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Research Article

Safflower (*Carthamus tinctorius* Linn.) Inhibits Cell Proliferation and Induces Apoptotic in Breast Cancer Cell Lines T47D

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Abstract

Background and Objective: Safflower (*Carthamus tinctorius* Linn.) is one of the medicinal plants that contain secondary metabolites that have the potential to as anti-cancer by inducing apoptosis. This study aims to determine the content of secondary metabolite compounds and the induction activity of apoptosis from ethanol extract of safflower in the T47D breast cancer cell line. **Materials and Methods:** Safflower was extracted using 96% ethanol and assayed for phytochemical screening, cytotoxic tests by cell counting kit-8 to determine inhibitory concentration and apoptosis induction activity by flow cytometry to determine the ability of samples induce the programmed cell cancer in death. The data collected was analyzed with the PRISM GraphPad version. **Results:** The ethanol extract of safflower contains flavonoid compounds, alkaloids, saponins, tannins and terpenoids. The results of the anticancer activity test showed an IC_{50} value of $479 \mu\text{g mL}^{-1}$ and the best percentage of apoptosis at a concentration of $200 \mu\text{g mL}^{-1}$ was 16.61% at the beginning of apoptosis and 10.52% at the end of apoptosis. **Conclusion:** The safflower can be developed as a breast anticancer agent that works through the induction of apoptosis to improve the effectiveness of breast cancer treatment.

Key words: Safflower, T47D cell line, IC_{50} , apoptosis, breast cancer, flow cytometry

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cancer is the cells of body tissues that turn malignant, characterized by rapid and uncontrolled cell division to form cells similar to their original cells¹. Based on Globocan data released by WHO in 2020, the most common cancer cases in women in Indonesia are breast cancer, namely 65,858 (30.8%)². Breast cancer (*Carcinoma mammae*) is cancer that affects the mammary glands, glandular ducts and supporting tissues of the breast³. Breast cancer occurs due to DNA damage and genetic mutations affected by estrogen exposure. In addition, it can be caused by precancerous genes such as the *BRCA1* and *BRCA2* genes¹.

Therapy in breast cancer aims to reduce symptoms, improve quality of life and prolong survival⁴. Chemotherapy agents became a standard cancer treatment. However, it also targets normal cells in the body, such as the spinal cord, hair follicles and digestive tract cells thus leading to side effects and drug resistance⁵. This failure in chemotherapy is usually related to the inability of such anticancer agents to induce apoptosis. Therefore, efforts to find new chemotherapy agents capable of inducing apoptosis, namely by developing compounds from natural ingredients as chemotherapy agents⁶.

Safflower (*Carthamus tinctorius* Linn.) belongs to the family Asteraceae which is widely grown worldwide. This plant has been used empirically as traditional medicine by the people of South Sulawesi for treating measles, which is given by infused in hot water to improve the immune system⁷. It contains polyphenols, flavonoids⁸, glycosides⁹, quinochalones, safflower polysaccharides (SPS)¹⁰, polyacetylenes¹¹, kinobeaone A, HSYA (hydroxysafflower yellow A), tinctormine¹², safflower yellow A, safflomin, cartormine and alkaloids¹³. Recent studies showed that safflower has broad pharmacological activity, including cardiovascular protective effect, immunomodulator, antioxidant, anti-inflammatory and antibacterial¹⁰. In addition, antitumor¹¹, arteriosclerosis, anti allergy and antagonist fibrosis¹³, suppresses the proliferation of cancer cells in human breast cancer cells MCF-7 and HeLa cells¹⁴, as well as decreases the viability, proliferation, migration and apoptosis of HepG2 cells¹⁵ and others also reported.

Apoptosis is a programmed, genetically regulated, active process of cell death and is characterized by the presence of chromatin condensation, cell fragmentation and phagocytosis of these cells by neighboring cells¹⁶. Apoptosis becomes the body's natural defense mechanism against cancer. Typically, cell damage due to radiation, trauma or oxidative damage will

initiate apoptosis first¹. However, cancer cells avoid apoptosis by limiting the mechanism of apoptosis, thus allowing excessive proliferation¹⁷. Based on the description above, this study aims to determine safflower (*Carthamus tinctorius* Linn.) ethanol extract induces apoptosis in the T47D breast cancer cell line.

