ORIGINAL ARTICLE

Cytotoxic Potential of *Uncaria cordata* (Lour) Merr. Ethyl Acetate Subfraction on T47D Breast Cancer Cells

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ABSTRACT

Uncaria cordata is a plant native to Indonesia, commonly known locally as Akar kaik-kaik. The local communities have traditionally used it to treat various conditions, including cancer. This study aims to determine the cytotoxic activity of subfractions obtained from ethyl acetate extracts of *U. cordata* leaves on T47D breast cancer cells. The dried leaves were extracted using ethyl acetate and then fractionated using vacuum liquid chromatography (VLC). The extracts and subfractions were determined their toxicities using the Brine Shrimp Lethality Test (BSLT) and their anticancer activity in vitro on T47D brest cancer cells using MTT assay. The BSLT assay showed that the cytotoxic activity subfractions (SF3-SF14) have LC_{50} values of 24.0, 67.1, 98.8, 161.5, 102.6, 119.8, 18.3, 16.8, 43.1, 186.1, 54.3, and 29.9 µg/mL. The MTT assay revealed that the IC_{50} of the fractions SF9, SF10, and SF14 were 15.81, 15.12, and 17.43 µg/mL, respectively. The three subfractions exhibited a strong cytotoxic effect on T47D breast cancer cells. Therefore, it can be concluded that the three fractions, SF 9, SF10, and SF14, have a high cytotoxic effect on T47D breast cancer cells.

Keywords: akar kaik-kaik; MTT assay; T47D breast cancer; *Uncaria cordata* (Lour.) Merr.

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Introduction

Cancer is a disease characterized by uncontrolled and abnormal cell proliferation, with the ability to invade surrounding tissues and metastasize to other organs. Cancer originates in a single cell and usually results from mutations caused by carcinogenic agents. This agent causes genetic mutations, so it cannot repair itself. Data from the Global Cancer Observatory (Globocan) in 2025 shows that the incidence of breast cancer is 2.45 million [1]. Lifestyle patterns such as smoking, lack of physical activity, exposure to free radicals from the environment, etc., are the causes of the increase in the number of cancer sufferers [2], [3].

Cancer treatments, such as anticancer drugs, chemotherapy, X-rays, and surgery, are expensive; only a few patients have recovered from cancer. This treatment is still not specific because it is not selective in killing cancer cells, but other normal cells are also killed. It encourages the development of new drugs with selective therapeutic effects as anticancer drugs by exploring natural compounds derived from plants [4], [5]. The genus *Uncaria* has one plant that is utilized as medicinal in several tropical nations. Species of *Uncaria* have been utilized in the treatment of inflammation, analgesics, diabetes, neurological diseases, spasmolytics, hypertension, and cancer [6–8]. Several species of *Uncaria* that have been reported to be used for cancer are *U. gambier*, *U. tomentosa*, and *U. nervosa* [9], [10].

U. cordata (Lour) Merr. is another species in the *Uncaria* genus whose potential as an anticancer agent has not been investigated. Local people have long used the leaves of this plant as a cancer treatment.This plant contains phenolic acids, coumarins, flavonoids,

terpenes and iridoid glycosides, namely 2-hydroxybenzoic acid or salicylic acid; 2,4-dihydroxybenzoic acid; 3,4-dihydroxybenzoic acid; scopoletin or 7-hydroxy-6-methoxy-coumarin; 3,4-dihydroxy-7-methoxycoumarin; quercetin; kaempferol; taxifolin; loganin; and β-sitosterol [11]. In this study, we used *U. cordata* leaves from Indonesia. The leaves were extracted in stages using ethyl acetate, terpenoids and steroids were obtained. As known terpenoid compound have a cytotoxic thus research focused on isolation of cytotoxic compound from the ethyl acetate extract of *U. cordata* leaves in an attempt to develop new treatments for cancer that came from plants [12]. The VLC method was used to separate the ethyl acetate extract. Fraction and ethyl acetate extract was screened for cytotoxicity using the BSLT and active fraction, followed by cytotoxic testing using T47D breast cancer cells. The study of cytotoxic activity of *U. cordata* (Lour) Merr leaf subfractions on T47D breast cancer cells is the first to be reported.

