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Original Research Article

Cytotoxic Activity, Cell Migration and Apoptosis Effects of *Uncaria nervosa* Elmer Leaf Fractions on MCF-7 HER 2 Cells

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ABSTRACT

Uncaria nervosa Elmer is a medicinal plant distributed in Indonesia, New Guinea and the Western Pacific. Traditionally, this plant is used for cancer treatment. This study aims to determine the cytotoxic activity, cell migration and apoptosis of n-hexane, ethyl acetate and butanol fractions in MCF-7 HER 2 breast cancer cells. The ethanol extract of *U. nervosa* Elmer leaves was fractionated using a separating funnel. Fractionation used solvents with varying polarities, n-hexane, ethyl acetate and butanol. The three fractions were tested for their cytotoxic activity by the MTT assay method. The concentrations of the fractions were 100, 10 and 1 µg/ml. The effect of inhibiting cell migration on the fractions using the scratch wound healing assay was observed at 0, 24 and 48 hours and the apoptosis effect was observed using the double staining assay at 48 hours. The results showed that n-hexane and ethyl acetate fractions had IC_{50} values31,44 and 16,422 µg/ml, respectively and the butanol fraction had no activity on MCF-7 HER 2cells at a concentration of 100 µg/ml. Wound healing assays explained the potency of fraction to decrease the cell migration after 24 hours. Apoptosis effect of the fraction of the double staining test results showed MCF-7 HER 2 cells experiencing yellow and orange fluorescence. The n-hexane and ethyl acetate fractions having moderate and strong cytotoxic activity and were able to inhibit cell migration as well as caused death of MCF-7 HER 2 breast cancer cells by apoptosis.

Keywords: *Uncaria nervosa* Elmer**,** MTT assay, scratch wound healing assay, double staining assay

Introduction

Cancer is a non-communicable disease characterized by very rapid and uncontrolled growth and development of cells and tissues. One of the causes of cancer is gene mutations. Gene mutations can be caused by several factors, including UV light, physical, chemical and natural factors.[1]Based on the data obtained by Globocan (2021), breast cancer is the cancer with the most new cases with 11.7%.[2]Breast cancer is caused by the damage to the growth and differentiation genes so that cells can grow and develop uncontrollably, these cells also spread through the blood in the body.[1] Cancer drugs treatment has many side effects. Side effects that arise when using cancer drugs are something that cannot be avoided. Breast cancer patients who are given the anticancer drug combination of flourouracil, doxorubicin and cyclophosphamide will experience side effects such as hair loss, decreased appetite, nausea and abnormal nerves. [3,4] To overcome this problem, research is needed to obtain new candidates for anticancer drugs. Research is directed at testing natural ingredients used as chemo preventive agents.

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One plant that can be developed is *U. nervosa* Elmer. *U. nervosa* Elmer is a species of the genus *Uncaria*, family rubiaceae.[5] Traditionally, this plant is called Bajakah. Plants of the genus *Uncaria* have been widely reported for their anticancer activity. The *U. gambir* plant was reported to have an IC_{50} value of 1,086 µg/ml on T47D breast cancer cells.[6–9]*U. tomentosa* which contains oxindole alkaloids such as isopterpodine, pteropodine, isomitraphylline, uncarine F, and mitraphylline, showed that four of the five alkaloids tested were able to inhibit the proliferation of acute lymphoblastic leukemia cells.[8,9]Isolation from *U. macrophylla* found eight terpenoid compounds named uncaric acid C, uncaric acid D, uncaric acid E, uncaric A, 3β-hydroxy-27-p-(Z)-coumaroyloxyolean-12-en 28-oic acid, uncaric B, 3β-hydroxy-27 -p-(E)-coumaroyloxyurs-12-en-28-oic acid and 3β-hydroxy-27-p-(Z)-coumaroyloxyurs-12-en-28-oic acid. All of these compounds showed inhibition of PLCγ1 with an IC_{50} of 9.5-44.6 µM. [10–12]

There has not been much research regarding the cytotoxic activity of *U. nervosa* Elmer. Rahmawati (2023), reported the cytotoxic activity of *U. nervosa* Elmer ethanol extract against T47D breast cancer cells with strong activity category and IC_{50} of 64.42 µg/mL.[13]There have been no reports regarding the cytotoxic activity of polar and non-polar fractions of this plant. Based on a study of the chemical content of the polar and non-polar fractions, there are alkaloid, phenolic and terpenoid compounds. Based on the research reports on the genus *Uncaria*, compounds in this group are responsible for cytotoxic activity. This study evaluated the cytotoxic activity, cell migration and apoptosis of n-hexane, ethyl acetate and butanol fractions on MCF-7 HER2 breast cancer cells.