MATERIALS AND METHODS

Study area: This study was conducted from January, 2023 to June, 2023, located in the Laboratory of the Faculty of Pharmacy, Halu Oleo University, Indonesia.

Sample collection: Safflower (*C. tinctorius*) was collected from a safflower plantation in Waemppubu Village, Amali Subdistrict, Bone District, South Sulawesi. The T47D cell line was obtained from the collection of a research laboratory at Airlangga University, Surabaya, Indonesia.

Extraction: Safflower powder (1 kg) was macerated with 96% ethanol (Mercks®, Darmstadt, Germany) for 3×24 hrs. The macerate was then concentrated with a rotary evaporator (BUCHI®, Bangkok, Thailand) at 50°C with 77 mbar. A total of 107.8 g of concentrated extract was yielded⁷.

Phytochemical screening: Phytochemical screening of ethanol extract of safflower, including flavonoids, alkaloids, tannins, saponins, steroids and terpenoids by colorimetric¹⁸⁻²¹.

Culture cell: The T47D cell lines, an epithelial human breast cancer for the hormonal model which were obtained from ATCC®, Virginia, USA. They were cultured in RPMI-1640 complete medium composed of RPMI-1640 supplemented with 10% fetal bovine serum/FBS and 1% antibiotic (10,000 U mL⁻¹ penicillin-streptomycin) which was obtained from Gibco™, New York, USA).

Cytotoxic test: Cell suspension in complete media (100 µL) was seeded in 96-well plates and incubated for 24 hrs at 37°C in a controlled atmosphere (5% CO₂). Then, the media was discarded and replaced with 100 µL various concentrations of the sample containing medium (50, 100, 250, 500, 1000, 1500 and 2000 µg mL⁻¹) and continued incubating. After 24 hrs, 10 µL of CCK-8 reagents (Dojindo®, Rockville, MD) were added to the well, then incubated for 4 hrs at 37°C until an orange formazan complex was formed. The absorbances were

measured with a microplate reader (Infinite® 200 Pro, Tecan, Swiss) at wavelength 450 nm²². The following equation calculates the cell viability (%) of cells:

$$\text{Viability (\%)} = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{medium}}}{\text{Abs}_{\text{cell control}} - \text{Abs}_{\text{medium}}} \times 100$$

From the viability (%), the inhibitory concentration (IC₅₀) values were determined using GraphPad PRISM® version 5 software.

Apoptosis induction test using flow cytometry: In the apoptosis induction assay, the cells were stained with Fluorescein Isothiocyanate (FITC)-annexin V/propidium iodide (PI) which was obtained from BioLegend™, San Diego, USA. Briefly, 100 µL of T47D cells (5 × 10⁵ cells/well) were seeded into a 24-well plate and incubated for 24 hrs at 37°C in a controlled atmosphere (CO₂). The old media was discarded and washed using phosphate buffer saline/PBS (Sigma Aldrich®, USA). The cells were treated with 50 µg mL⁻¹ of 5-fluorouracil, 0.5% of DMSO/dimethyl sulfoxide (Merck®, Darmstadt, Germany) and various ethanol extracts of safflower at a concentration of 100, 1 and 50, 200 µg mL⁻¹ contained medium and complete medium, then re-incubated. After 24 hrs, the medium was discarded and washed with PBS and the cells were harvested with 0.25% trypsin-EDTA (Sigma Aldrich®, St Louis, USA).

The trypsinized cells were added with medium and PBS and transferred into the tube. The tubes were then centrifuged for 5 min at 5000 rpm (25°C) and the supernatant was discarded and resuspended with 1 mL of PBS. The cell suspensions were then re-centrifuged and the supernatant was discarded. Then, 2 µL of FITC-annexin V-PI was suspended to cells and mixed. Then, they were incubated for 10 min in darkness at 25°C. The flow cytometer (BD FACSCalibur™, San Jose, California) with program FlowJo™ v.10 Software (Vancouver, British Columbia) was used to determine and analyze the level of early apoptotic cells (annexin V+/PI-), late apoptotic cells (annexin V+/PI+), necrotic cells (annexin

V-/PI+) and live cells (annexin V-/PI-). Each experiment was conducted in triplicate. The data collected was analyzed with PRISM GraphPad version 5²³⁻²⁷.