METHODS

Materials

The materials used in this study consisted of methanol p.a, ethyl acetate p.a, hexane p.a, (Merck), lask T-75 (Nest®), microtube centrifuge (Nest®), micro pipette 0,5-10 µL (Eppendorf®), micropipette 100-1000 µL (Accuhance®), cryotube (Tpp®), autoklaf (Hirayama®), cryocube freezer (Eppendorf ®), incubator 37°C/ 5% CO2 (Thermo Scientific®), waterbath (Memmert®), hemacytometer (Neubauer®), centrifuge (Thermo Scientific®), microscope inverted (Nikon®), microbial safety cabinet air flow class II (Thermo Scientific®).

Sample Collection

Plant samples were obtained from forest in Riau Province Indonesia. Samples were collected in November 2022 and identified at Herbarium Andalas (ANDA), Universitas Andalas, Padang, West Sumatra, Indonesia, with sample code NR-04. The leaves were separated from the stems, cleaned and air-dried, then sorted and ground.

Preparation of Ethyl Acetate Extract

Dried leaves of *U. cordata* (Lour.) Merr. (700 g) were soaked in n-hexane (5 L) for five days, and stirred daily, then filtered. The dregs were macerated again with n-hexane. The process was repeated three times until the macerate was clear. Next, the dregs remaining from maceration with n-hexane were macerated again with ethyl acetate solvent. The same process was carried out when maceration with n-hexane. The ethyl acetate macerate was collected and thickened using a rotary evaporator.

TLC Profile Determination of Ethyl Acetate Extract

The TLC profile was determined using n-hexane and ethyl acetate mobile phases in the ratios of 7:3 and 8:2. The stationary phase is silica gel 60 GF254. Observation of TLC profiles at UV 254 and 366 nm

Separation of Ethyl Acetate Extract with VLC

Using a VLC column and filter paper of the same size as the column, 30 grams of thick ethyl acetate extract and 30 grams of silica gel 60 (230-400 mesh) were mixed to create pre-adsorption. Elution was carried out using Step Gradient Polarity (SGP) eluent, which was produced in 21 different eluent ratios, including n-hexane 100%, n-hexane: ethyl acetate (9:1), (8:2), (7:3), (6:4), (5:5), (4:6), (3:7), (2:8), (1:9), 100% ethyl acetate, ethyl acetate: methanol (9:1), (8:2), (7:3), (6:4), (5:5), (4:6), (3:7), (2:8), (1:9), and 100% methanol. Separation was done with gravity and a vacuum. Fractions with the same spot separation pattern were combined into 1 fraction to obtain 14 VLC fractions and labeled SF1 - SF14.

Screening Cytotoxic Activity Using Brine Shrimp Lethality Test (BSLT)

The extract and subfraction were weighed at 50 mg and then dissolved in 5 mL of ethyl acetate to obtain a sample stock solution with a concentration of 10,000 µg/mL then diluted, to obtain concentration 1000, 100 dan 10 µg/mL. *Artemia salina* larvae (10) and 5 mL of saltwater were added to each vial after 50 µL of DMSO had been applied to the samples. The selection of DMSO solvent aims to dissolve the extract and subfractions in the vial. In this study, the negative control used was DMSO (50 µL) added with 2 mL of seawater and 10

Artemia salina larvae. The observations were made by counting the dead larvae after 24 hours. The cytotoxic activity of the extract and subfractions was determined using statistical calculations using probit analysis. This calculation was performed by comparing the number of dead larvae to the total number of larvae to obtain the mortality percentage, which was then displayed in a probit table. The probit value was then entered into a regression equation to obtain the LC_{50} value [13].

