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RESEARCH ARTICLE

REVISED *Uncaria nervosa* Elmer, a new herbal source for betulinic acid and ursolic acid: Metabolites profiling, isolation, and in vitro cytotoxicity studies against T47D breast cancer

[version 2; peer review: 2 approved]

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Abstract**Abstract****Background**

Uncaria nervosa Elmer is an Indonesian herbal plant that is traditionally used for breast cancer. The results of phytochemical screening contained alkaloids, flavonoids, and terpenoids in the ethanol extract of this plant. Based on literature searches, reports regarding the bioactive compounds responsible for breast cancer have not been found. Further research is needed to understand the potential of *Uncaria nervosa* Elmer as a breast cancer treatment and to identify the specific compounds responsible for its effects

Methods

This study aims to determine the metabolite profiling of ethanol extract, the isolation, characterization of bioactive compounds, and their bioactivity in T47D breast cancer cells. The research began by extracting the leaves by maceration using 70% ethanol, and then solid phase extraction was carried out using the solid phase extraction (SPE) method. In this study, the sorbent used was polyamide. The extract was analyzed using a tandem analysis technique based on LCMS using the MZmine and SIRIUS platforms. Isolation was carried out using column chromatography, and preparative recycling HPLC.

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Bioactive compounds were characterized using UV, HPLC, NMR, and 2D NMR, as well as bioactivity tests using the MTT method.

Results

The results show that the extract contained N-[(1,3-dimethyl-2,6-dioxo-7-prop-2-ynylpurin-8-yl) amino] formamide, N-(3-phenylbutyl)hexan-2-amine, 1,1-Dichloro-1-nitrosopropane, ceratodictyol, betulinic acid, ursolic acid, 7-methyl-N-[6-[(7-methyl-6-oxooctanoyl) amino] hexyl]-6-oxononanamide, nervisterol and 3,5,10-tris (acetyloxy)-2-hydroxy-4,14,16,16-tetramethyl-8-methylidene-13-oxo-15oxatetracyclo [9.4.1.0¹,¹⁴.0⁴,⁹] hexadecan-7-yl 3-phenylprop-2-enoate. The ethanol extract of *Uncaria nervosa* Elmer leaves contains nine compounds consisting of alkaloids, terpenoids, and fatty acid. The bioactive compounds that were successfully isolated were betulinic acid, and ursolic acid, with IC₅₀ values of >100 and $14,70 \pm 4,50$ $\mu\text{g/ml}$, respectively. These compounds were reported in this plant for the first time.

Conclusion

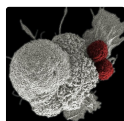
Betulinic acid, and ursolic acid have been successfully isolated from leaves *Uncaria nervosa* Elmer, and ursolic acid have moderate cytotoxic activity on T47D breast cancer cells.

Keywords

Isolation; cytotoxicity; LCMS/MS; T47D; *Uncaria nervosa* Elmer, MZMine, SIRIUS



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This article is included in the **Oncology** gateway.

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Competing interests: No competing interests were disclosed.

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REVISED Amendments from Version 1

In revision 2, I have completed the article according to the reviewer's suggestions. I have strengthened the background by adding literature related to the traditional use of plants. In the research methods, the amount of extract used and its yield are shown in Table 1, and an explanation of NMR has also been included in the research methods. A discussion of the use of ethanol extracts and fractions has also been added. The conclusion has been revised according to the reviewer's suggestions.

Any further responses from the reviewers can be found at the end of the article

Introduction

Breast cancer is cancer that forms in breast cells. This disease occurs in men and women, but women are more often affected by this disease. Breast cancer usually starts in the ductal carcinoma or lobular carcinoma of the breast. This disease spreads through the lymphatic system or bloodstream. Breast cancer is the most common cancer in women throughout the world, with incidence rates varying in each country. According to the World Health Organization (WHO), there were an estimated 2.3 million new cases of breast cancer diagnosed worldwide in 2020 (Sung et al., 2021). One of the current treatments for breast cancer is chemotherapy. These side effects will be different for each person. The type of drug, dosage, length of time using the drug and the medical history of each individual are factors that greatly influence the side effects that will occur. The cancer treatment continues to develop rapidly and drug development continues, but this disease is still an evolutionary process. Cancer cells can adapt to the treatment given. Many studies have shown that natural products can kill cancer cells and also limit their proliferation by targeting several target molecules and genes in cancer cells (Newman and Cragg, 2020; Zhu et al., 2022).

Plants are a source of medicinal compounds, due to their ability to synthesize bioactive compounds, especially secondary metabolites (Joshua et al., 2020). *Uncaria nervosa* Elmer, commonly known as "Akar Kaik Kaik" is a plant species belonging to the genus *Uncaria* within the family Rubiaceae. This botanical species is indigenous to Southeast Asia, particularly found in regions such as Indonesia, Malaysia, and the Philippines. *U. nervosa* is renowned for its medicinal properties and has been an integral part of traditional herbal medicine practices in the region for centuries. *U. nervosa* is a woody vine characterized by its distinctive climbing habit, with slender stems that can reach significant lengths as they twine around trees and other support structures in their natural habitat. The leaves are opposite, elliptical to lanceolate in shape, and possess prominent veins. The plant produces small, tubular flowers with whitish or yellowish hues, which eventually give rise to small fruits containing seeds (Anonim, n.d.; Maulina et al., 2019).

U. nervosa has been revered for its diverse medicinal properties and has been used by indigenous communities for various health purposes. In traditional herbal medicine, different parts of the plant, including the leaves, stems, and roots, are utilized to prepare decoctions, infusions, or poultices to address ailments such as fever, inflammation, digestive disorders, and respiratory conditions. Additionally, *U. nervosa* has been valued for its purported benefits in promoting overall wellness and vitality. The pharmacological activities of *U. nervosa* are attributed to its rich phytochemical composition, which includes alkaloids, flavonoids, phenolic compounds, tannins, and triterpenoids (Ridho, 2023). Triterpenoid is a terpenoid, often having a pentacyclic or tetracyclic structure (Hill and Connolly, 2020; Ling et al., 2022; Noushahi et al., 2022). Pentacyclic triterpenes consist of five isoprene units which are classified into lupane, oleanane, and ursane types (El-Dawy et al., 2022; Kaps et al., 2021; Yanlin Li, Jing Wang, Linyong Li, Wenhui Song, Min Li, Xin Hua, Yu Wang, Jifeng Yuan, 2023). These compounds have a wide spectrum of pharmacological properties including, cardiovascular activities, antioxidant, antimicrobial, antiviral, antidiabetic, anti-inflammatory, anti-ulcerogenic, anti-obesity, anti-aging, analgesic, immunomodulatory, hypolipidemic, neuroprotective, hepatoprotective, and anticancer (Adham et al., 2020; Chooluck et al., 2021; Wang et al., 2020). Research on the isolation and cytotoxic activity of *U. nervosa* has not been widely explored. The cytotoxic activity of ethanol extract of *U. nervosa* leaves on T47D breast cancer cells has been reported by previous researchers where the IC₅₀ value was obtained at 64.42 µg/ml (Rahmawati et al., 2023). In this study, the metabolites profiling of ethanol extract was determined using LCMS/MS, isolation using column chromatography and preparative recycling HPLC and cytotoxic testing of T47D breast cancer cells using the MTT method.

Methods

U. nervosa Elmer plant obtained from the Kampar Forest, Riau, Indonesia. The plant was identified in the ANDA Herbarium, Andalas University, Padang with voucher number NR-04. The chemicals used are ethanol (Merck 1.00983.2500), methanol (Merck 1.06007.4000), acetonitrile (Merck, 1000304000), n-hexane (Merck 1.04367.2500), ethyl acetate (Merck 1.09623.1000), butanol (Merck 1.01990.1000), DMSO, cells T47D, phosphate buffered saline (PBS) (Sigma, P5119), culture media, 20% MTT solution 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (Merck, M2003).