RESULTS

Phytochemical screening: Phytochemical screening is a preliminary test used in determining the secondary metabolite, which has the biological activity of a plant²¹. The results of the chemical content test showed that the ethanol extract of safflower positively contains flavonoid compounds, alkaloids, tannins, saponins and terpenoids (Table 1). It showed a similar result to a previous study conducted by Hamsidi *et al.*⁷, which ethanol extract of safflower positively contains flavonoids, alkaloids, tannins, saponins and terpenoids.

Cytotoxic activity: A cytotoxic test was conducted to determine the toxicity potency of compounds obtained from ethanol extract of safflower against the T47D breast cancer cell line. The IC₅₀ value is a parameter used for cytotoxic activity, which is the *in vitro* concentration required to inhibit the growth of cancer cells by 50% of the total population²⁸. If the IC₅₀ value obtained is smaller, it means that the compound has the potential to be used as an alternative for a better anticancer drug because only a low concentration is required to inhibit the proliferation activity of cancer cells by 50%²². According to the % cell viability, the IC₅₀ of ethanol extract of safflower was 479 µg mL⁻¹ (Fig. 1). The results of this study show that ethanol extract of safflower flowers can inhibit the growth of T47D cells by 50% at a concentration of 479 µg mL⁻¹.

Induction of apoptosis: Apoptosis induction tests are performed using the flow cytometry method to determine the cause of cell death, apoptosis and necrosis. The flow cytometry method distinguishes living cells, early apoptosis, late apoptosis and necrosis since annexin V and PI reagents selectively bind intact or incomplete cells (fragmentation)²⁹. Annexin V is a protein with a high affinity for negatively

Table 1: Results of the chemical content test of ethanol extract of safflower flowers

Secondary metabolites	Reagents	Result	Annotation
Flavonoid	Concentrated HCl+Mg Powder	Formed yellow color	+
Alkaloid	Dragendorff	Formed orange precipitate	+
Saponin	Hot water+2N HCl	Formed stable foam	+
Tannin	FeCl ₃ 1%	Formed blackish-green color	+
Steroid	Lieberman-Burchard	Unformed green color	-
Terpenoid		Formed reddish-yellow color	+

+: Contains secondary metabolite compounds and -: Does not contain secondary metabolite compounds