Cytotoxic activity using 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay

Determination of cytotoxic activity was carried out using the MTT test. The test began with culturing T47D breast cancer cells and making subfraction test solutions with concentrations of 1000, 100, 10,1 µg/mL. The test solution was placed in a 96-Well Plate containing 180 µL of cell suspension and had been incubated for 24 hours removed from the incubator. 20 µL of test solution for each concentration (1000, 100, 10,1 µg/mL) was put into each well except the control well and blank well to obtain test solutions with concentrations of 100, 10, 1, 0.1 µg/mL. The 96-Well Plate was incubated again for 48 hours in a 5% CO_2 incubator at 37ºC. After the incubation was complete, the MTT solution was placed into each well and incubated for another 4 hours in a 5% CO_2 incubator at 37ºC. After 4 hours, a purple precipitate of formazan crystals was seen. Then, the condition of the cells was observed using an inverted microscope. The MTT reagent was removed by pipetting, leaving only the purple precipitate of formazan crystals. The precipitate in each well was dissolved with 100 µL of DMSO. The absorbance was measured with an ELISA reader spectrophotometer at a wavelength of 550 nm [14].

RESULT AND DISCUSSION

In this study, the preparation of ethyl acetate extract of *U. cordata* (Lour.) Merr leaves was carried out using a multilevel maceration method, where the soaking was carried out with a solvent using the SGP (Step Gradient Polarity) system, an extraction system with its polarity increased in stages, from nonpolar-semi-polar-polar. This system aims to separate the compounds contained in plants based on their polarity levels. In this study, the simplicia used was 700 g and ethyl acetate extract was obtained as much as 38.908 g with a yield percentage of 5.55%.The yield value of ethyl acetate extract shows that there are not many chemical compounds that can be extracted by ethyl acetate solvent and this can be seen based on the results of phytochemical screening, only terpenoids are present [11].

Profile of ethyl acetate extract was determined by the Thin Layer Chromatography (TLC), and it showed in **Figure-1**. Observation of the TLC plate under UV light 254 nm aims to determine whether the compound has at least two conjugated double bonds, while observations under UV light 366 nm show that the compound has a longer conjugated double bond or is called a chromophore group and has a chromophore group axochrome in its structure [15]. TLC observed under UV light 254 nm did not give colour fluorescence, and when observed under UV light 366 nm gave fluorescence, which means the extract contains compounds with a longer conjugated double bond called chromophores. The extracts were separated using Vacuum Liquid Chromatography (VLC). This method involves the partitioning and adsorption of compound components, with separation assisted by pressure from a vacuum apparatus. The VLC results yielded 21 fractions. Fractions with similar separation patterns were combined to yield 14 subfractions **(Figure- 2)** [16].

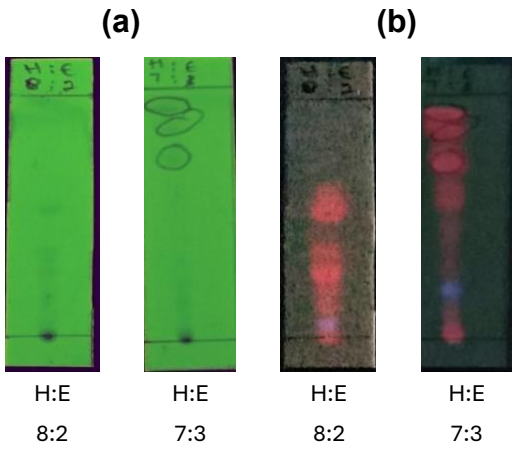

Figure 1. TLC Profile of ethyl acetate extract of U. cordata (Lour.) Merr and monitoring under UV lamp (a) 254 nm; (b) 366 nm. Mobile phase n-hexane (H) and ethyl acetate (E)

