INTRODUCTION

Breast cancer (BC) and cervical cancer (CC) are the first and second most diagnosed cancer in women in developing countries, and being the third main cause of cancer deaths (Bray, et al., 2018). The high prevalence and mortality by BC and CC along with weaknesses of existing management and prevention raise the urgency and need for discovery.

Inhibition Capacity of the n-Hexane Fraction of *Myrmecodia pendens* as a Potential Anti-Cancer in Breast and Cervical Cancer: *In Vitro* Study

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Abstract

Breast cancer (BC) and cervical cancer (CC) have a high prevalence and mortality rate worldwide. Despite the availability of advanced treatment, resistance to conventional chemotherapies has emerged. *Myrmecodia pendens*, one of the species of *Sarang Semut* (local name), possess a potential of antitumor effects by inducing cell death different cancer cell entities. This study aimed to assess anti-tumor activities of n-hexane fraction of *M. pendens* in inhibiting cell survival and cell migration in BC and CC cells. *M. pendens* was extracted in methanol then fractionated using n-hexane or ethyl acetate. BC cells including MCF-7 (luminal A), HCC-1954 (HER2+) cells and CC Hela cells were treated with *M. pendens* extracts to evaluate cytotoxic activity using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay as well as anti-cell migration using scratch assay. We also analyzed inhibitory concentration 50 (IC₅₀) of n-hexane fraction in BC and CC cells. *M. pendens* was extracted in methanol then fractionated using n-hexane or ethyl acetate. BC cells including MCF-7 (luminal A), HCC-1954 (HER2+) cells and CC Hela cells were treated with *M. pendens* extracts to evaluate cytotoxic activity using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay as well as anti-cell migration using scratch assay. We also analyzed inhibitory concentration 50 (IC₅₀) of n-hexane fraction in BC and CC cells. We started with comparing cytotoxicity activities of methanol extract, ethyl acetate and n-hexane fractions of *M. pendens*. Data showed that the n-hexane fraction was the most potent inducing BC cell death. Therefore, we used the n-hexane fraction for further experiments. Interestingly, IC₅₀ of this fraction in HCC-1954 and Hela cells were lower than in MCF-7 cells, 16; 13 and 60 ppm, respectively. Moreover, the low concentrations of n-hexane fraction inhibited HeLa cells migration, compared to control group (p<0.05). The n-hexane fraction of *M. pendens* shows promising anti-cancer agent, by inhibiting BC and CC cell survival as well as inhibiting CC cells migration.

Keywords: breast cancer, cervical cancer, MTT assay, Sarang Semut, scratch assay
of novel drugs (Aungsumart, et al., 2007; Bray, et al., 2018). Indonesia as an archipelago country possesses botanical biodiversity potential that incompletely developed for alternative cancer treatment. Sarang Semut is a local Indonesian plant which abundantly found in Papua island, in the central mountains of Jayawijaya, Tolikara, Puncak Jaya, Gunung Bintang, and Paniai (Soeksmanto, et al., 2010). From all documented Sarang Semut species, only Hydnophytum formicarum, Myrmecodia pendens and Myrmecodia tuberosa are regarded as useful as medications (Soeksmanto, et al., 2010). In Papua island, Sarang Semut was used traditionally for treating ulcers, hemorrhoids, epistaxis, backache, allergy, uric acid disorders, stroke, coronary heart problems, tuberculosis, and others (Soeksmanto, et al., 2010). This plant contains abundant bioactive substances including flavonoids, tocopherols, tannins, and many essential minerals (Sanjaya, et al., 2014; Engida, et al., 2015). Our published data reported the antibacterial terpenoids from Sarang Semut against pathogenic oral bacteria E. faecalis ATCC 29212, S. mutans ATCC 25175 and P. gingivalis TACC 10556 (Kurnia, et al., 2017).

In particular cancer research, Sarang Semut plant has been shown cytotoxic effects in different cancer cell lines from different type of extractions (Soeksmanto, et al., 2010; Fatmawati, Puspitasari & Yusuf, 2011; Rifaian, et al., 2015; Bashari, et al., 2018). Sarang Semut extract has been shown to have a cytotoxic effect in HeLa and MCM-B2 cells (Soeksmanto, et al., 2010; Fatmawati, Puspitasari & Yusuf, 2011). The n-hexane fraction of M. pendens has been appraised to have the strongest cytotoxic activity compared with other extracts of M. pendens on colon cancer cells (Bashari, et al., 2018). Previous studies showed ethyl acetate fraction, water extract as well as ethanol extract of Sarang Semut trigger BC and CC cell death (Famtawati, Puspitasari & Yusuf, 2011; Mudjahid & Budiani, 2015; Andriani, et al., 2017). However, whether the n-hexane fraction of Sarang Semut demonstrates cytotoxic and anti-cell migration in breast and cervical cancer cells is still unclear. The ability of cancer cells to migrate plays a crucial role in metastasis. Hence, assessment of the capability of natural products to inhibit metastasis needed to be conducted. This study was intended to reveal antitumor activities of the n-hexane fraction of M. pendens in inhibiting cell survival and cell migration in BC and CC cells.