Materials and Methods

Plant collection and identification
Leaves of *U. nervosa* Elmer were obtained in forest areas in Kampar Regency Riau Province, Indonesia. Sample identification was conducted at the Herbarium ANDA Andalas University Indonesia in November 2022, with the voucher number NR 004. Materials used included cryo tube (Tpp®), autoklaf (Hirayama®), cryo cube freezer (Eppendorf ®), falcon tube (Nest®), inkubator 37°C/5% CO2 (Thermo Scientific®), microscope inverted (Nikon®), microbial safety cabinet air flow class II (Thermo Scientific®), 24 well plate (Tpp®), RPMI (Roswell Park Memorial Institute), trypan blue, akridin orange and propidium iodid.

Extraction of plant materials
Ethanol extract (2 g) was fractionated using n-hexane, ethyl acetate and butanol. The three fractions were evaporated using a rotary evaporator. All fractions were then tested for cytotoxic activity, cell migration and apoptosis.

Cytotoxicity assay
Platting solution test
The test plate, which contains cells and were incubated for 24 hours, was divided into 3 parts. Each section was designed for three repetitions. The test solution was placed starting from the lowest concentration. 20 µL test solution was transferred into each well except the control well and blank well. The plates were incubated for 24-48 hours in a 37ºC 5% CO_2 incubator, after which the media was removed using a pipette and washed by adding 100 µL of PBS to each well.[14–16]

Platting MTT solution
Solution of MTT (100 µL) was pipetted into each well, then incubated for 3-4 hours at 37ºC, 5% CO_2 after 3-4 hours, a purple precipitate of formazan crystals was visible. The medium containing the MTT reagent was discarded using a pipette, so that only a purple formazan crystal precipitate remained. The precipitate in each well was dissolved with 100 µL DMSO. The absorbance was measured with a microplate spectrophotometer at λ 550 nm.[17–19]

Scratch wound healing assay
MCF-7 HER2 cells were grown into 24 well plates. Cells were observed using an inverted microscope to see their distribution, then incubated for one night until the cells reached 80% confluence. After that, a scratch was made on each well using a sterile yellow tip.All media in each well was removed using a pipette and the cells were washed using PBS. Medium containing each fraction was added to each well, then the plate was incubated in an incubator at 37° C, 5% CO_2 for 48 hours. Cell observations were carried out after incubation for 0, 24 and 48 hours. The test was carried out in three repetitions, then the width of the coverage of the cell scratch area and the percentage of coverage of the cell growth area were measured using ImageJ software.[18–20]

Apoptosis assay
The cover slip was inserted into a 6 well plate, then the cells were planted and left for 30 minutes so that the cells could stick to the cover slip. Cells were added with 3 ml of media and incubated overnight in a 5% CO_2 incubator at 37°C, then added to the well the test solution with a concentration of 1x $IC_{50}$and it was incubated again for 24 hours. After incubation, the media was discarded and the cover slip was taken and placed on the glass object. Acridine orange (AO) with a concentration of 10 µg/mL and propidium iodide (PI) with a concentration of 10 µg/mL were dropped on the cover slip. Cells were observed under an inverted microscope with a fluorescent setting.[21–23]

Data Analysis
Cytotoxicity assay
The absorbance obtained with the ELISA reader was converted into % live cells. The value of % live cells and the concentration used were entered into graphPad software and the IC_{50} value was obtained.

Scratch wound healing assay
The width of the images obtained was measured using imageJ software and the percentage of cell coverage at various incubation times was determined. The percentage was obtained by comparing the scratch area before and after treatment with each fraction.