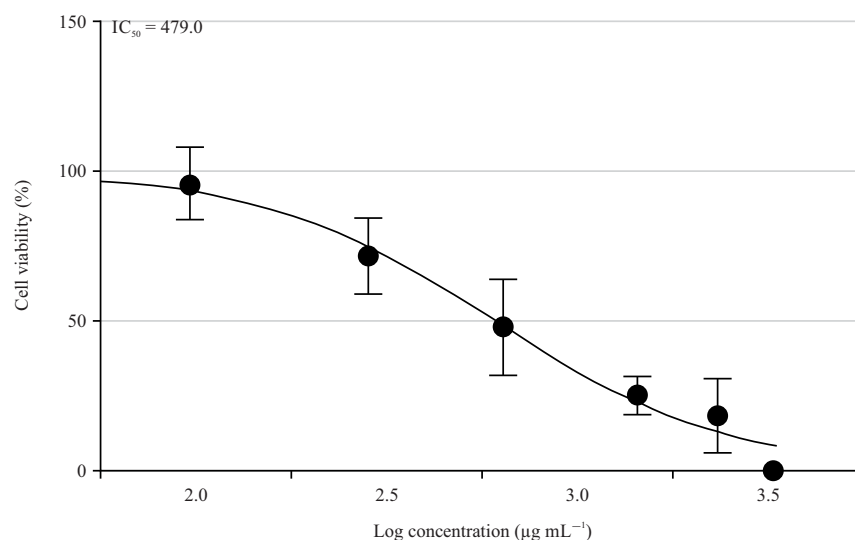


Fig. 1: IC_{50} value ethanol extract safflower

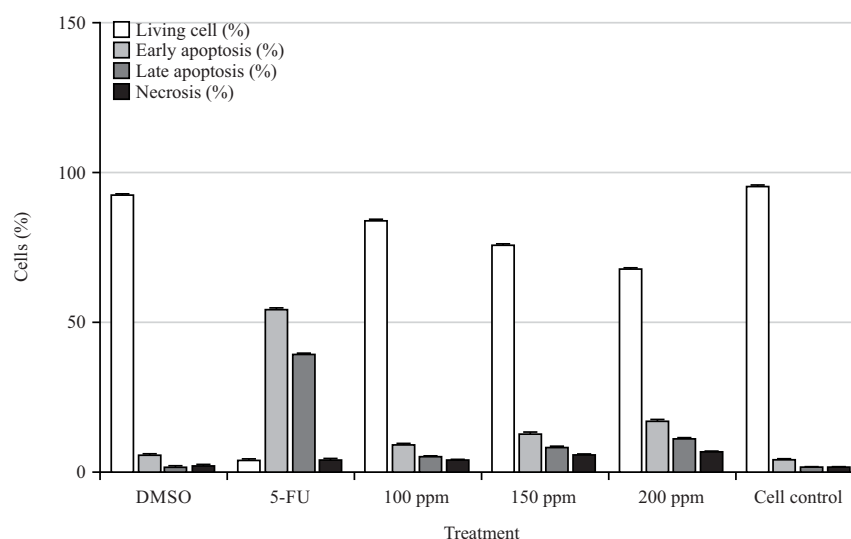


Fig. 2: Graph value % cell death of T47D

Table 2: Results of absorbance value and % cell viability of *C. tinctorius* flower ethanol extract

Concentration ($\mu\text{g mL}^{-1}$)	Cell viability (%)
50	87.53 $\pm$ 0.43
100	86.35 $\pm$ 6.26
250	79.22 $\pm$ 6.28
500	72.10 $\pm$ 8.26
1000	65.49 $\pm$ 3.54
1500	63.30 $\pm$ 6.59
2000	57.91 $\pm$ 1.01
Medium	0.00 $\pm$ 0.98
Cell	100 $\pm$ 0.09

charged phospholipids in the presence of Ca^{2+} ions. The calcium contained in annexin V binds to phosphatidylserine (PS) apoptotic cells' outer plasma membrane. The PS translocation on the cell surface makes

annexin V bonds a marker of apoptosis and the addition of propidium iodide (PI) in cancer cells is used as a marker of necrosis²⁵.

The concentration used for the apoptosis induction assay was 100, 150 and 200 $\mu\text{g mL}^{-1}$. The apoptotic assay can be seen in Table 3 and Fig. 2. Ethanol extract of safflower at a concentration of 200 $\mu\text{g mL}^{-1}$ induces significant apoptosis with 16.61% early apoptosis and 10.52% late apoptosis in breast cells T47D, respectively. The cell death percentage through apoptosis after the administration of ethanol extract of safflower (concentrations of 100, 150 and 200 $\mu\text{g mL}^{-1}$) showed a significant difference ($p < 0.05$) with negative control, thus concluding that all of the concentrations can induce apoptosis.

Table 3: Apoptotic percentage of T47D cancer cells using flow cytometry

Treatment	T47D cells (%)			
	Living cells	Early apoptosis	Late apoptosis	Necrosis
DMSO ^a	92.01	5.21	1.08	1.72
5-FU ^b	3.49	54.13	38.99	3.4
100 µg mL ⁻¹	83.38	8.76	4.37	3.49
150 µg mL ⁻¹	75.06	12.36	7.58	5.03
200 µg mL ⁻¹	66.92	16.61	10.52	5.95
Control T47D	95.28	3.25	0.63	0.83

