_{10}H_{16}O$	152	32.19	2.29
10	**Naphtalene**	Monoterpene	$C_{10}H_8$	128	33.26	0.52
11	**Gamma-muurolene**	Sesquiterpene	$C_{15}H_{24}$	204	33.28	0.46
12	**Alpha-amorphene**	Sesquiterpene	$C_{15}H_{24}$	204	33.31	1.45
13	**Nerol**	Monoterpene	$C_{10}H_{18}O$	154	33.77	2.62
14	**Citronellol**	Monoterpene	$C_{10}H_{20}O$	156	34.44	14.05
15	**Geraniol**	Monoterpene	$C_{10}H_{18}O$	154	38.63	35.21
16	Methyleugenol	Eter	$C_{11}H_{14}O_2$	178	46.64	1.00
17	**Germacrene**	Sesquiterpene	$C_{15}H_{24}$	204	47.36	0.41
18	2-butanone	Ketone	C_4H_8O	72	48.46	0.23
19	Anisylaceton	Ketone	$C_{10}H_{12}O_2$	164	49.60	2.05
20	Phenol	Alcohol	C_6H_6O	94	51.97	0.59

The bolded are secondary metabolite volatile compounds

The results of GC-MS show that the majority of volatile compounds in *C. nardus* were from the terpenoid group, monoterpenes (45.00%) or sesquiterpenes (20.00%). Other volatile compounds were derived from the group of hydrocarbons

(5.00 %), carboxylic acids (5.00%), esters (5.00%), ethers (5.00%), ketones (10.00%) and alcohols (5.00%).

The dominant volatile compounds in *C. nardus* essential oil that were alleged to have potential to inhibit the proliferation of cancer cells were geraniol, citronellol, citronella, cis-citral, trans-caryophyllene, limonene, naphthalene and germacrene. Such compounds were alleged to inhibit the proliferation of cancer cells by certain inhibitory mechanisms.

Geraniol belongs to the monoterpene class of acyclic alcohols and is commonly contained in drugs for cancer therapy such as 5-fluorouracil and docetaxel (Carnesecchi et al., 2002). Geraniol can suppress the growth of MCF-7 cells by reducing protein levels of cyclin D1, cyclin-dependent kinase 4 (CDK-4), cyclin E and cyclin A and increasing P27 levels so that cell cycle containment occurs in phase G1 and cell proliferation stops (Duncan et al., 2004). Citronellol compound according to (Zhuang et al., 2009) has anticancer potential but the inhibition mechanism is not yet known. Antiproliferation test found that the essential oil of *C. nardus* had the potential to inhibit the growth of MCF-7 cancer cells but also inhibits the growth of normal Vero cells, in other words is also toxic to Vero cells. This is due to the presence of citronella volatile compound, a monoterpenoid compound $C_{10}H_{18}O$. As reported by (Stone et al., 2013), citronella is toxic in Vero cells because with 0.74.10-2% it can inhibit 50% of cell proliferation and has a Selectivity Index (SI) of 1.6 whereas if a compound has an SI below 3 it can be said that the compound is non-selective (Prayong et al., 2008). *C. Nardus* essential oils also contain cis-citral (neral) which is alleged to have antiproliferation potential for cancer cells. (Zeng et al., 2015) reported that citral compounds can inhibit the proliferation of 4TI cancer cells (rat breast cancer) in vivo but the mechanism of inhibition is not yet known with certainty. Trans Caryophyllene is also a compound found in *C. nardus* that is alleged to be able to inhibit the proliferation of cancer cells. According to Dahham et al., (2015) trans-caryophyllene can inhibit the proliferation of bowel cancer cells HCT-116 and HT-29 in-vitro by inducing the process of apoptosis of the mitochondrial pathway.

Limonene is alleged to inhibit cancer cell proliferation by activating the apoptotic pathway through the activation of caspase 3 and 9 and increasing the cleaved PARP protein expression. These mechanisms help to establish Limonene as a powerful pro-apoptotic agent to serve as an important therapeutic target (Araújo-Filho et al., 2021). Naphthalene, according to Luo et al., (2021) had in-vitro cytotoxic activity by arresting the cell cycle in the S phase and inducing apoptosis in MDA-MB-231 cells. The percentage of MDA-MB-231 cells in the G1 phase decrease significantly from 74.39% to 39.14% and 55.31%. Whereas, the cells in the S phase increased from 19.84% to 36.99% and 21.86%. According to (Cao et al., 2018) germacrene had anti-cancer activity toward T24 cells. The amount of T24 cells was reduced drastically. Whereas the shape of the cells gradually changed from wedge-shaped and polygonal into a round then the cells soon fell off, atrophied and

disintegrated. Cells then were observed to be dead after the administration of germacrene. The inhibitory mechanism of germacrene needs to be explored further.