Apoptosis assay
The data obtained was a microscopic picture of viable cancer cell morphology, apoptosis and necrosis. Viable cells, apoptosis and necrosis were counted using NIS software and the percentage of viable cells, apoptosis and necrosis was determined in MCF-7HER2 breast cancer cells**.**

Results and Discussion

Cytotoxic activity of fractions against MCF-7 cells
The results of the percent cell viability at each concentration are shown in Figure 1. At a concentration of 100 µg/ml, the viable cells in the n-hexane and ethyl acetate fractions were below 20%, while the butanol fraction had more than 60% viable cells (Figure 1).
The IC_{50} values obtained for the n-hexane and ethyl acetate fractions were 31.44 and 11.00 µg/ml respectively (Table 1, Figure 2).
The butanol fraction could not be calculated because it had more than 50% viable cells at a concentration of 100 µg/ml. Based on the research conducted by Yuliastri, et al (2022), cytotoxic activity category 4, namely $IC_{50} \leq 20$ µg/ml = highly active, IC_{50} of 21 - 200 µg/ml = moderately active, IC_{50} 201 - 500 µg/ml = weakly active, and IC_{50}> 501 µg/ml = inactive.[24]Based on this category, the n-hexane fraction is categorized as moderately active and the ethyl acetate fraction is categorized as highly active.
The IC_{50} for the butanol fraction was not calculated because at a concentration of 100 µg/ml the % of viable cells was above 60%. To the best of our knowledge, we have not found any reports on the cytotoxic activity of n-hexane and ethyl acetate fractions on MCF-7 HER 2 cancer cells. In other species, *U. cordata*, the cytotoxicity of leaf ethanol extract on MCF-7 cells was tested and obtained at the concentrations of 10, 40, 70 and 100 µM, this reduced the number of viable cells by 20-50 %. Similar research on the ethyl acetate fraction was also carried out on *Carica papaya* leaves. It was reported that this plant had moderate activity on MCF-7 cancer cells with an IC_{50} of 22.74µg/ml.[25,26]

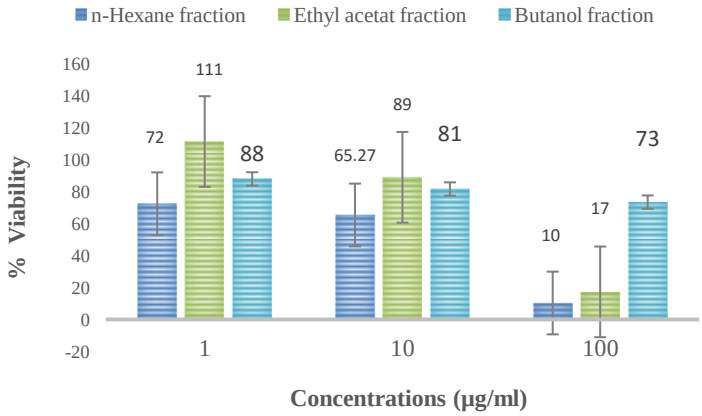

Figure 1: Percentage cell viability of the fraction concentrations

Table 1: IC_{50}value of fractions

Sample	IC_{50} (µg/ml)
n-Hexane fraction	31.44
Ethyl acetate fraction	11.00
Butanol fraction	Not active