**MATERIALS AND METHODS**

**Chemicals and Reagents**

RPMI 1640 medium (cat No. 11875093), fetal bovine serum (FBS) (cat No. 10270106), phosphate buffered saline (PBS) (cat No. 10010031) and penicillin-streptomycin (cat No. 15140122) were purchased from Gibco, New York, USA. Dimethyl sulfoxide (DMSO) (cat No. D8418), 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) (cat No. M2128), and trypan blue (cat No. T8154) were purchased from Sigma-Aldrich, St. Louis, USA. All the other chemicals were of analytical grade purchased from Merck, New York, USA.

**Preparation of Extract and Fractions of Myrmecodia pendens**

The M. pendens tuberous stems were collected from Papua Island, Indonesia and stored before preparation at Laboratory of Plants Taxonomy, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Indonesia. The stems were then sorted, cleaned, cut and sliced into small pieces, before dried and grounded into dry powder. This grounded M. pendens was later macerated with methanol for 2-3 days followed by filtered then evaporated and dried into dry powder. This methanol extract was partitioned using n-hexane or ethyl acetate to yield n-hexane fraction or ethyl acetate fraction (Bashari, et al., 2018).

**Cell Culture Condition**

BC cells were used in this study are the ER-/PR-/HER2+ BC (HCC-1954) cells and the Luminal A ER+/PR+HER2- BC (MCF-7) cells.
addition, CC cells were used Hela cells. HCC-1954 cells were from Prof. Stefan Wiemann (DKFZ, Heidelberg, Germany), while MCF-7 and HeLa cells were from Ahmad Faried, Ph.D. (Faculty of Medicine, Universitas Padjadjaran, Indonesia). These cell lines were cultured in RPMI1640 medium supplemented with 10% heat inactivated FBS and 1% penicillin/streptomycin. Cells were incubated in a humidified incubator with controlled 5% CO\textsubscript{2} at the temperature 37°C.

**Cytotoxic Assay**

Cell survival inhibition of extract of *M. pendens* were assessed in BC and CC cells using MTT assay (Meerloo, Kaspers & Cloos, 2011). Cells were seeded on a 96-well plate then treated with indicated concentrations on the next day. After that, the cells were placed in an incubator for 72 h with 5% CO\textsubscript{2} at 37°C. Cells containing medium with 1% DMSO was used as control. The MTT solution was added and the mixture was incubated for 4 h. Next, DMSO was added to dissolve the formazan crystal. Absorbance was measured at the wavelength of 550 nm for percentages of viable cells on treated cells, compared to the control cells.

**Migration or Scratch Assay**

The inhibition effect of n-hexane fraction of *M. pendens* toward cell migration was evaluated in HeLa cells using scratch/wound healing assay as published elsewhere (Liang, Park & Guan, 2007). Briefly, the procedure was started by creating a straight line across the plate using a cutter on the back bottom of each well. Cells were seeded in RPMI1640 supplemented by FBS and 1% Penstrep in the flask. The cells were then stored in an incubator with 5% CO\textsubscript{2} at 37°C for 24 h to reach 70-80% confluency as a monolayer. Cells were scratched using a 200 µL sterile pipette tip crossing the plate center perpendicular to the outside line. Cells were later washed twice with PBS. Wells were refilled with fresh complete medium followed by treated or untreated with n-hexane fraction as indicated concentration then incubated in an incubator. The 0\textsuperscript{th}, 24\textsuperscript{th}, 48\textsuperscript{th}, and 72\textsuperscript{nd} observation were done under the microscope, using the cross between gap and marker line as a checkpoint.

The gap was captured for picture at the microscope using the camera connected with a computer and Touview Software (version x64, 3.7.7892) (ToupTek Photonics Co., Ltd., China) at each time point. The gap area was evaluated quantitatively using the software ImageJ. The data were conducted from at least triplicate data from 3 individual experiments with micrometer (µm) as unit of measurement. The image analysis was applied to measure and plot from gap area computationally as a function of time. Standard format such as TIFF was used for saving images. The TIFF images were named and coded (Jonkman, et al., 2014). Images were converted into 8-bit grayscale image and analyzed for respective gap areas using MRI Wound Healing Tool macro for ImageJ (http://dev.mri.cnrs.fr/projects/imagejmacros/wiki/Wound_Healing_Tool).