D. CONCLUSIONS AND SUGGESTIONS

The essential oil of *C. nardus* has the potential to inhibit the proliferation of MCF-7 cancer cells in vitro with a percent inhibition of 44.49% in 170 ppm (highest concentration). In inhibiting the proliferation of normal cells of Vero, the essential oil of *C. nardus* was better than doxorubicin with a percent inhibition of 29.04%. This inhibition was caused by the presence of volatile compounds contained in essential oils such as geraniol (35.21%), citronellol (14.05%), citronella (10.59%), cis-citral (2.29%), trans-caryophyllene (2.16%), limonene (0.56%), naphthalene (0.52%) and germacrene (0.41%) that were alleged to inhibit cell proliferation through several pathways such as the cell cycle arrest until triggering the apoptosis process. It is necessary to conduct an induction test of cell apoptosis, analysis of mitochondrial damage and analysis of flow cytometry to find out the exact mechanism in the process of inhibition of cancer cells by essential oils.

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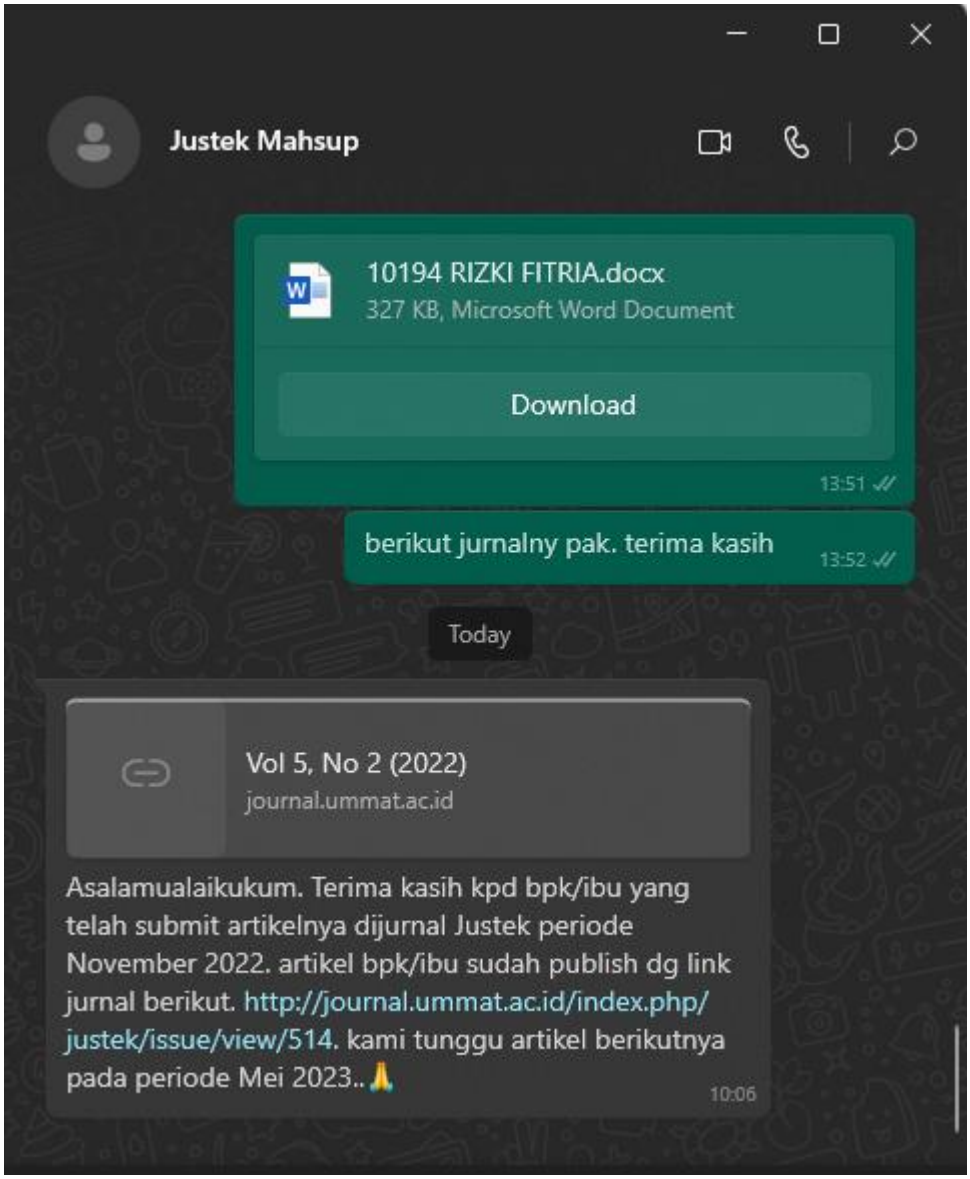
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Cytotoxic Activity of Volatile Compounds in *Cymbopogon nardus*' Essential Oils