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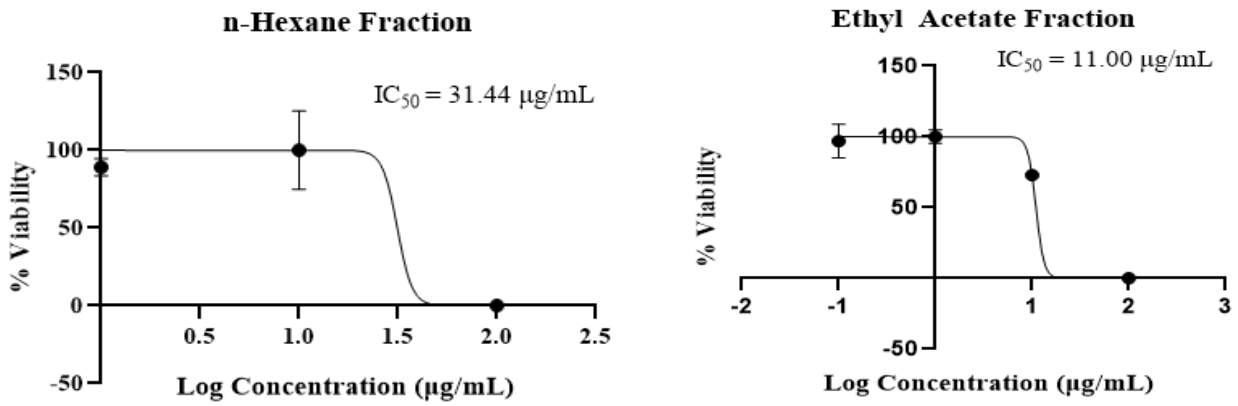
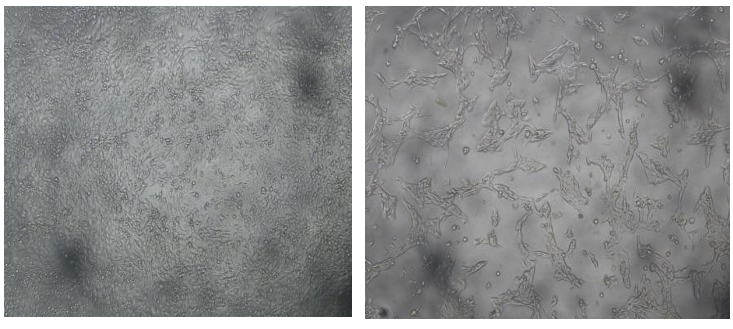


Figure 2: Graph of log concentration and percent viability of n-hexane fraction and ethyl acetate fraction

Evaluation of morphological changes after treatment with fractions
Evaluation of morphological changes in MCF-7HER2 cells was carried out after 24 hours. Changes in cell morphology after being given the fraction are presented in Plate1. The ethyl acetate fraction had the lowest IC_{50} value among the three fractions. At a concentration of 100 µg/ml, cells were seen detaching from the surface of the well, which indicated cell death.

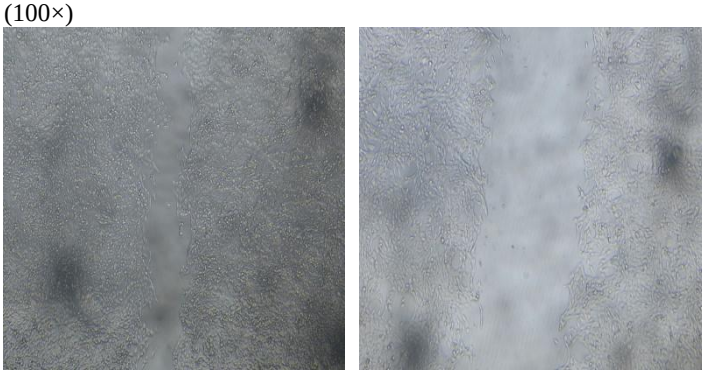
Scratch wound healing assay of fractions
Cell migration is associated with many cellular processes. The method that can be used to determine the ability of cells to migrate in vitro is wound healing. One of the advantages of the wound healing assay is that it is cheap and easy to carry out. Migration testing with the scratch wound healing assay is carried out by scratching the cell monolayer using a sterile yellow tip until a scratch of a certain size is formed. Cell migration ability was determined by measuring the width of the streak at 24 and 48 hours. In this study, a negative control which was not given any treatment was used. Fractions were made with 2 serial concentrations of $1xIC_{50}$ and $2xIC_{50}$ (Plate 2). Figure 3, shows that at a concentration of $1x\ IC_{50}$ after incubation for 24 hours, the ethyl acetate fraction showed the lowest % cell closure, below 10% and after 48 hours of incubation it increased to 20%. However, when compared with the n-hexane and butanol fractions, the % closure of the ethyl acetate fraction at 48 hours remained the lowest. At 48 hours of incubation, all fractions experienced an increase in % cell closure. In theory, scratches applied to a cell monolayer can activate an intercellular response to the breakdown of intercellular contacts. The presence of growth factors at the scratch border triggers cells to close the scratch.[27–29]