The equipment used were LCMS/MS (Thermo Scientific™ Orbitrap Fusion™ mass spectrometer), recycling preparative HPLC (Japan analytical industry™), column Jaigel-ODS-AP.SP-120-15 20x250 mm, liquid nitrogen tank, 96 hole microplate, hemocytometer, biosafety cabinet (Faster), micropipettes (10, 20, 200, 1000 µL), culture flask, 1.5 mL centrifuge tube, inverted/phase contrast microscope (Olympus), centrifugator, CO₂ incubator, spectrophotometric microplate reader (Thermo), ELISA reader, Laminar Air Flow and FACS-Calibur.

Crude extraction and liquid-liquid fractionation

U. nervosa dry leaves (1.932 g) leaves were macerated using the 2000 ml ethanol (Merck 1.00983.2500). The ethanol extract was fractionated using a separating funnel starting with 500 ml n-hexane (Merck 1.04367.2500), 500 ml ethyl acetate (Merck 1.09623.1000), and 500 ml butanol (Merck 1.01990.1000) as solvents. The three fractions were thickened using a rotary evaporator. The ethyl acetate fraction was selected to continue the isolation process.

Metabolites profiling of ethanol extract

Ultra-High-Performance Liquid Chromatography (UHPLC)

The ethanol extract (20 mg) was cleaned up using SPE-polyamide and obtained 5.7 mg (28.5% recovery). SPE is carried out by rinsing using 3 ml methanol (Merck 1.06007.4000) and a conditioning process with water which aims to eliminate interference in the sample (2:98, v/v). The sample was filtered with a 0.45 µm UHPLC filter, then placed in an HPLC vial. The crude was transferred to the Eppendorf tube and dissolved in 1 mL of solvent mixture (80% methanol and 20% water). The tube was sonicated and the solution was filtered (0.22 µm) then put into an HPLC bottle. The stationary phase used was a Luna Omega C18 column (100 x 2.1 mm, 1.6 µm) with a diode array detector (DAD). The sample injected was 1 µL, the column temperature was 30°C, and the system flow rate was 0.28 mL/minute. Elution was carried out using deionized water (% A) and the organic solvent, acetonitrile (Merck, 1000304000), (% B) and solvent system: 0-30 minutes (5-100% B) and 30-40 minutes (100% B) (Ammar et al., 2024).

Liquid Chromatography Mass Spectrometry (LCMS)

LCMS analysis at a temperature of 30 °C. The sample (1000 ppm), was dissolved in a mixture of methanol and water, then injected 1 µL and the flow rate was set to 0.2 mL/min. LCMS grade water (% A) and methanol (% B) were used in the analysis, with elution systems of 0-30 minutes (10-100% B) and 30-35 minutes (100% B). The samples were filtered using positive ionization mode and analyzed using multistage mass spectrometry (MS/MS) data analysis. The ethanol extract data processing was carried out using the MZmine platform and compound identification using the Sirius platform (Pang et al., 2024; Zheng et al., 2023).

Isolation and elucidation of bioactive compound

The ethyl acetate fraction was weighed as much as 20 g, preabsorbed using silica gel and then put into a chromatography column. The fraction was eluted using a step gradient polarity system solvent, using n-hexane 250 mL (Merck 1.04367.2500), ethyl acetate 250 mL (Merck 1.09623.1000), and methanol 250 mL (Merck 1.06007.4000). There were 11 sub-fractions obtained and the one that was continued for purification was sub-fraction E (HE 5:5). Purification was carried out using recycling preparative HPLC with 1000 ml acetonitrile (Merck, 1000304000) and distilled water as eluents (98:2) (Ammar et al., 2024). Elucidation of the structure of the isolated compound was carried out using a mass spectrometer, 1H- and 1D and 2D-NMR. Samples for NMR (nuclear magnetic resonance) experiments were performed in DMSO solvent, analyzed using NMR Bruker 500 MHz for 1H and NMR 125 MHz for 13 C NMR.

Cytotoxic assay against T47D cells

Cell lines and culture condition

T47D breast cancer cells are a collection of the Cancer Chemoprevention Research Center (CCRC) Gajah Mada University (UGM) Indonesia. T47D cancer cells were taken from the liquid nitrogen tank in a sterile manner, and then the cancer cells in a cyro tube were thawed over a wet bath at a temperature of 37°C for 2-3 minutes. Cancer cells were transferred into a falcon tube containing 9 mL of DMEM and centrifuged at 2000 rpm (10 minutes). The supernatant was removed by pipetting it, leaving a layer of pellets. Next, 2 mL of medium was added to the pellet layer and transferred into a flask, then incubated in a 5% CO₂ incubator at 37°C for 3-4 hours. The flask was observed under an inverted microscope to see whether the cells adhered to the bottom of the flask and formed a monolayer. The growth medium was changed once every two days, and when the number of cells in the flask reached 70-85% confluent, cell sub-culture was carried out.

Cell seeding

Cell of suspension (180 μ L) was made in cell suspension medium, and put into each well except the wells in the first and last columns and the first and last row. The first and last columns, as well as the first and last rows, are blanks, which only contain 200 μ L of medium, while the second and seventh columns are controls, which contain 200 μ L of cell suspension. Incubate in a 5% CO₂ incubator at 37°C for 24 hours.

Cytotoxicity assay

Determination of cytotoxic activity was carried out on betulinic acid, ursolic acid and doxorubicin. The 96-well plate, which contains 180 μ L of cell suspension and has been incubated for 24 hours, is removed from the incubator. The test solution (20 μ L) for each concentration of 0.1-67 μ g/mL was transferred into each well except the control well and blank well to obtain a test solution with a concentration of 100, 10, 1, and 0.1 μ g/mL. The 96-well plate was again incubated for 48 hours in a 5% CO₂ incubator at 37°C. After that, the medium was discarded, then 100 μ L of PBS (Sigma, P5119) was added to each well, and the PBS was removed by pipetting. 100 μ L of 0.5 mg/mL MTT (Merck, M2003) solution was pipetted into each well and incubated for 4 hours in a 5% CO₂ incubator at 37°C. After 4 hours, a purple precipitate of formazan crystals will form. Then the cell condition is observed using an inverted microscope. The MTT reagent is discarded, so that what remains is only a purple precipitate of formazan crystals. The precipitate in each well was dissolved in 100 μ L of DMSO, and its absorbance was measured using an ELISA reader spectrophotometer at λ 550 nm. Based on the absorbance value between the sample and control cells, the % viability value can be determined. The data is processed using graphPad prism software to obtain the IC₅₀ value. The cytotoxicity results for the chemicals strong cytotoxicity: IC₅₀<4 μ g/mL (or IC₅₀<10 μ M); moderate cytotoxicity: 4 μ g/mL< IC₅₀<20 μ g/mL (or 10 μ M< IC₅₀<50 μ M); low cytotoxicity: 20 μ g/mL< IC₅₀<100 μ g/mL (or 50 μ M< IC₅₀<250 μ M); no cytotoxicity: IC₅₀>100 μ g/mL (or IC₅₀>250 μ M) (Bonsou et al., 2022).

Statistical analysis

Data analysis of extract metabolites profiles and structure elucidation in the form of tables, graphs and images. IC₅₀ values of samples and controls were determined using statistical analysis GraphPad Prism Software V 9.0.0 (GraphPad Software LLC).

Results

Crude extraction and liquid-liquid fractionation

The leaves of *Uncaria nervosa* Elmer were extracted using the maceration method. A total of 1,932 g of dried leaves were soaked in 70% ethanol, producing 514 g of extract with a yield of 26.6%. Ethanol extract (261.75 g) was further separated using solvents of increasing polarity, n-hexane, ethyl acetate, and butanol and resulting in fractions weighing 10.15 g, 31.34 g, and 19.21 g, respectively (Table 1).