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ABSTRACT

Abstract: *Plants produce a variety of secondary metabolites, one of which is essential oils that contain a variety of volatile compounds and are useful for humans. Cymbopogon nardus contains volatile compounds that can inhibit the proliferation of cancer cells. This research aims to explore the antiproliferation activity of C.nardus' essential oils with different concentrations against breast cancer Michigan Cancer Foundation-7 (MCF-7) cells. Antiproliferation test was carried out with total cells method using trypan blue and cells were calculated using a microscope. Gas Chromatography-Mass Spectrometer (GC-MS) was performed to identify the volatile compounds. The results showed that the Inhibitory Concentration 50 (IC_{50}) value was 359.6 ppm with an inhibition percent of 44.49% at 170 ppm. Meanwhile, inhibition percent against Vero normal cell was 29.04%, compared to Doxorubicin 35.23%. The dominant volatile compound in C. nardus' essential oil were Geraniol and Citronellol.*

Abstrak Tanaman menghasilkan berbagai macam metabolit sekunder, salah satunya dalam bentuk minyak atsiri yang mengandung berbagai macam senyawa volatil yang berguna bagi manusia. *Cymbopogon nardus* mengandung senyawa volatil yang dapat menghambat proliferasi sel kanker. Penelitian ini bertujuan untuk mengetahui aktifitas antiproliferasi minyak atsiri *C. nardus* dengan beberapa konsentrasi terhadap sel kanker payudara *Michigan State Foundation-7* (MCF-7). Uji antiproliferasi dilakukan dengan metode total sel menggunakan *trypan blue* dan sel dihitung menggunakan mikroskop. *Gas Chromatography-Mass Spectrometer* (GC-MS) dilakukan untuk mengidentifikasi senyawa volatil. Hasil menunjukkan bahwa konsentrasi penghambatan 50 (IC_{50}) sebesar 359.6 ppm dengan persen penghambatan sebesar 44.49% pada konsentrasi 170 ppm. Sementara itu, persen ihibisi terhadap sel normal Vero sebesar 29.04%, dibandingkan dengan Doksorubisin yang sebesar 35.23%. Senyawa volatil dominan yang terdapat pada *C. nardus* adalah Geraniol dan Citronellol.

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A. INTRODUCTION

Cancer ranks as the main purpose of death and an essential barrier to growing existence expectancy in every country of the world. In line with estimates from the World Health Organization (WHO) in 2019, cancer is the primary or second leading cause of death before the age of 70 years in 112 of 183 countries. Cancer is an ailment that results from genetic or epigenetic alterations within the somatic cells and has ordinary cell increases which can be spread to other body components (Saini et al., 2020). Breast cancer in women ranks first in the most common cancers (11.7%) with a mortality rate of 6.9% (Sung et al., 2021). Breast cancer is metastatic cancer and may typically transfer to distant organs including the bone, liver, lung and brain, which particularly accounts for its incurability. Most breast cancers arise in women and the wide variety of instances is one hundred times higher in women than that in men. Breast tumors usually start from ductal hyperproliferation, after which grow to be benign tumors or even metastatic carcinomas after continuous stimulation through diverse carcinogenic factors (Sun et al., 2017).

Most cancer cells are very similar to cells of the organism from which they originated and have comparable (but not identical) DNA and RNA. This is the reason why they are not very often detected through the immune system, in particular, if it is weakened. Few attempts that are normally used for most cancer treatment therapies are surgical procedures, radiation therapy, and chemotherapy (Brunton et al., 2018). Recent procedures in the management of breast cancer are gene therapy, oncogenes inactivation, augmentation of tumors suppressor genes, cell-target suicide, chemoprotection method, virus-mediated oncolysis and immunomodulation (Sharma et al., 2010). These strategies purpose to destroy most cancer cells and manipulate the growth of tumors within the breast tissue affected by cancer as well as control tumors increase in different tissues that are not affected by most cancers.