10µg/ml 100µg/ml

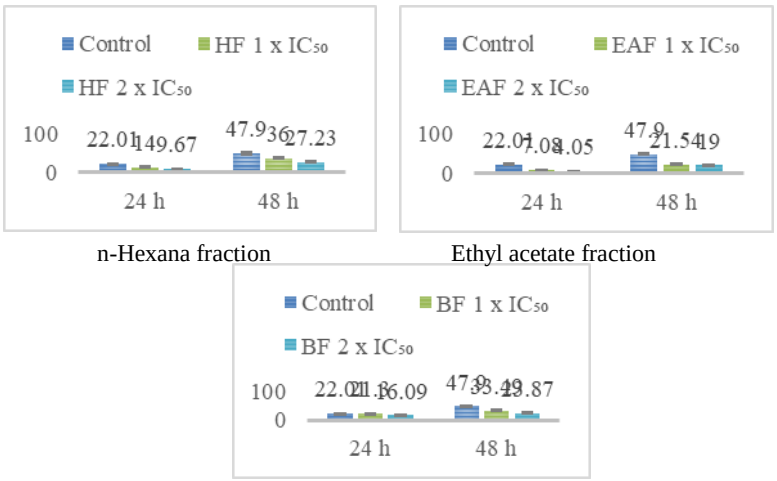
Plate 1: Morphological changes of the MCF-7 HER2 cells after treatment with 10 and 100 µg/mlethyl acetate fraction for 24 h

(100×)



Negative control (48h) Ethyl acetate fraction $2xIC_{50}$ (48h)

Plate 2: Comparison of % cell closure of $1xIC_{50}$ and $2xIC_{50}$control and fractions



n-Hexana fraction Ethyl acetate fraction

Butanol fraction

Figure 3: Percentage of cell closure of fractions at 0, 24 and 48 hours

Apoptosis assay
Table 2 shows that the viable cells used in the apoptosis assay for the n-hexane, ethyl acetate and butanol fractions they were 581, 662 and 371 respectively. It can be seen that the ethyl acetate fraction shows the lowest % of viable cells (4.83 %) compared to the n-hexane and butanol fractions. The ethyl acetate fraction also showed a high value

when compared to other fractions, that is 92.7% (Plate 3). So far, there have been no reports regarding apoptosis from the ethyl acetate fraction of *U. nervosa* Elmer leaves. Research related to apoptosis of the ethyl acetate fraction was reported by Hadisaputri, *et al*,[3] showing that the ethyl acetate fraction of *Anona muricata* had cytotoxic activity on MCF-7 breast cancer cells of 2.86 µg/ml. The cytotoxic and apoptotic activity of the ethyl acetate fraction of *U. nervosa* Elmer leaves is thought to be due to the presence of semi-polar compounds such as steroids and terpenoids which are the second major component after alkaloids in the *Uncaria* genus.[30]

Table 2: Cell viability, apoptosis and necrosis cell of fraction

Sample	Cell viability	Apoptosis	Necrosis	Amount of cell
n-hexane raction	58 ± 3.10	457 ± 4.99	66 ± 2.50	581 ± 3.69
Ethyl acetate fraction	32 ± 0.94	614 ± 0.82	16 ± 3.74	662 ± 3.68
Butanol fraction	76 ± 2.45	270 ± 4.92	25 ± 4.11	371 ± 1.63

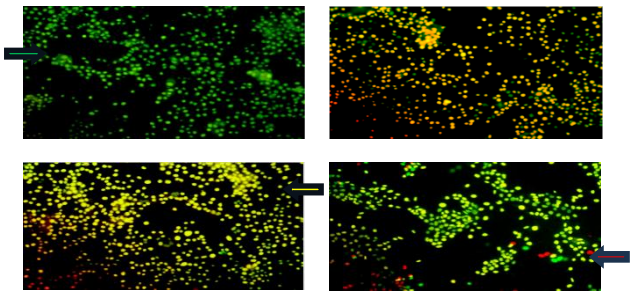


Plate 3: Microscopic picture of viable cancer cell morphology, apoptosis and necrosis at → 1 x IC_{50}. Viable cells with fluorescen green(),apoptosis cells with fluorescen yellow (→), andnecrosis cells with fluorescen red (→).

Conclusion

The n-hexane and ethyl acetate fractions had moderate and strong cytotoxic activity and were able to inhibit cell migration as well as cause the death of MCF-7 HER 2 breast cancer cells by apoptosis.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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