Metabolites profiling of ethanol extract

Determination of the metabolite profile of ethanol extract begins with the clean-up process of ethanol extract using the solid phase extraction (SPE) method. After the clean-up is complete, the extract is prepared for chromatogram determination using UHPLC. The chromatogram profile was monitored under a UV 254 lamp (Figure 1). The extract was prepared to determine the metabolite profile using LCMS. Raw data obtained from the instrument is processed by carrying out data conversion, data processing and metabolite annotation using the Sirius platform. In the ethanol extract, it is predicted that there are 9 compounds (Figure 2, Table 2), contained N-[(1,3-dimethyl-2,6-dioxo-7-prop-2-ynylpurin-8-yl) amino] formamide, N-(3-phenylbutyl) hexan-2-amine, 1,1-Dichloro-1-nitrosopropane, ceratodictyol, betulinic acid, ursolic acid, 7-methyl-N-[6-[(7-methyl-6-oxooctanoyl) amino] hexyl]-6-oxononanamide, nervisterol and 3,5,10-tris (acetyloxy)-2-hydroxy-4,14,16,16-tetramethyl-8-methylidene-13-oxo-15oxatetracyclo [9.4.1.0¹,¹⁴.0⁴,⁹] hexadecan-7-yl 3-phenylprop-2-enoate.

Table 1. Yield of ethanol extract and fractions.

Sample	Yield (%)
Ethanol extract	26.60
n-Hexane fraction	3.88
Ethyl acetate fraction	11.97
Butanol fraction	7.34

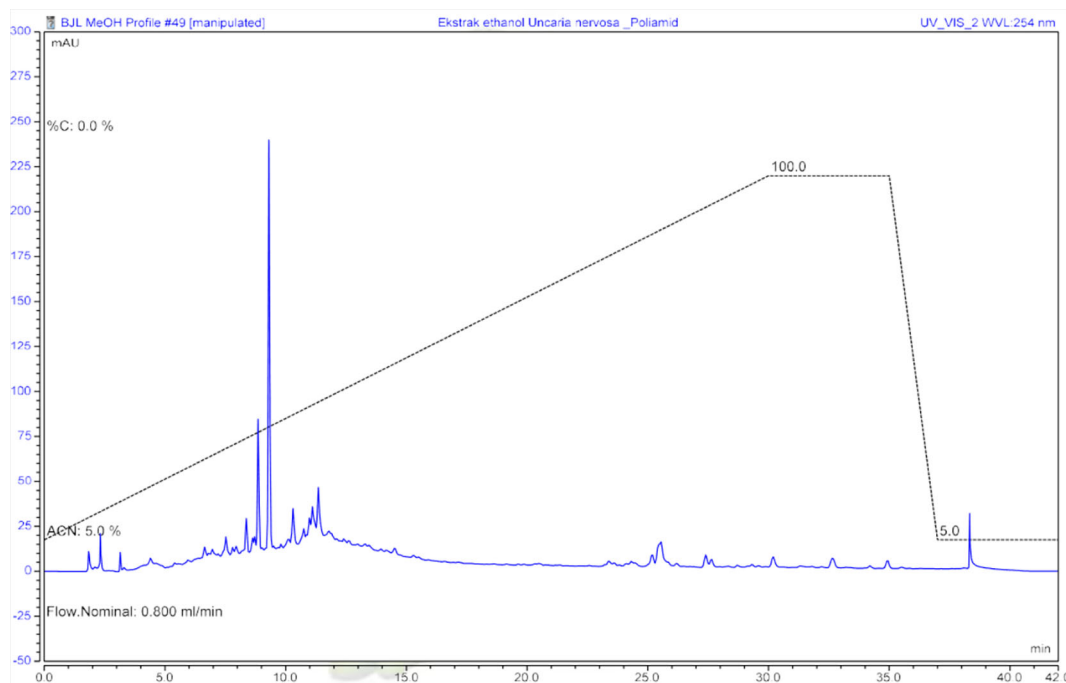


Figure 1. Chromatogram of ethanol extract of *U. nervosa* leaves at UV 254 nm.

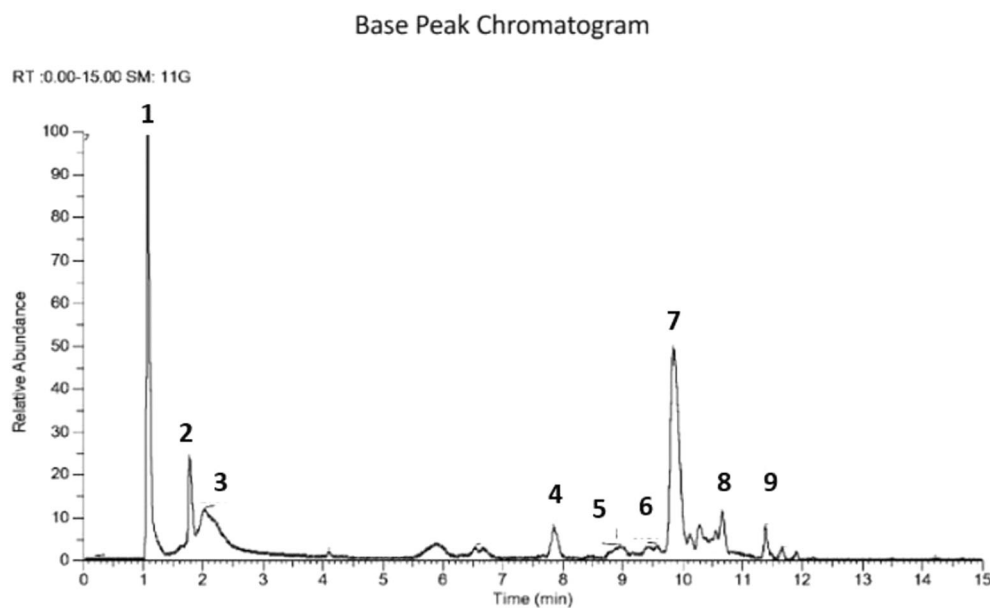


Figure 2. Base peak chromatogram of ethanol extract of the leaves of *U. nervosa*.

Isolation and structural elucidation of the isolated compounds

The ethanol extract of *U. nervosa* Elmer was separated using a fractionation method using n-hexane, ethyl acetate, and butanol as solvents. The isolation process was carried out on the ethyl acetate fraction using column chromatography. The column chromatography results obtained 11 subfractions, and based on monitoring the stain pattern using thin layer chromatography, the isolation was directed to subfraction E. The HPLC chromatogram of subfraction E can be seen in Figure 3.

Table 2. Predicted compounds from the ethanol extract of the leaves of *U. nervosa*.