Treatment that is carried out in the early stages of cancer is surgery combined with other treatments to reduce recurrences such as radiation therapy, chemotherapy, hormone therapy and targeted drugs. For cancers that have undergone metastatic, the treatment is carried out more with systematic therapies including chemotherapy, hormone therapy and targeted drugs. The purpose of surgery is to remove cancer and determine the stage. This surgery is called a mastectomy where only tissue is affected by cancer and a slight edge of normal tissue that omitted/removed. However, this mastectomy is not recommended for cancer with a large tissue ratio and multicentric cancer. Regular surgery followed by radiation, patients who have undergone radiation are not recommended to perform a mastectomy. Radiotherapy is the use of high-energy particles to kill cancer cells and is commonly used after surgery to destroy cancer cells remaining in the breast, walls chest or lower area of the arms. Radiotherapy is non-selective that can also kill normal cells and have no effect on sufferers who have undergone hormone therapy for 5 years. Chemotherapy uses drugs that work by attacking cancer cells. Long-term use of chemotherapy drugs causes cardiomyopathy and congestive heart failure. Whereas hormone therapy works by blocking the body's natural hormones or lower hormone levels which can trigger the growth of some

cancers. Side effects of this hormone therapy include the risk of blood clots, bone thinning and vision changes. Targeted drugs work by attacking specific molecules in cells that have the potential to become more active in cancer cells. An example of these drugs are Trastuzumab and Herceptin. Side effects of using these drugs include heartbeats, shortness of breath, fever to bleeding that is not ordinary (McDonald et al., 2004).

To reduce the side effects of the use of such cancer drugs, now widely used substitutes in the form of herbal medicines. Pharmacology characteristic of common herbal ingredients found in its essential oils which contains a wide range of potential secondary metabolite compounds inhibits the proliferation of cancer cells. Essential oils are hydrophobic so it can enter the cell membrane and damage the cell.

Essential oils are secondary metabolites with a key function in plant protection, consisting commonly of terpenes with a volatile nature and a various array of chemical structures (Blowman et al., 2018). Essential oils have been proven to own anticancer properties via numerous mechanisms, including cancer preventative mechanisms, as well as appearing at the established tumors cellular itself and interaction with the microenvironment (Sitarek et al., 2017). Key hallmarks of cancer embrace resisting cell death, sustained proliferative signal and evading growth suppressors. therefore, therapeutic techniques focused on inducing apoptosis and cell arrest are of clear importance. Essential oils were proven to result in both the intrinsic (or mitochondria-based) and extrinsic (or death receptor-dependent) apoptosis pathways (Amin et al., 2009).

C. nardus is known to act as an insect repellent with some studies as antiproliferation of cancer cells (Akhila, 2009). There is still few research on antiproliferation activity in *C. nardus*. Among them, it is reported that essential oils from *C. nardus* have antiproliferation potential and contain active compounds in the form of geraniol and citronellal. *C. nardus* essential oil is also reported to have cytotoxic activity against MCM-B2 breast cancer cells (Hasim et al., 2020).

To the best of our knowledge, cytotoxic potential and antiproliferative activity of *C. nardus* essential oil against MCF-7 breast cancer cells in vitro have not been investigated previously. Therefore, the present study was focused on understanding *C. nardus* essential oil as an antiproliferative substance and figuring out its volatile compound.

B. RESEARCH METHOD

Material

The ingredients used in this study were *C. nardus* leaves taken from the Biopharmaceutical Cultivation Conservation Unit Center for Biopharmaceutical Studies Tropics with latitude (S) 6°34'37.95", longitude 106°47'20.37" and altitude (M) 238, Bogor, Indonesia. Other materials were n-hexane, water, seawater, tween 80, Artemia salina Leach, distilled water, MCF-7 cell, Vero cell, trypan blue, DMEM (Dulbecco's Modified Eagle's Medium), trypsin, gentamicin, FCS (Fetal Calf Serum), DMSO (Dimethyl Sulfoxide) and doxorubicin.