**Statistical Analysis**

Drug curves were created and IC\textsubscript{50} were determined using four parametric logistic regression by SigmaPlot ver.12 (SYSTAT Software Inc., San Jose, California, USA). While gap areas of each time points were compared to initial gap area (day 0) and calculated for percentage of area in day 0. Data were initially sorted into tables and processed into graph in Microsoft Excel 2007 (Microsoft Office).

The hypothesis was tested using the parametric ANOVA and then continued using the Post-hoc Bonferroni. The statistical analysis and hypothesis testing were processed using the software GraphPad Prism (version 6.01) (GraphPad Software, Inc., San Diego, California, USA). The data was considered significantly different if \( p < 0.05 \).
RESULT

The n-hexane Fraction of *M. pendens* Inhibits Cell Survival in BC Cells

First, bioassays were conducted on three different extractions of *M. pendens* in BC cells using cytotoxic assay. The methanol extract, and n-hexane as well as ethyl acetate fraction of *M. pendens* were tested in HCC-1954 and MCF-7 cells. Data showed that the n-hexane fraction of *M. pendens* inhibits BC cell survival than the methanol extract or the ethyl acetate fraction (Figure 1A). Therefore, for further experiments we used n-hexane fraction for remaining experiments. Next, we aimed to determine the IC\textsubscript{50} of the n-hexane fraction of *M. pendens* in these BC cells. Interestingly, our data showed that IC\textsubscript{50} of the n-hexane fraction of *M. pendens* in HCC-1954 and MCF-7 are 16 ppm and 60 ppm, respectively (Figure 1A-C). It means that the n-hexane fraction of *M. pendens* has cytotoxic activity in these two cell lines.

Figure 1. The n-hexane fraction of *M. pendens* induces cell death in BC cells. (A) The n-hexane fraction triggers more cell death than the methanol extract as well as ethyl acetate extract of *M. pendens*. Drug curves of n-hexane fraction *M. pendens* in HCC-1954 cells (B) and MCF-7 cells (C) as well as determination of IC\textsubscript{50} were conducted by treated BC cells with n-hexane fraction *M. pendens* for 72 h followed by cytotoxic assay using MTT assay. Data was analyzed using SigmaPlot ver 12. Data was presented as mean of three different experiments.
The n-hexane Fraction of *M. pendens* Inhibits Cell Survival in CC HeLa Cells

Next, the n-hexane fraction of *M. pendens* was tested in HeLa cells. Data showed that the n-hexane fraction of *M. pendens* inhibits survival of HeLa cells. The IC\(_{50}\) is 13 ppm (Figure 2). It also means that the n-hexane fraction of *M. pendens* has cytotoxic activity in HeLa cells.

![Graph showing cell death percentage against concentration (ppm)](image)

**Figure 2.** The n-hexane fraction of *M. pendens* induces cell death in HeLa cells. Drug curves and IC\(_{50}\) of n-hexane fraction *M. pendens* in HeLa cells were conducted by treated BC cells with n-hexane fraction *M. pendens* for 72 h followed by cytotoxic assay using MTT assay. Data was analyzed using SigmaPlot ver 12. Data was presented as mean of three different experiments.

The n-hexane Fraction of *M. pendens* Inhibits Cell Migration in CC HeLa Cells

Next, we conducted the migration assay in HeLa cells. To avoid bias of cytotoxic activity of n-hexane fraction of *M. pendens* toward cell migrations, we used very low concentrations of n-hexane fraction, 2 and 5 ppm which did not trigger cell death. Our data showed that this low concentration n-hexane fraction of *M. pendens* do not significantly (p>0.05) trigger cell death in HeLa cells (Figure 3A). Therefore, we could evaluate these concentrations for migration assay. The data implicated that n-hexane fraction of *M. pendens* inhibits HeLa cells movement (Figure 3C). We then analyzed gap area in each group in every time points compared to the initial gap area (day 0). This was shown that the gap area of n-hexane fraction of *M. pendens* are larger than the control suggesting that the treated cells moved slower than in control group (Figure 3B). This reveals that the cervical cancer cells movement and migration are inhibited by the n-hexane fraction of *M. pendens*.