Peak	RT (min)	Compound identification	Molecular formula	Precursor ion (m/z)	MS/MS fragmentation	Group	Sirius similarity (%)
1	1.09	N-[(1,3-dimethyl-2,6-dioxo-7-prop-2-ynylpurin-8-yl) amino] formamide	C ₁₁ H ₁₂ N ₆ O ₃	277.10	139.01, 121.05	Alkaloid	61
2	1.76	N-(3-phenylbutyl) hexan-2-amine	C ₁₆ H ₂₇ N	234.22	205.18, 204.17, 190.15, 177.15, 163.13, 176.14, 162.12, 149.11, 148.11	Alkaloid	99
3	2.09	1,1-Dichloro-1-nitrosopropane	C ₃ H ₅ Cl ₂ NO	141.98	113.96, 100.95, 97.96	Fatty acid	99
4	7.85	Ceratodictyol	C ₁₉ H ₃₈ O ₄	331.28	257.24, 239.23, 221.22	Fatty acid	100
5	9.00	Betulinic acid	C ₃₀ H ₄₈ O ₃	455.33	453.77, 353.67, 99.46	Triterpenoid	70
6	9.57	Ursolic acid	C ₃₀ H ₄₈ O ₃	455.65	450.54, 407.67, 345.45, 316.83, 187.46	Triterpenoid	100
7	9.86	7-methyl-N-[6-[(7-methyl-6-oxooctanoyl) amino] hexyl]-6-oxononanamide	C ₂₅ H ₄₆ N ₂ O ₄	439.35	394.35, 270.22, 269.22, 244.21, 242.19, 243.20, 241.19	Diterpenoid	100
8	10.66	Nervisterol	C ₃₀ H ₄₈ O	425.37	407.36, 131.08, 95.08	Triterpenoid	100
9	11.38	3,5,10-tris (acetyloxy)-2-hydroxy-4,14,16,16-tetramethyl-8-methylidene-13-oxo-15oxatetracyclo [9.4.1.0 ^{1,14} .0 ^{4,9}]hexadecan-7-yl 3-phenylprop-2-enoate	C ₃₀ H ₄₈ O ₁₂	639.28	621.27, 595.28, 579.26	Triterpenoid	59

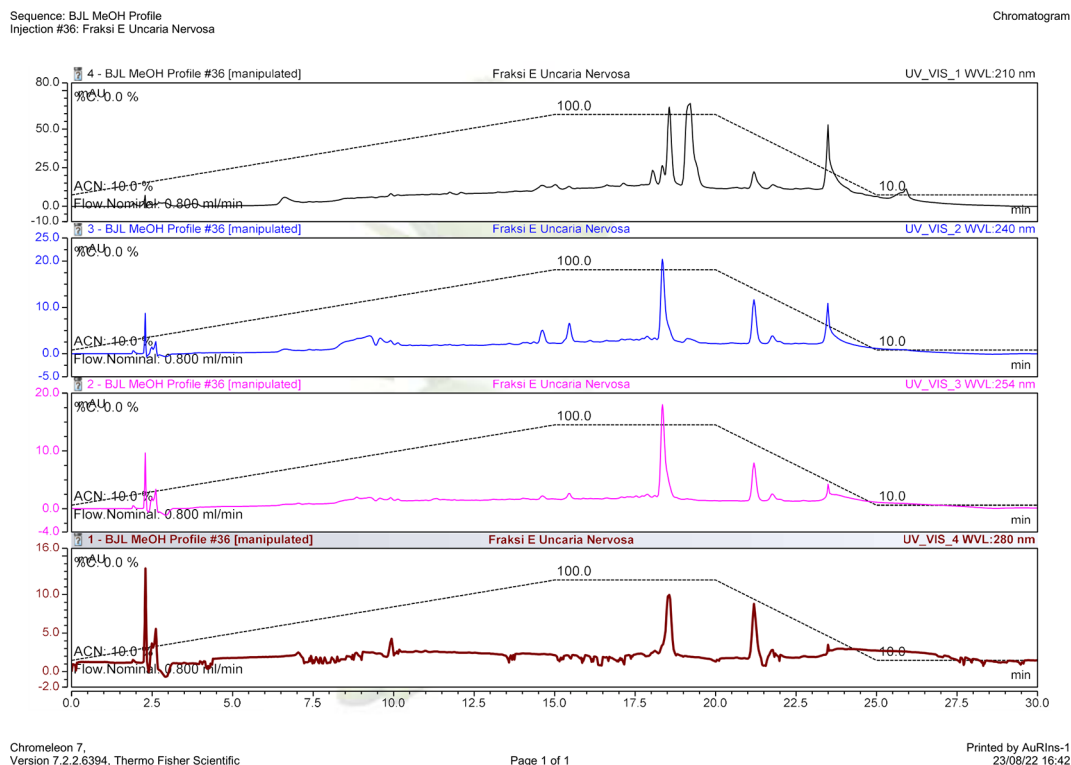


Figure 3. HPLC profile of *U. nervosa* Elmer subfraction E.



Figure 4. Six pure compounds from subfraction E.

The isolation process for subfraction E was continued using a recycling preparative HPLC instrument, where the subfraction was dissolved in methanol and water using a C-18 column. The results of preparative HPLC recycling obtained six pure compounds (Figure 4), and those that were elucidated were UnE-3 and UnE-4. Compounds UnE-3 and UnE-4 were monitored by ¹H and ¹³C NMR, 1D NMR, and 2D NMR (Figures 5, 6, 7, 8). The compounds that have been isolated are betulinic acid (UnE-3) and ursolic acid (UnE-4), with chemical structures as shown in Figure 9.

Cytotoxic activity betulinic acid and ursolic acid against T47D cells

The cytotoxic activity of betulinic acid (BA) and ursolic acid (UA) on human breast cancer cells (T47D) was determined using the MTT method. As a positive control, the reference standard doxorubicin was used (Numonov et al., 2020). Doxorubicin is one of the most widely used chemotherapy agents in the treatment of breast cancer. The results of the cytotoxic test showed that the IC₅₀ values for UA and doxorubicin were 14.70 ± 4.50 µg/ml and 0.15 ± 0.02, respectively. The IC₅₀ value for BA was not determined because the % viability at the highest concentration tested (53 µg/ml) was greater than 50%. The % viability values of UA and doxorubicin can be seen in Figures 10 and 11.

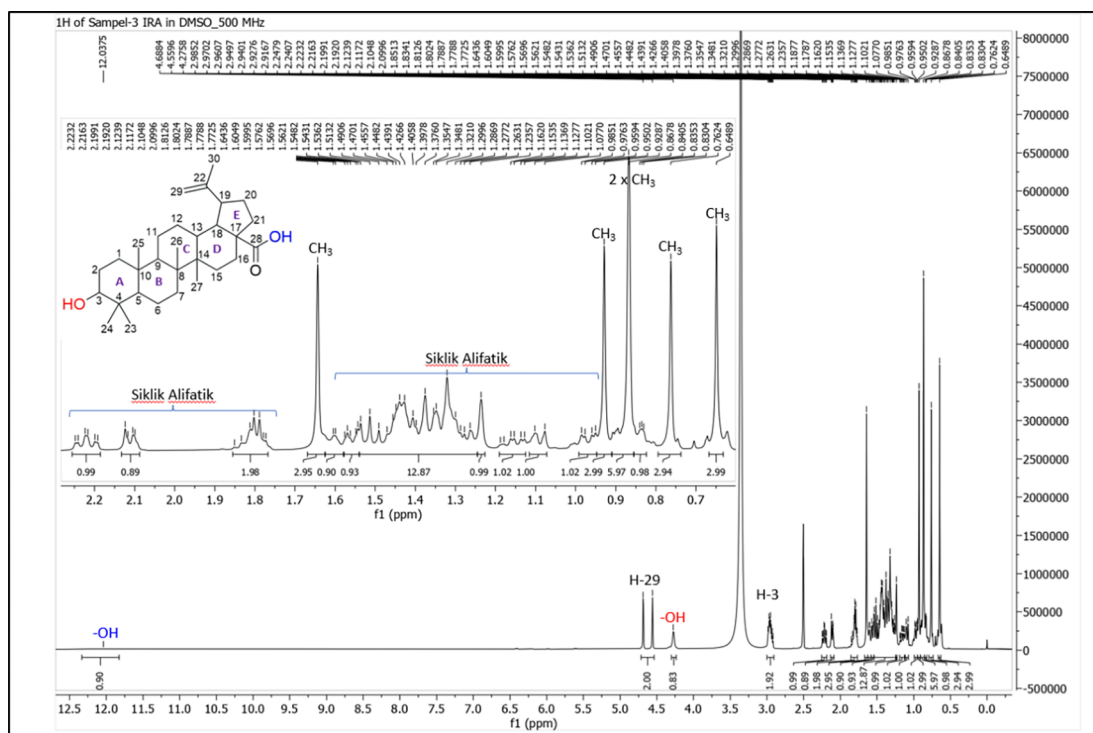


Figure 5. 1H NMR compound UnE-3.