Distillation of Essential Oils

Extraction of essential oil was carried out by the method of steam distillation in a single way. A total of 3000 grams of small sliced sample were put into the distiller steam and added to distilled water in a ratio of 1:5 (sample: water). Distillation was carried out for 4 hours at a temperature of 100-105°C. Distilled essential oils then accommodated and stored at 4°C for further analysis. The percent of oil yield obtained is calculated by the formula (1):

$$\text{Yield Percent} = \frac{Oil\ Weight}{Dry\ Sample\ Weight}\ x\ 100\% \qquad (1)$$

Cytotoxicity Test of Essential Oils with Brine Shrimp Lethality Test (BSLT)

Artemia salina Leach shrimp eggs and aerators were put into a container and seawater was added, allowed to stand for 24 hours until it turned into larvae. A solution of essential oil extract was dissolved in seawater with tween 80. The concentrations of the extract solution made were 1000, 500, 100, 10µg/mL and 0µg/mL (control). The test plate was prepared and stamped with seawater as much as 2 mL and then marked with a marker. A total of 1 mL of seawater was put into the test plate and then added 10 shrimp larvae and 0.5 mL of test solution for concentrations of 1000, 500, 100, 10µg / mL and 0µg / mL (control) respectively. The larvae were incubated for 24 hours and the number of dead larvae was calculated. Control was carried out by the same procedure without the addition of extracts. The LC_{50} value was determined using probit analysis using SPSS 16.0.

Antiproliferation Test of Essential Oil against MCF-7 cells and Vero

Preparation of Media and Extracts. MCF-7 cells and Vero cells were grown on 850µL of DMEM growing media in microplates of 24 wells to which 10µL of antibiotics (gentamicin), 10µL of fungizon, 30µL of FCS and extracts were added. The stock solutions of the extract with 5 different concentrations based on the results of the BSLT test were made using 10% DMSO as the solvent. Variations in extract concentrations were determined based on LC_{50} values and tested for three repetitions.

Cell Planting. The suspension of MCF-7 and Vero cells was pre-liquefied and homogenized with a vortex. Cell planting was carried out on a 24-well tissue culture plate containing a growth medium with 5 different concentrations of extracts, without the addition of essential oils as a negative control and doxorubicin as a positive control. A total of 50µL cell suspensions were added to each hole. Performed as many as three repetitions. The cell suspension was grown by incubating it in a 37°C incubator (5% CO_2).

Harvesting and Counting cells. Cell harvesting was carried out when the cells in the control hole had grown optimally covering about 70% of the hole surface (confluence) or approximately after 3 days. Cell suspensions were homogenized using micropipettes by sucking and removing them. A total of 80µl of homogeneous cell suspensions were placed in a microplate that already contains 20µl of trypan blue dye, so that the cells are visible under the microscope, then homogenized. The suspension was dripped on the Neubauer hemacytometer and a count of the number of cells under a light microscope

with a magnification of 100x was carried out. The calculated cells were those that are in the middle box of the counting room. All cells were counted, both living and dead cells.

The formula used to calculate the percentage of activity cell growth (2) and inhibition (3) are as follows :

$$\% \text{ growth activity} = \frac{Average\ number\ of\ treatment\ cells}{Average\ number\ of\ negative\ control\ cells}\ x\ 100\% \qquad (2)$$

$$\% \text{ of inhibitory activity} = 100\% - (\% \text{ growth activity}) \qquad (3)$$

GC-MS Essential Oil Analysis

Analysis of essential oils volatile compounds using the GC-MS tool Agilent Technologies with selective mass detectors. A total of 20µL of oil plant essentials were dissolved into 2000µL of n-hexane solvent (1:100 v/v). Sample separated using HP INNOWAX capillary columns (30 m x 0.25 mm, 0.25µm film thickness) with helium gas as a carrier (0.6 mL/min). Temperature the initial for the analysis was 60°C and raised to 150°C in increments by 2°C/min for about 1 minute, then increased to 210°C with a rate of increase of 20°C/min and kept constant for 10 minutes. The ratio of the separation rate was 250:1. The temperature at injection was 250°C by 1µL of essential oils for injection.

Statistical Analysis

Cytotoxicity data of LC_{50} BSLT were obtained using SPSS probit analysis 16.0, and IC_{50} inhibition activity of essential oils on cells was analyzed using regression analysis (log it). ANOVA analysis was carried out through the Duncan test at $p \leq 0.05$ to see the differences in each treatment.

C. RESULTS AND DISCUSSION

1. Essential Oils Productivity

The yield of essential oils produced was less than the previous studies (Table 1). This difference was due to several factors such as the condition of the material and the distillation time. The condition of the withered material will produce a higher yield of essential oils compared to fresh ingredients because the moisture content in the material glands has been reduced and extraction is easier to do. A longer distillation time will result in more essential oil yields because volatile compounds that are difficult to evaporate will require a longer heating time to evaporate, in addition to the growing place and soil conditions also affect the yield of essential oils (Cassel & Vargas, 2006).