**DISCUSSION**

Exploring natural resources for anti-cancer candidates remains promising for novel cancer agents. Previous studies subjected *M. pendens* by using different extract and fractions in various cancer entities including breast, ovarian, tongue, colon, and cervical cancer cells (Rifayani, et al., 2015; Achmad, et al., 2014; Bashari, et al., 2018; Mudjahid & Budiani, 2015; Supriatno, 2014; Fatmawati, Puspitasari & Yusuf, 2011). However, the n-hexane fraction of *M. pendens* had not been well studied in breast and cervical cancer cells. Here our data demonstrated promising anti-tumor activities of the n-hexane fraction *M. pendens* in BC and CC cells. Specifically, the n-hexane fraction of *M. pendens* inhibits cell survival of HCC-1954 and MCF-7 cells as well as HeLa cells in dose-dependent manner (Figure 1B-C, Figure 2). Importantly, regarding BC cells this data indicates that the n-hexane fraction of *M. pendens* is more active in HCC-1954 cells, which is HER2+ BC subtype than to the MCF-7, Luminal A BC subtype. Previous study has shown that the HER2+ BCs are more aggressive BC subtype, responsible for poor prognosis and correlated with tumor metastasis (Guo, et al., 2017). Importantly, HCC-1954 cells are intrinsically resistant to trastuzumab, a primer targeted therapy for HER2+ BC patients (Brien, et al., 2010). This indicates a potent cytotoxic activity of n-hexane fraction of *M. pendens* in CC cells. According to previous studies, *M. pendens* extracts
had anti-proliferation capability and repress cell proliferation markers (Achmad, et al., 2014; Mudjahid & Budiani, 2015). In addition, *M. pendens* evokes lymphocyte proliferation and macrophage phagocytic activity (Hertiani, 2010; Sumardi, 2013).

Having known that of n-hexane fraction of *M. pendens* strongly induces Hela cell death, we then analyzed its effect on cell migrations. As we know, CC cells have the capability to migrate and invade, permitting them to alter their position in the tissues or among different organs (Friedl & Wolf, 2003; Justus, et al., 2014). These processes enable neoplastic cells to infiltrate into the circulation and then thrive in distant organs. To spread in the tissues, tumor cells use migration mechanisms like in the normal cells during physiological processes (Friedl & Wolf, 2003; Justus, et al., 2014). In experimental cell biology, migration is interpreted as cell movement aimed to substrates such as basal membrane, extracellular matrix (ECM) fibers, or plastic plate/dish (Justus, et al., 2014). Migration is one component of cancer metastasis (Friedl & Wolf, 2003; Aungsumart, et al., 2007; Justus, et al., 2014).

There are limited available drugs which are designed to target cell migration and metastasis (Dunton, 2008; He, et al., 2010; Dasari & Bernard Tchounwou, 2014). Cisplatin and topotecan which standard chemotherapeutic agents for CC are only designed to interfere with cell cycle and induce cell death (Dunton, 2008; Dasari & Bernard Tchounwou, 2014). Bevacizumab, a monoclonal antibody, is the first approved VEGF inhibitor which inhibits
angiogenesis (Tewari, et al., 2014). However, there are limited therapeutic agents that have double effects which inhibit cell proliferation and cell migration (He, et al., 2010; Kang, et al., 2012).

Our data demonstrated that n-hexane fraction of *M. pendens* strongly inhibits cervical cancer cell migration (Figure 3). According to the previous study, Sarang Semut plant has rich in bioactive substances including flavonoids, toco-pherols, tannins, and many essential minerals (Sanjaya, et al., 2014; Engida, et al., 2015). Molecular mechanisms of flavonoids in cancer treatment remain unclear. Flavonoids may demonstrate as anti-proliferation, cell cycle inhibition, apoptotic induction, inhibition of angiogenesis, antioxidants, or the combination of such mechanisms (Chahar, et al., 2011; Liao, et al., 2015). Some types of flavonoids having antioxidant characteristics including myricetin, quercetin, rutin, catechin, kaempferol, fisetin, and naringenin (Hamsar & Mizaton, 2012). Moreover, kaempferol inhibits metastatic cancer development by inhibiting activities of matrix metalloproteinase-3, MET and AKT signaling pathways (Lee & Kim, 2016).

This study was limited to a functional assay of cytotoxic and anti-cell migration of n-hexane fraction of *M. pendens* in BC and CC cells. Further study will be conducted to discover the exact inhibitory mechanisms of *M. pendens* on inhibiting cell survival and cell movement. Moreover, we will also aim to look for any active compounds in the n-hexane fraction. Of course, in vivo data will be interesting to be done later.

**CONCLUSION**

The n-hexane fraction of *M. pendens* demonstrates promising anti-cancer activities through inhibiting BC and CC cell survival as well as inhibiting CC cell migration.

**ACKNOWLEDGMENT**

This study was supported by Universitas Padjadjian under a Research Grant scheme (No. 3855/UN6.C/LT/2019) for MHB and Academic Leadership Grant (ALG) 2017 for MHS. We thank Harold Atmaja for the technical assistance.

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