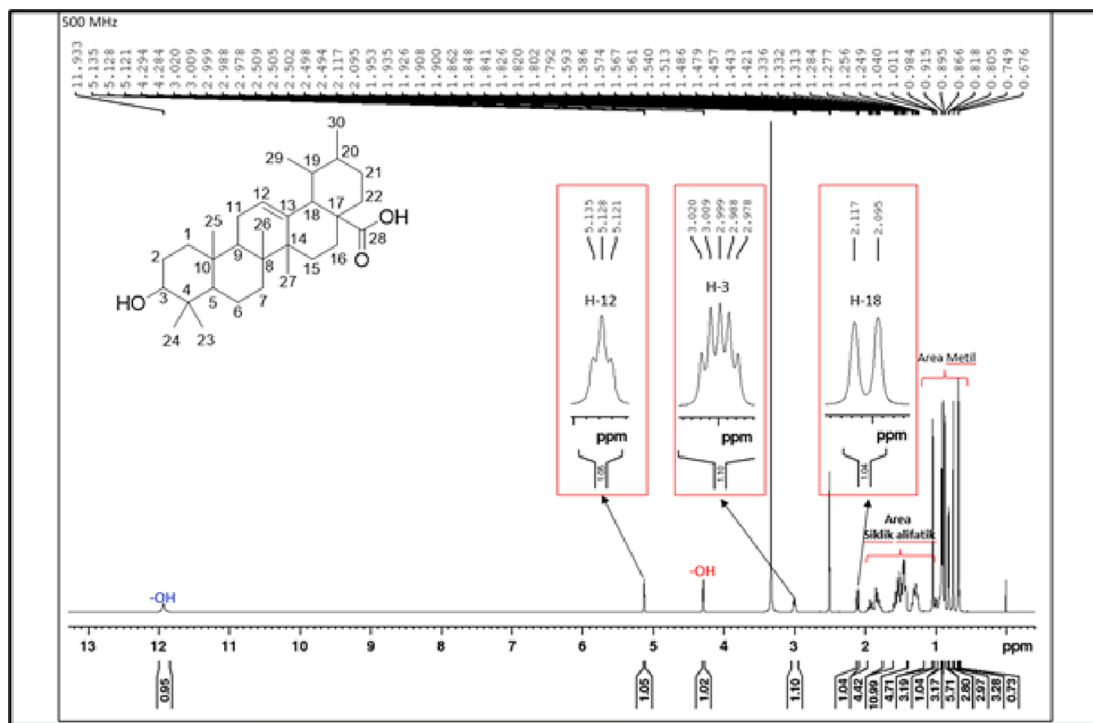


Figure 6. 1H NMR compound UnE-4.

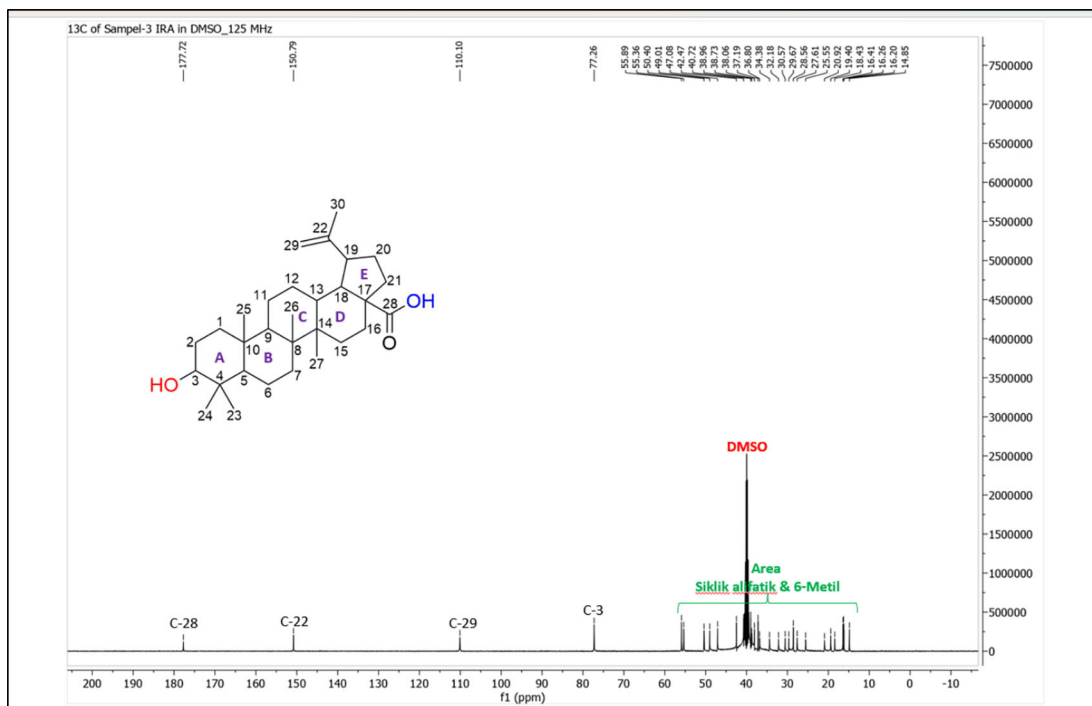


Figure 7. ¹³C NMR compound UnE-3.

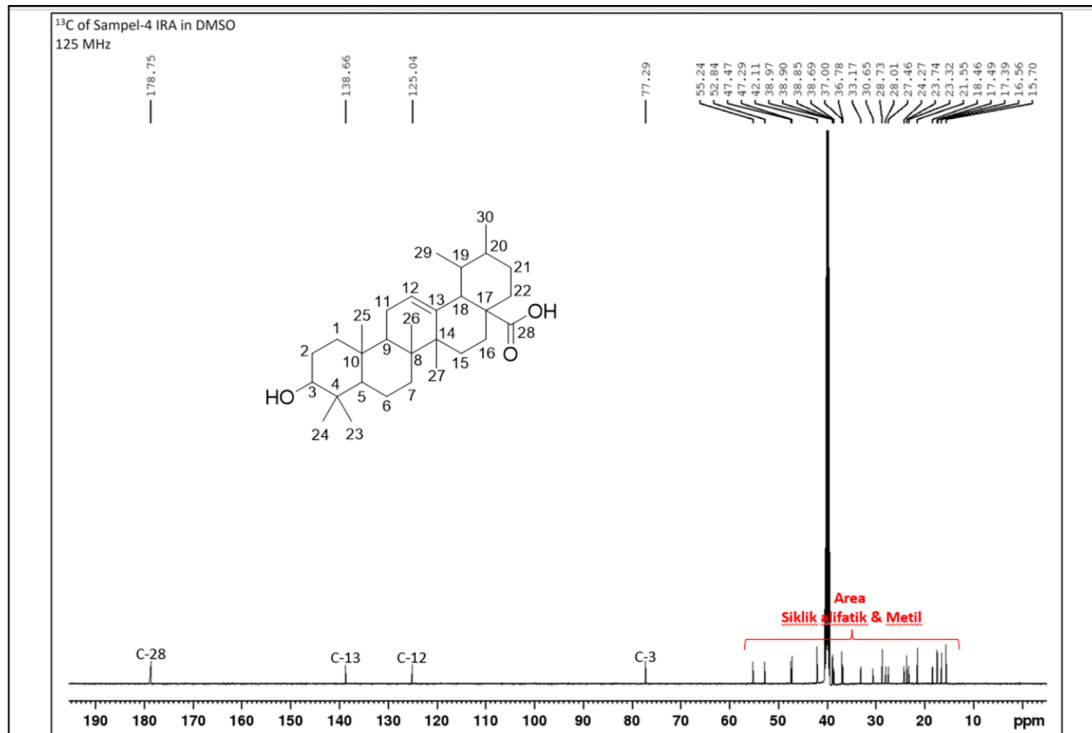


Figure 8. ¹³C NMR compound UnE-4.