Table 1. Distilled Essential Oils Yield

Plant	Yield Experiment (%)	Yield Reference (%)	Reference
C. nardus	0.4	0.79	Cassel and Vargas 2013

2. Cytotoxicity Test of Essential Oils with BSLT

BSLT is an initial pharmacological screening method that is easy and relatively inexpensive and does not require certain specialization in its implementation (Ghosh et al., 2015). BSLT can be used as a simple bioassay to examine the toxicity level of a compound by determining the value of LC_{50}. The test was carried out using *Artemia salina* in the napoli (larval) phase. The parameter measured was the mortality rate of shrimp larvae due to the administration of test solutions, in this study it was essential oil. The cytotoxicity of essential oils was determined in the LC_{50}. The results of the BSLT test showed that *C. nardus* had toxic potential because the LC_{50} calculation results were obtained at 98.5 ppm. (Meyer et al., 1982) classify the toxicity level of a compound that is said to have potential bioactivity if the LC_{50} is less than 1000 ppm so that the essential oils of *C. nardus* had the potential to be toxic and had the potential to be antiproliferation compounds for cancer cells.

3. Antiproliferation Test of Essential Oil against MCF-7 cells and Vero

The antiproliferation activity of *C. nardus* essential oil was tested against MCF-7 cancer cells. Using the total cell method according to (Priosoeryanto et al., 1995)with the principle of negative control in the plate containing cancer cells and growth media is considered to proliferate as much as 100% so that the total treatment cells (which were given essential oil) and positive control (doxorubicin) will be compared with the total cells in the negative control and obtained a percent of proliferation and percent inhibition. Cells were incubated in the incubator for 3 to 4 days because it takes 3-4 days for the cells to grow and proliferate optimally so that cell counting can be done on day 3 or 4. If less than 3 days the cell has not grown optimally and for more than 4 days it is feared that the cell will experience death due to lack of growth nutrients. The calculation of total cells was carried out with a hemacytometer under a microscope with a magnification of 100 times. The cells were dripped with trypan blue to clarify the appearance of the cells under the microscope. The results showed that the proliferation of cancer cells decreased as the concentration of the administered extract increased. As shown in Figure 1, the administration of 170 ppm of *C. nardus* essential oil can inhibit the proliferation of cancer cells by 44.5% with an IC_{50} value of 359.6 ppm. (Koba et al., 2008) report that citronella essential oil at a concentration of 150 ppm can inhibit the growth of HaCaT cells by 23% with IC_{50} of 450 ppm.

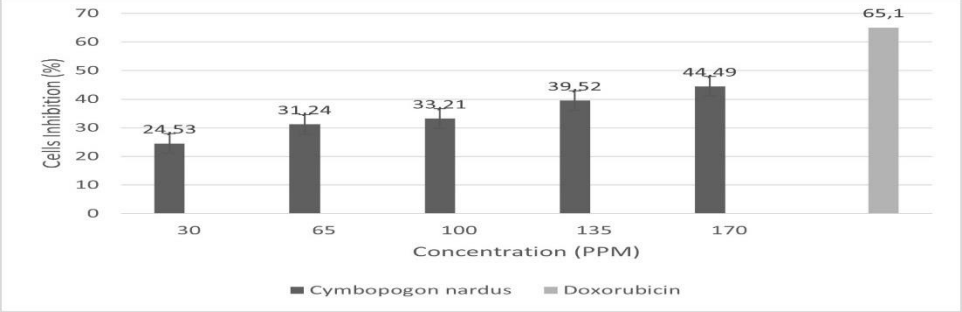

Figure 1. Effect of essential oils and doxorubicin administration on inhibition MCF-7 cells.