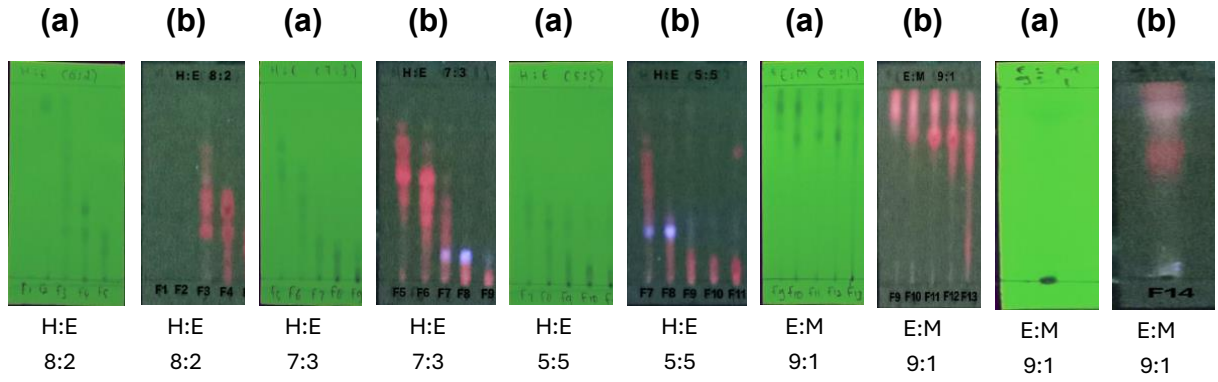

Figure 2. TLC profile of fractions 1-14 and monitoring under UV lamp (a) 254 nm; (b) 366 nm. Mobile phase n-hexane (H), ethyl acetate (E) and methanol (M)

The TLC profile shows that SF1 - 4 with the n-hexane:ethyl acetate (8:2) elution system have spots with relatively high Rf values. This indicates that SF 1- 4 contain nonpolar to semipolar compounds. In SF 5 - 9, with the eluent systems n-hexane:ethyl acetate (7:3) and (5:5), an increase in the number of spots with a wider variation in Rf values was observed. This indicates the presence of a mixture of compounds with varying levels of polarity. Subfractions 10 - 14 with an ethyl acetate:methanol (9:1) eluent system showed spots with low to moderate Rf values and indicated the presence of polar compounds, such as flavonoid glycosides or polar alkaloids. Sharp spot intensities in some subfractions indicated higher concentrations of active compounds.

All fractions were subjected to cytotoxicity tests using Artemia salina Leach larvae. The use of Artemia salina Leach larvae in initial anticancer screening is a highly effective method because it is fast, easy, and can be used as a bioassay to detect toxic compounds in plant extracts or fractions [17]. In this test, DMSO was also used as a negative control, with the aim of ensuring that the death of *Artemia salina* Leach larvae was truly caused by the influence of the sample, not by the solvent. In the vial containing the negative control, all larvae were found to be alive in the control solution. The results of the determination of the cytotoxic activity of subfractions 3 to 14 obtained LC_{50} values of 24.0; 67.1; 98.8; 161.5; 102.6; 119.8; 18.3; 16.8; 43.1; 186.1; 54.3 and 29.9 µg/mL. Based on this value, SF 3-SF14 can be categorized as moderate to very toxic **(Table 1,2)** [18].

The subfraction with a substantial LC_{50} value (9, 10, and 14) was continued with the cytotoxic determination of T47D breast cancer cells using the MTT assay. The principle of the MTT assay is the reduction of the yellow MTT tetrazolium salt [(3(4,5-dimethylthiazol-2-yl)-2,5-

Table 1. Classification of LC_{50} toxicity values

LC_{50} value (µg/mL)	Toxicity classification
< 20	Highly toxic
20-100	Toxic
100-500	Moderate
500-1000	Weak
>1000	Non toxic

Table 2. The results of the cytotoxic activity sub-fraction

Sample	LC_{50} (µg/mL)
Ethyl acetate extract	41
SF- 3	24.07
SF- 4	67.1
SF- 5	98.8
SF- 6	161.5
SF- 7	102.6
SF- 8	119.8
SF- 9	18.3
SF- 10	16.8
SF- 11	43.1
SF- 12	186.1
SF- 13	54.3
SF- 14	29.9