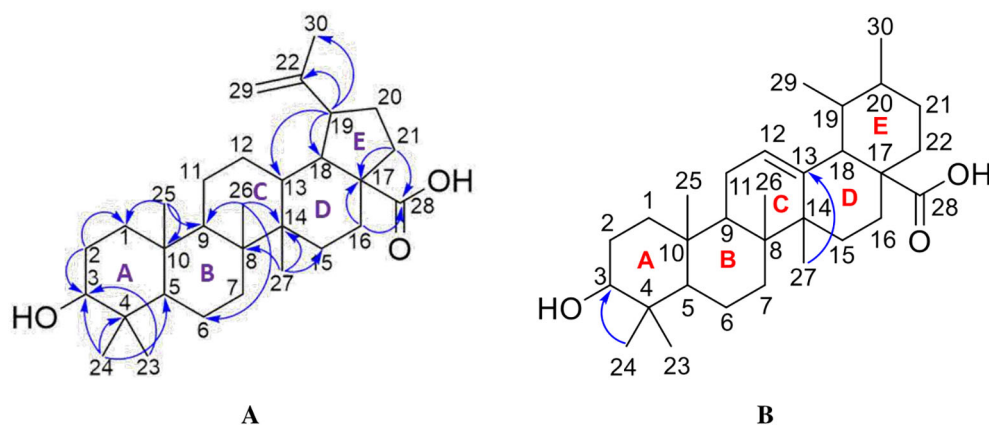


Figure 9. The structure of betulinic acid (A) and ursolic acid (B).

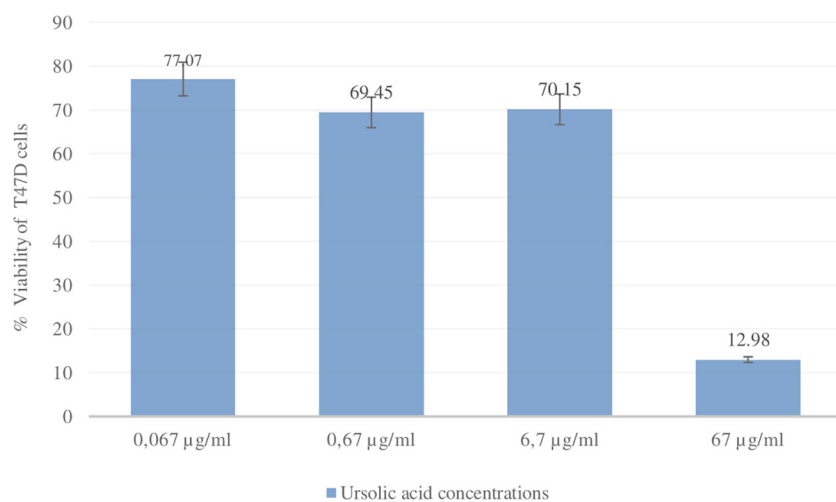


Figure 10. Graph of ursolic acid concentration ($\mu\text{g/ml}$) and % viability of T47D cells.

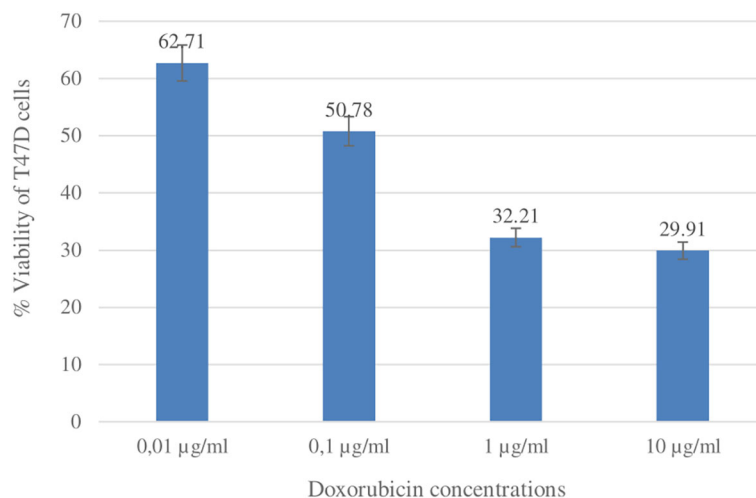


Figure 11. Graph of doxorubicin concentration ($\mu\text{g/ml}$) and % viability of T47D cells.

Discussion

This research began with the preparation of ethanol extract from *U. nervosa* Elmer leaves. Ethanol was chosen as a solvent because ethanol is a polar solvent (lower polarity than water) so that it can attract bioactive compounds found in *U. nervosa* Elmer leaves such as alkaloids, flavonoids, and phenolics which can be extracted more optimally with ethanol (Lin et al., 2020). Determining the metabolite profile of the extract begins with clean-up of the ethanol extract using the Solid Phase Extraction method (SPE). The extraction method that can be used for the analysis, separation, and purification of samples in the industrial, pharmaceutical, and toxicological fields. In this research, a cartridge containing a polyamide stationary phase was used. The stages carried out are conditioning, equilibration, sample loading, elution, evaporation of solvent, and yield recovery (Abd Karim et al., 2022; Farré-Segura et al., 2022). Samples that have been cleaned up are prepared to determine the UHPLC profile and observed at 254 nm. In the chromatogram, it can be seen that in the ethanol extract, there are polar compounds with higher peaks than semi-polar and non-polar compounds. The metabolite profile of the ethanol extract was determined using LCMS/MS.

The ethanol extract data processing was carried out using the MZmine platform and compound identification using the Sirius platform (Pang et al., 2024; Zheng et al., 2023). The ethanol extract is predicted to contain 9 compounds consisting of alkaloids, terpenoids, and fatty acids among them N-[(1,3-dimethyl-2,6-dioxo-7-prop-2-ynyl)purin-8-yl] amino] formamide, N-(3-phenylbutyl) hexan-2-amine, 1,1-dichloro-1-nitrosopropane, ceratodictyol, betulinic acid, ursolic acid, 7-methyl-N-[6-[(7-methyl-6-oxooctanoyl) amino] hexyl]-6-oxononanamide, nervisterol, and 3,5,10-tris (acetyloxy)-2-hydroxy-4,14,16,16-tetramethyl-8-methylidene-13-oxo-15-oxatetracyclo [9.4.1.0¹,¹⁴.0⁴,⁹] hexadecan-7-yl 3-phenylprop-2-enoate. Based on the compounds predicted in the ethanol extract, there are 2 compounds that have been found in other species but have only just been discovered in this species, namely BA and UA. These two compounds are reported to have cytotoxic activity on several cancer cell (Alam et al., 2021; Lou et al., 2021).

Ethanol extract was fractionated using n-hexane, ethyl acetate and butanol solvents. Furthermore, the isolation process was carried out on the ethyl acetate fraction. The ethyl acetate fraction was chosen because it contains semi-polar compounds, such as flavonoids, phenolics, alkaloids, certain terpenoids, and glycosides. Many bioactive compounds from plants that have the potential to be anticancer are in this polarity range, so they are more widely distributed in the ethyl acetate fraction (Zhang et al., 2021). The separation of ethyl acetate fractions was carried out using column chromatography, a separation technique based on adsorption events. This method allows for the separation of a sample in the form of a mixture weighing several grams (Koseki et al., 2025).