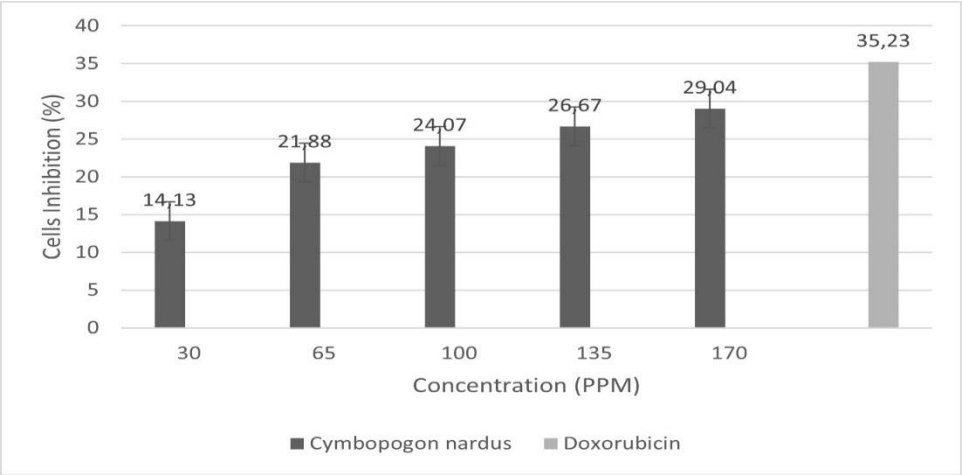

Figure 2. Effect of essential oils and doxorubicin administration on inhibition Vero normal cells.

Figure 2, the administration of *C. nardus* essential oils to Vero normal cells also shows a noticeable difference compared to doxorubicin. Testing of Vero normal cells was also carried out to determine the effect of essential oils administration on normal cell growth. *C. nardus* essential oils gave an inhibitory result of 29.0% at a concentration of 170 ppm for Vero normal cells while doxorubicin inhibited the growth of Vero normal cells by 35.23%.

As a comparison, doxorubicin (a drug used for cancer therapy) was used against MCF-7 cells and Vero cells. Figures 1 and 2 show that doxorubicin concentrations of 100 ppm inhibited MCF-7 cells by 65.1% but also damage Vero normal cell proliferation by 35.2%, this is in contrast to inhibition by *C. nardus* at the same concentration which only inhibited Vero cell proliferation by 29% and indicated that the essential oil of *C. nardus* was safer against normal cells compared to doxorubicin.

4. GC-MS Essential Oil Analysis

The results of GC-MS obtained a total of 20 volatile compounds contained in the essential oil of *C. nardus*. It consists of 13 secondary metabolite compounds and 7 hydrocarbon compounds. The dominant volatile compounds found in *C. nardus* include geraniol (35.2%), citronellol (14%) and citronella (10.6%). The types of volatile compounds found in the essential oil of *C. nardus* will be presented in Table 2.

Table 2. Volatile Compounds Found In Essential Oils

No	Compound	Faction	Chemistry Structure	Molecule Weight (g mol^{-1})	Retention Time (min)	Peak Area (%)
1	Benzene	Hydrocarbon	C_6H_6	78	3.83	3.62
2	Acetic acid	Carboxylic acid	CH_3COOH	60	4.23	18.46
3	**Limonene**	Monoterpene	$C_{10}H_{16}$	136	7.10	0.56
4	**Cis-ocimene**	Monoterpene	$C_{10}H_{16}$	136	8.24	0.46
5	**Citronella**	Monoterpene	$C_{10}H_{18}O$	154	18.96	10.59

6	**Trans-caryophyllene**	Sesquiterpene	$C_{15}H_{24}$	204	24.66	2.16
7	Citronellyl propionate	Ester	$C_{13}H_{24}O_2$	212	28.70	0.96
8	**Z-Citral**	Monoterpene	$C_{10}H_{16}O$	152	29.45	1.73
9	**Cis-citral**	Monoterpene	$C_{10}H_{16}O$	152	32.19	2.29
10	**Naphtalene**	Monoterpene	$C_{10}H_8$	128	33.26	0.52
11	**Gamma-muurolene**	Sesquiterpene	$C_{15}H_{24}$	204	33.28	0.46
12	**Alpha-amorphene**	Sesquiterpene	$C_{15}H_{24}$	204	33.31	1.45
13	**Nerol**	Monoterpene	$C_{10}H_{18}O$	154	33.77	2.62
14	**Citronellol**	Monoterpene	$C_{10}H_{20}O$	156	34.44	14.05
15	**Geraniol**	Monoterpene	$C_{10}H_{18}O$	154	38.63	35.21
16	Methyleugenol	Eter	$C_{11}H_{14}O_2$	178	46.64	1.00
17	**Germacrene**	Sesquiterpene	$C_{15}H_{24}$	204	47.36	0.41
18	2-butanone	Ketone	C_4H_8O	72	48.46	0.23
19	Anisylaceton	Ketone	$C_{10}H_{12}O_2$	164	49.60	2.05
20	Phenol	Alcohol	C_6H_6O	94	51.97	0.59

The bolded are secondary metabolite volatile compounds

The results of GC-MS show that the majority of volatile compounds in *C. nardus* were from the terpenoid group, monoterpenes (45.00%) or sesquiterpenes (20.00%). Other volatile compounds were derived from the group of hydrocarbons (5.00 %), carboxylic acids (5.00%), esters (5.00%), ethers (5.00%), ketones (10.00%) and alcohols (5.00%).