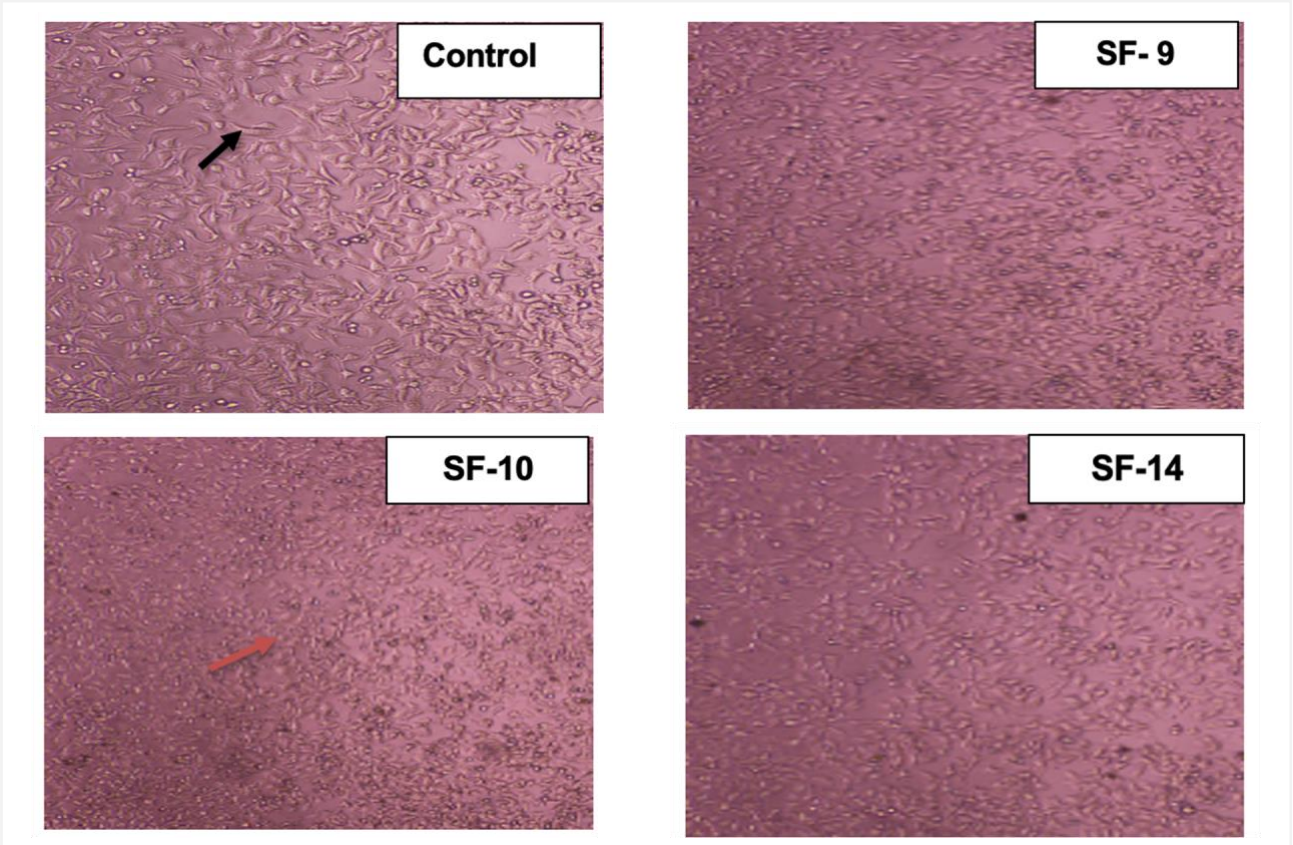

Figure 3. Microscopic photos of T47D cells as control and microscopic photos of T47D cells after the given sample 100 µg/mL (⟶ , alive cells and ⟶ , dead cells)