Ethyl acetate fraction, preadsorbed using silica gel. The elution system used is an elution system with graded polarity. The eluent comparison starts from non-polar solvents (n-hexane), semi-polar (ethyl acetate) to polar solvents (methanol). The eluent will be increased in polarity if the band that descends with the previous eluent has all been eluted and collected in a vial. The results of column chromatography are collected in a vial and allowed to evaporate. Vials with the same TLC stain pattern are combined and 11 subfractions are obtained which are labeled SF A, SF B, SF C, SF D, SF E, SF F, SF G, SF H, SF I, SF J and SF K. Based on the separation pattern and the number of subfractions available, subfraction E is selected to proceed to the isolation stage. Purification of subfraction E was carried out using a recycling preparative HPLC instrument. The results of the purification of subfraction E, obtained 6 isolates and were labeled UnE-1, UnE-2, UnE-3, UnE-4, UnE-5 and UnE-6 (Pereda & Gómez, 2024). UnE-1, UnE-2, UnE-5 and UnE-6 were obtained in small amounts so that structure elucidation was not carried out.

The UnE-3 compound that has been isolated is a white powder. The molecular formula (C₃₀H₄₈O₃) has an M/Z of 456,684 MS (-mode) along with its 1H NMR and 13C NMR spectral data. The compound has a UV spectrum that only has one peak at a maximum absorption of 195.18 nm. This indicates that this compound does not have a conjugated chromophore. To support the UV data, 1D NMR analysis (1H, 13C, and APT NMR in DMSO as well as 2D (HSQC and HMBC) solvent) was carried out. 13C NMR, 125 MHz analysis was carried out to see the total number of carbons. 30 signals were seen, which were divided into 4 types. chemical shift, namely 1 signal at δ C 177.72 ppm carbonyl area, 2 signals at δ C 150.79 and 110.10 ppm sp² area, 1 signal at δ C 77.26 ppm, and the remaining 26 signals in sp³ area. From the results of the analysis of all spectroscopic data and comparison with the literature, it can be concluded that this compound is classified as a pentacyclic triterpenoid, which has the name betulinic acid (Rahmawati et al., 2024a).

BA is a pentacyclic triterpenoid compound and in *U. nervosa* leaves, it is the first to be reported. BA is a naturally occurring compound found primarily in the bark of certain trees, particularly in the white birch tree (*Betula alba*). However, it's not present in high concentrations in most plants. Apart from white birch, BA has also been found in other plants such as white or silver birch (*Betula pendula*), sweet birch (*Betula lenta*), yellow birch (*Betula alleghaniensis*), white lupin (*Lupinus albus*), *Syzygium* species (Pai and Joshi, 2014), *Paronema canescens* (Muharni et al., 2021), *Feretia canthioides* (Egbubine et al., 2020), and *Tetracarpidium conophorum* (Kelly et al., 2021).

The second compound that has been isolated is UnE-4, which is a white powder and has the molecular formula ($C_{30}H_{48}O_3$) with m/z 456.66. This compound has a UV spectrum that only has one peak at a maximum absorption of 195.18 nm. This indicates that this compound does not have a conjugated chromophore. Furthermore, to support the UV data, 1D (^1H and ^{13}C NMR in DMSO solvent) and 2D (HMBC) NMR analysis was carried out. ^{13}C NMR analysis, 125 MHz was carried out to see the total number of carbons which showed 30 signals which were divided into 4 types of chemical shifts, namely 1 signal at δC 178.75 ppm carbonyl area, 2 signals at δC 138.66 and 125.04 ppm sp^2 area, 1 signal at δC 77.29 ppm and the remaining 26 signals in the sp^3 area.

The ^1H -NMR spectrum, 500 MHz there are several specific signals such as two hydroxy protons whose chemical environments are very different, namely at δH 11.93 (s, 1H) which usually bonds directly with the carbon sp^2 carbonyl at C-28 while δH 4.29 (d, 1H) possibly binding to the sp^3 carbon of methine at C-3. One sp^2 area proton signals at δH 5.13 (t, $J = 3.25$ Hz, 1H) and the rest are aliphatic protons. HMBC data shows a long between H-24 and C-3 which confirms the position of the hydroxy methine which is in ring A. Furthermore, a long between H-27 and C-13 which turns off its position splits rings C and D. Based on Index calculations of Hydrogen Deficiency (IHD) obtained a value of 7 which is in good agreement with NMR data where 5 IHD comes from rings and 2 IHD from double bonds. From the results of the analysis of all spectroscopic data and comparison with the literature, it can be concluded that this compound is classified as a pentacyclic triterpenoid which has the name ursolic acid. UA has reportedly been isolated from several plants including *Salvia officinalis* (Jedinák et al., 2006), Apples peels (Yamaguchi et al., 2008), *Arbutus pavarii* (Groshi et al., 2022), and *Uncaria macrophylla* (Sun et al., 2012).

Compounds that have been isolated from the ethanol extract of *U. nervosa* Elmer leaves are BA and UA. These two compounds were subjected to a cytotoxic test on T47D breast cancer cells using the MTT method and doxorubicin was used as a positive control. The test results show that ursolic acid has strong activity when compared with betulinic acid. The IC_{50} values for ursolic acid and doxorubicin were 14.70 ± 4.50 $\mu g/ml$ and 0.15 ± 0.02 , respectively (Rahmawati et al., 2024b). BA and UA are the active compounds of pentacyclic triterpenoids which is a triterpene acid that is found in various fruits, vegetables and medicinal plants (Zafar et al., 2022; Maniyamma et al., 2022) and has been reported to have antidiabetic, anti-inflammatory, immunomodulatory, and anticancer activities (Alam et al., 2021; Numonov et al., 2020; Sianipar, 2021; Wang et al., 2020). BA and UA are from natural products against metastasis and was also investigated in studies conducted in the last decade (Khwaza et al., 2020; Naeem et al., 2022; Zafar et al., 2022).

Research related to the cytotoxic activity of BA includes the effects of skin carcinogenesis, where it was reported that BA can inhibit 70% of tumor development (Agame-Lagunes et al., 2021). Research on MDA-MB-231 breast cancer cells gave an IC_{50} value of 38.81 ± 4.9 mg/mL (Qi et al., 2021). BA was also reported to inhibit the viability of HuCCA and BHK-21 cells and induce neoplastic cell apoptosis (Phonarknguen et al., 2022). Research related to the cytotoxic activity of UA has also been widely reported. UA is active in T47D breast cancer cells with an IC_{50} value of 1.63 ± 0.62 μM (Numonov et al., 2020). UA increases cell sensitivity Triple-negative breast cancer (TNBC) to doxorubicin via signaling inactivation ZEB1-AS1/miR-186-5p/ABCC1 (Lu et al., 2022). UA formulated into nanoparticles provides an IC_{50} value below 30 μM against pancreatic cancer cells AsPC-1 dan BxPC-3 (Markowski et al., 2021) and UA can significantly increase the drug sensitivity of human breast cancer cells MCF-7/MDA-MB-231 against Epirubicin (Wang et al., 2022).

Conclusion

BA and UA were successfully isolated and identified for the first time from the leaves of *U. nervosa* Elmer, an indigenous Indonesian medicinal plant. Metabolite profiling using LC-MS/MS confirmed the presence of nine secondary metabolites, including triterpenoids, alkaloids, and fatty acids. The isolated compound UA demonstrated strong cytotoxic activity against T47D breast cancer cells, with an IC_{50} value of 14.70 ± 4.50 $\mu g/mL$, while BA showed moderate activity. These findings suggest that *U. nervosa* Elmer holds significant potential as a novel natural source of pentacyclic triterpenoids with anticancer properties and may contribute to the development of plant-based chemotherapeutic agents.