^aDMSO: Dimethyl sulfoxide and ^b5-FU: Fluorouracil

DISCUSSION

The dried flowers are brewed with hot water to increase endurance⁷. Safflower is often used in traditional Chinese medicine to improve hormonal balance, remove blemishes, regenerate new cells, promote blood circulation, remove bruises, strokes, gynecological diseases, coronary heart disease, angina pectoris, hypertension, amenorrhea, gastric tumors, relieve pain and remove blood stasis. In Mongolian and Tibetan medicine, safflower treats liver metabolic disorders, such as hepatomegaly and liver injury³⁰⁻³². In other countries like India, safflower is commonly used for scurvy, arthritis and mastalgia, while in Iran, safflower is commonly used for skin patches, baldness, diabetes, phlegmatic fever, melancholy and dropsy³³. In addition, Arab countries use safflower during the puerperium after childbirth or even miscarriage, helping to cope with postpartum³³.

The induction of apoptosis might occur through a tumor protein p53 (p53)-dependent mechanism of apoptosis. It is because the T47D cell line has the characteristic p53 mutant³⁴. Apoptosis in breast cancer cells undergoing p53 mutations through the Fas/caspase 8 and Akt/Bad pathways. The Fas activated the 8/10 pro-caspase, then continued activating caspases 3 and 6, which executed apoptosis. Caspase 8 also starts t-Bid, which spurs mitochondria to release cytochrome-c, further forming an apoptosome with APAF-1 (Apoptotic Protease Activating Factor-1) and dATP (deoxyadenosine triphosphate)^{35,36}.

The T47D cell's cell death through apoptotic mechanisms, both early and late apoptosis post administration of *C. tinctorius* flower extract at concentrations of 100, 150 and 200 µg mL⁻¹ showed differences from the negative control so that it could be concluded that the three concentrations had potential as anticancer agents. Anticancer activity, cytotoxic and induction of apoptosis shown by ethanol extract of safflower are inseparable from the secondary metabolite contained in it. One of them is flavonoids²⁷. Flavonoids in safflower are Hydroxysafflor Yellow A (HSYA), hydroxysafflor yellow B (HSYB), safflower yellow (SY) and safflower polysaccharides (SPS). These compounds show their potential as anticancer by inhibiting proliferation in

various human cancer cells. The anticancer activity of flavonoids is also demonstrated through the induction of apoptosis and is non-toxic in normal human cells³⁷. According to Zhang *et al.*³⁸, HSYA can induce apoptosis in the MCF-7 breast cancer cell line by blocking the Nuclear Factor-kappa β (NF-κB) pathway and interfering with the transmembrane potential of mitochondria. According to Mani *et al.*³⁷, HSYB may induce apoptosis in the MCF-7 breast cancer cell line. It was also reported that SPS can increase BAD expression and decrease Bcl-2 expression. The BAD is a significant member of the Bcl-2 family that controls apoptosis by regulating intrinsic pathways or mitochondria and engaging in positive and negative regulation of apoptotic cell death.

CONCLUSION

Extract ethanol safflower contains secondary metabolites, including flavonoids, alkaloids, saponins, tannins and terpenoids. These compounds might contribute to its anticancer activity in inhibiting cell proliferation with an IC₅₀ value of 479 µg mL⁻¹ and inducing apoptosis in T47D breast cancer cell lines at a concentration of 200 µg mL⁻¹ of 13.36% at early apoptosis, 9.89% at late apoptosis and 5.12% at necrosis. The results of this study indicate that the ethanolic extract of *C. tinctorius* flower has anticancer potential through the induction of apoptotic signals. However, it is not known precisely what genes trigger apoptosis, so further research is needed on gene expression profiles as markers of apoptosis.

SIGNIFICANCE STATEMENT

The purpose of this study was to evaluate the *Carthamus tinctorius* extract as an anti-cancer, specifically inducing apoptosis in T47D breast cancer cell lines. The current finding suggested that *C. tinctorius* has activity as an anticancer with IC₅₀ of 479 µg mL⁻¹ and exhibit apoptosis ability at a concentration of 200 µg mL⁻¹. This result might be developed to find novel anti-cancer, especially from natural product like *C. tinctorius*.

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