The dominant volatile compounds in *C. nardus* essential oil that were alleged to have potential to inhibit the proliferation of cancer cells were geraniol, citronellol, citronella, cis-citral, trans-caryophyllene, limonene, naphthalene and germacrene. Such compounds were alleged to inhibit the proliferation of cancer cells by certain inhibitory mechanisms.

Geraniol belongs to the monoterpene class of acyclic alcohols and is commonly contained in drugs for cancer therapy such as 5-fluorouracil and docetaxel (Carnesecchi et al., 2002). Geraniol can suppress the growth of MCF-7 cells by reducing protein levels of cyclin D1, cyclin-dependent kinase 4 (CDK-4), cyclin E and cyclin A and increasing P27 levels so that cell cycle containment occurs in phase G1 and cell proliferation stops (Duncan et al., 2004). Citronellol compound according to (Zhuang et al., 2009) has anticancer potential but the inhibition mechanism is not yet known. Antiproliferation test found that the essential oil of *C. nardus* had the potential to inhibit the growth of MCF-7 cancer cells but also inhibits the growth of normal Vero cells, in other words is also toxic to Vero cells. This is due to the presence of citronella volatile compound, a monoterpenoid compound $C_{10}H_{18}O$. As reported by (Stone et al., 2013), citronella is toxic in Vero cells because with 0.74.10-2% it can inhibit 50% of cell proliferation and has a Selectivity Index (SI) of 1.6 whereas if a compound has an SI below 3 it can be said

that the compound is non-selective (Prayong et al., 2008). *C. Nardus* essential oils also contain cis-citral (neral) which is alleged to have antiproliferation potential for cancer cells. (Zeng et al., 2015) reported that citral compounds can inhibit the proliferation of 4TI cancer cells (rat breast cancer) in vivo but the mechanism of inhibition is not yet known with certainty. Trans Caryophyllene is also a compound found in *C. nardus* that is alleged to be able to inhibit the proliferation of cancer cells. According to Dahham et al., (2015) trans-caryophyllene can inhibit the proliferation of bowel cancer cells HCT-116 and HT-29 in-vitro by inducing the process of apoptosis of the mitochondrial pathway.

Limonene is alleged to inhibit cancer cell proliferation by activating the apoptotic pathway through the activation of caspase 3 and 9 and increasing the cleaved PARP protein expression. These mechanisms help to establish Limonene as a powerful pro-apoptotic agent to serve as an important therapeutic target (Araújo-Filho et al., 2021). Naphthalene, according to Luo et al., (2021) had in-vitro cytotoxic activity by arresting the cell cycle in the S phase and inducing apoptosis in MDA-MB-231 cells. The percentage of MDA-MB-231 cells in the G1 phase decrease significantly from 74.39% to 39.14% and 55.31%. Whereas, the cells in the S phase increased from 19.84% to 36.99% and 21.86%. According to (Cao et al., 2018) germacrene had anti-cancer activity toward T24 cells. The amount of T24 cells was reduced drastically. Whereas the shape of the cells gradually changed from wedge-shaped and polygonal into a round then the cells soon fell off, atrophied and disintegrated. Cells then were observed to be dead after the administration of germacrene. The inhibitory mechanism of germacrene needs to be explored further.

D. CONCLUSIONS AND SUGGESTIONS

The essential oil of *C. nardus* has the potential to inhibit the proliferation of MCF-7 cancer cells in vitro with a percent inhibition of 44.49% in 170 ppm (highest concentration). In inhibiting the proliferation of normal cells of Vero, the essential oil of *C. nardus* was better than doxorubicin with a percent inhibition of 29.04%. This inhibition was caused by the presence of volatile compounds contained in essential oils such as geraniol (35.21%), citronellol (14.05%), citronella (10.59%), cis-citral (2.29%), trans-caryophyllene (2.16%), limonene (0.56%), naphthalene (0.52%) and germacrene (0.41%) that were alleged to inhibit cell proliferation through several pathways such as the cell cycle arrest until triggering the apoptosis process. It is necessary to conduct an induction test of cell apoptosis, analysis of mitochondrial damage and analysis of flow cytometry to find out the exact mechanism in the process of inhibition of cancer cells by essential oils.

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