Ethical considerations

No animals were harmed in this research.

Data availability

Zenodo: (Data set) Isolation of Pentacyclic Triterpenoids from The Leaves of *Uncaria nervosa* Elmer. <https://doi.org/10.5281/zenodo.11544271>

This project contains the following underlying data:

- Dataset of this research

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC4).

Zenodo: (Data set) Cytotoxic activity of betulinic acid and ursolic acid againsts T47D breast cancer cells. <https://zenodo.org/doi/10.5281/zenodo.11545104>

This project contains the following underlying data:

- Dataset of this research

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CCO).

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Suhuan Mei 

Bioscience and Engineering, Jiangxi Agricultural University, Nanchang, Jiangxi, China

The authors have thoroughly addressed the reviewers' comments and made improvements in the revised manuscript. I recommend acceptance.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: 1. Separation, characterization, and evaluation of bioactive components from foods or plants. 2. The impact of natural products on gut health. 3. Safety evaluation of natural products. 4. Chemical and functional changes of foods during processing

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 16 October 2025

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Subiya Showkat

Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

The authors have revised the manuscript as per my previous comments. I have no further suggestions, and the manuscript may be considered for indexing.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Phytochemistry, pharmacology, Microbiology and computational studies.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 07 March 2025

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Suhuan Mei

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This paper illustrates the cytotoxicity against T47D breast cancer of betulinic acid and ursolic acid from *Uncaria nervosa* Elmer. The authors have performed LC-MS, UV, HPLC, NMR, and 2D NMR to isolate and characterize bioactive compounds as well as the MTT method to evaluate the cell viability. The rationale of the proposal is logical and the manuscript is written well but the manuscript should be revised considering the following points which should be incorporated for its improvement.

1. In the title, 'Elmer' does not need italics.
2. The weight of 1.932 g leaves is dry weight or wet weight should be described in the section of methods. Please address the manuscript thoroughly.
3. Details of the NMR should be described in the method section.
4. Is there any data about the yield of the different fractions and pure compounds?
5. Results and discussion have many similar descriptions, for example,
"Results
Metabolites profiling of ethanol extract
Determination of the metabolite profile of ethanol extract begins with the clean-up process of ethanol extract using the solid phase extraction (SPE) method."
"Discussion
Metabolites profiling of ethanol extract
Determining the metabolite profile of the extract begins with clean-up of the ethanol extract using the Solid Phase Extraction method (SPE)."

6. An acronym of the full name is necessary when they appear first.
7. Please don't use the same subheading in the result and discussion.
8. The sentence 'The ethanol extract data processing was carried out using the MZmine platform and compound identification using the Sirius platform (Pang et al., 2024; Zheng et al., 2023)' in the discussion seems more appropriate when it appears in the method.
9. As mentioned above, please check the manuscript carefully, don't repeat the result, and add more in-depth discussions.
10. Is the ability of betulinic acid and ursolic acid from *Uncaria nervosa* Elmer any different compared with other herbal plants?

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: 1. Separation, characterization, and evaluation of bioactive components from foods or plants. 2. The impact of natural products on gut health. 3. Safety evaluation of natural products. 4. Chemical and functional changes of foods during processing

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 17 Mar 2025

Noveri Rahmawati

Thank you very much for all the comments from the reviewers. We will thoroughly revise

this article and send it back to the editor. This is a response to the reviewer's comments.

1. Thank you. We will revise it.
2. The weight of 1.932 g leaves is dry weight. We will revise it. Thank you.
3. We will include the NMR in the method. Thank you.
4. We have data on the results of different fractions and pure compounds. We will include them in the article.
5. Thank you. We will revise it.
6. Thank you. We will revise it.
7. Thank you. We will revise it.
8. Thank you. We will revise it.
9. Thank you. We will revise it.
10. The ability of betulinic acid and ursolic acid from *Uncaria nervosa* Elmer on cancer cells is no different when compared to other herbal plants. Betulinic acid and ursolic acid have been reported to have potent bioactivity on several T47D breast cancer cells.

Competing Interests: No competing interests were disclosed.

Reviewer Report 18 February 2025

<https://doi.org/10.5256/f1000research.167034.r364783>

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Subiya Showkat

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After carefully reviewing the manuscript and providing my comments, I believe that the manuscript has potential but requires substantial revisions. I recommend that the authors address the issues raised before the manuscript can be reconsidered for indexing.

Comments for authors:

1. In the introduction, several statements are made about the medicinal properties of *U. nervosa*, such as its wide range of health benefits and its traditional use by indigenous communities. *U. nervosa* has been revered for its diverse medicinal properties and has been used by indigenous communities for various health purposes. In traditional herbal medicine, different parts of the plant, including the leaves, stems, and roots, are utilized to prepare decoctions, infusions, or poultices to address ailments such as fever, inflammation, digestive disorders, and respiratory conditions. Additionally, *U. nervosa* has been valued for its purported benefits in promoting overall wellness and vitality. However, there is no reference provided to support these claims. It would be helpful to include appropriate citations to validate the medicinal properties attributed to the plant.

2. In the methods section, the ethyl acetate fraction is mentioned as being weighed and then subjected to chromatography. However, in the results, it mentions that the ethanol extract was fractionated and then the ethyl acetate fraction was used for isolation. This creates some confusion. Could you clarify whether the isolation was performed on the ethanol extract or the ethyl acetate fraction? Additionally, 20 g of the ethyl acetate fraction was obtained, which seems questionable, as 20 g appears too large. Was this fraction liquid? If so, was it dried before weighing?
3. There are many places in the manuscript where the grammar should be checked. Additionally, there are several sentences that are not well connected.
4. What was the rationale for choosing ethanol extract of *U. nervosa* for analysis in this study, and how does it compare to other possible extraction methods or solvents in terms of efficiency and bioactivity?"
5. The methodology for NMR studies is missing.
6. The conclusion is very short and not satisfactory.
7. The results and discussion section seems very weak and lacking in depth.
8. Have the authors used a standard for the HPLC analysis? If so, it should be properly labeled in the figure.
9. Ensure all scientific names are italicized throughout the manuscript.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Phytochemistry and pharmacology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 17 Mar 2025

Noveri Rahmawati

Thank you for all the suggestions for our article. We will revise the article thoroughly and send it back to the editor. Herewith we convey some responses to the reviewer's comments.

1. Thank you. We will provide references related to the benefits of plants.
2. Isolation was carried out on the ethyl acetate fraction. Ethanol extract 70% (514 g) was fractionated in stages using n-hexane, ethyl acetate and butanol solvents. The dry ethyl acetate fraction obtained was 20 g.
3. Thank you. We will revise it.
4. **The basis for selecting ethanol:** ethanol is a polar solvent (lower polarity than water) so it can attract bioactive compounds found in *U. nervosa* elmer leaves such as alkaloids, flavonoids, and phenolics.

Comparison with other extraction methods or solvents in terms of efficiency and bioactivity:

Extraction using the maceration method is a simple extraction without heating so it is more efficient and allows the bioactive compounds contained in plants to remain stable.

The use of other solvents such as water will only be able to attract polar compounds and is less effective for lipophilic compounds.

Methanol solvents can extract phenolic compounds more strongly, but are more toxic than ethanol so they are less suitable for biomedical applications.

n-hexane and dichloromethane solvents will be effective in attracting non-polar compounds such as essential oils.

5. Thank you. We will revise it.

6. Thank you. We will revise it.

7. Thank you. We will revise it.

8. Yes, we use standards in HPLC analysis and we will label them correctly in the images. Thank you.

9. Thank you. We will revise it.

Competing Interests: No competing interests were disclosed.

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