diphenyl tetrazolium bromide)] by the enzyme succinate dehydrogenase to form insoluble formazan crystals, dissolved in a solvent that is then the absorbance was measured using an ELISA reader. T47D breast cancer cells were isolated from the breast ductal tumour tissue of a 54-year-old woman. T47D breast cancer cells have an epithelial cell-like morphology. These cells express a mutant of the p53 protein. This tumour suppressor gene is essential in cell cycle regulation and apoptosis (cell death). It often undergoes mutations in cancer, and these cells are sensitive to the stimulatory effects of estradiol. T47D breast cancer cells are susceptible to chemotherapeutic agents, so they are often used in cancer research because they are easy to handle, have unlimited replication capabilities, have high homogeneity and are easy to replace with frozen stock in case of contamination. This method was chosen because it can measure many samples at once in a microplate with a relatively fast, sensitive, and accurate time. It is used to measure cell proliferation quantitatively [19], [20].

Based on our research, cell viability for SF 9, 10, and 14 showed a correlation between concentration and cell viability. The higher the sample concentration, the lower the cell viability. The lowest cell viability of 13.17 ± 1.09 was shown by SF 10. The relationship between concentration and cell viability was expressed by a graph between the log of concentration and cell viability (GraphPad Prism 9 application). Based on the percent of cell viability value, the IC_{50} of SF 9, 10, and 14 were 15.81, 1.12, and 17.43 µg/mL, respectively **(Table 3).** The IC_{50} values obtained from these three subfractions were less than 20 µg/mL and can be categorized as strong. The cytotoxic criteria are as follows: IC_{50} < 20 µg/mL (high), 21-200 µg/mL (moderate), 201-500 µg/mL (weak), and >500 µg/mL (no cytotoxic activity). Doxorubicin was used as a positive control. Doxorubicin was used as a control due to its widespread use in cancer therapy [21].

Microscopic photos of T47D cells as control and microscopic photos of T47D cells after being given the test compound are shown in **Figure-3**. Subfractions 9, 10, and 14, at a concentration of 100 µg/mL, showed reduced viable cells compared to the control. The intensity of the purple colour is proportional to the number of living

Table 3. IC_{50} value for sub-fractions and doxorubicin

Sample	Concentration	Log C	Cell viability (%)	IC $_{50}$ (µg/mL
SF-9	100	2	16.64 ± 0.57	**15.81**
	10	1	74.44 ± 2.89	
	1	0	85.27 ± 0.68	
	0.1	-1	91.84 ± 0.25	
SF-10	100	2	13.17 ± 1.09	**15.12**
	10	1	73.93 ± 2.24	
	1	0	89.48 ± 1.35	
	0.1	-1	94.58 ± 1.99	
SF-14	100	2	14.55 ± 0.20	**17.43**
	10	1	86.91 ± 2.85	
	1	0	88.37 ± 1.32	
	0.1	-1	97.03 ± 2.25	
Doxorubicin	10	1	31.32 ± 0.15	**0.11**
	1	0	31.70 ± 0.18	
	0.1	-1	51.4 ± 0.01	
	0.01	-2	62.43 ± 0.02	

cells. If the intensity of the purple colour is greater, it shows that the number of living cells was increased. The results indicate that the higher the extract concentration, the lower the absorbance value. It indicated a decrease in the number of living cells or an increase in the number of dead T47D breast cancer cells, along with an increase in the concentration of *U. cordata* (Lour.) Merr ethyl acetate extract [22].

These three fractions have the potential to be developed as anticancer. Several studies have reported that the terpenoid compound group gives the role anticancer activity. Uncarinic acids A and B isolated from *U. rhynchophylla* by bioassay guided isolation are pentacyclic triterpenes that can inhibit phospholipase Cc1 (PLCc1) (IC_{50} = 35,66 and 44,55 lM), enzymes that induce proliferation of human cancer cells [23]. Loganin compounds have also been reported to be isolated from *U. cordata* and *Strychnos nux-vomica* Linn. Loganin is reported to activate E2F-1 to regulate survivin expression [24].

CONCLUSION

The three subfractions 9, 10, and 14 from *Uncaria cordata* (Lour) Merr. have a high cytotoxic effect on T47D breast cancer cells.

CONFLICT OF INTEREST

The authors have no conflicts of interest regarding this investigatio

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