Pharmacogn J. 2022; 14(2): 374-378
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
www.phcogj.com

Research Article

Cytotoxic Effects of Kirinyuh Herb (*Austroeupatorium inulaefolium* (Kunth) R. d. King & H. Robinson) Extracts and Fractions on BSLT, MCF-7 Cells and T-47D Cells

Riskianto[1,*], Andreanus Andaja Soemardji[2], Marselina Irasonia Tan[3]

ABSTRACT

Breast Cancer is the second-highest prevalence of cancer disease in Indonesia, with the number of new cases and deaths continues to increase. *Kirinyuh or Austroeupatorium inulaefolium* (Kunth) R. D. King & H. Robinson is a shrub plant, which are widely grown in Indonesia. Traditionally, it is used for wound-healing and information about anticancer potential of *A. inulaefolium* is still very limited. This study aims to assess the potential of cytotoxic activity of *A. inulaefolium* on MCF-7 and T-47D breast cancer cells. Tests that carried out in this study were phytochemicals screening, Brine Shrimp Lethality Test (BSLT) and Cytotoxicity assay. The phytochemicals screening showed that EA (leaves extracts levels 1-3), EB (leaves extracts levels 4-6) and EC (stalk extracts) contained tannin, steroids/triterpenoids, flavonoids and phenols. The BSLT results showed that the EB has the smallest LC_{50} value of 12.86 µg/mL. MTT assay results showed the smallest IC_{50} value of the extract and the fraction on MCF-7 cells were EA 204.96 µg/mL and FEA 205.43 µg/mL and T-47D cells were EB 217.03 µg/mL and FEA 192.36 µg/mL. The conclusion of toxicity and cytotoxicity of the herb (leaves and stalks) ethanol 96% extracts of *A. inulaefolium* had a very toxic effect on BSLT however had less potential cytotoxicity on MCF-7 cells and T-47D cells.

Key words: *Kirinyuh* (*Austroeupatorium inulaefolium* (Kunth) R. D. King & H. Robinson), BSLT, Cytotoxicity, MCF-7, T-47D.

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History

- Submission Date: 03-02-2022;
- Review completed: 18-02-2022;
- Accepted Date: 01-03-2022.

DOI : 10.5530/pj.2022.14.48

Article Available online

http://www.phcogj.com/v14/i2

INTRODUCTION

Most tumors emerge from the division of a mother cell in which mutation results in insufficient activation of proto-oncogenes and often genes controlling development, as well as the inactivation of tumor suppressor genes and differentiation genes that control developmental growth and tissue repair. Tumors may be benign or malignant. Benign tumors are small, circumscribed tumors that do not spread to other parts of the body, do not recur after resection and do not result in death. Malignant tumors are ill-defined tumors that infiltrate underlying tissue and can metastasize to other areas of the body, recur after resection and invariably result in death if left untreated.[1] In Indonesia, based on data from Basic Health Research in 2013, the prevalence of breast cancer reached 0.5 per 1000 women.[2]

Indonesia has a very diverse and abundant biological wealth, both from land and marine. Scientists are now trying to do various research efforts to obtain natural materials that have efficacy as antitumor and considered relatively safe.[3,4] One of the plants used in herbal medicine in Indonesia is *Kirinyuh* (*Austroeupatorium inulaefolium* (Kunth) R. D. King and H. Robinson). *A. inulaefolium* is a type of bush plant that has a lot of population and grows wildly.

Ethnopharmacological studies conducted on *A. inulaefolium* plants have shown that used as a wound treatment[5] and there was information that also uses *A. inulaefolium* as a cure for breast cancer in Indonesia (5 leaves from the shoots mixed with 4 liters of water and spoon of salt then boiled until boiling, filtered, and ready to drink). In Caldas (Colombia) for the treatment of throat disorders.[6] Insecticide for the prevention of ectoparasites (Fleas) in livestock, particularly in bovine (Colombia),[7,8] as a mosquito repellent, as an anti-flea where the surface of the space covered with leaves that repel fleas.[9] In Brazil, used to alleviate swelling of the eye[10] and sometimes used as a contraceptive and abortion.[11,12] Leaves infusion as a digestive drug.[13] In Argentina, *A. inulaefolium* has the common name of "Sanalotodo" or "Yerba de Santa María" this species is used in traditional medicine for the treatment of skin infections because of its efficacy as an antimicrobial[14] and treatment for sore throat.[15]

Several studies were conducted to determine the efficacy of kirinyuh leaves such as Dichloromethane extract from *A. inulaefolium* leaves shows anti-inflammatory activity caused by carrageen inductions.[16] Indika *et al.* (2015)[17] also indicated antioxidant activity and antimicrobial activity in *A. inulaefolium*.[15,18] Simoes et al. (1999) evaluated antiviral efficacy, possibly finding involvement in two of the five types of viruses examined, namely HSV-1 (type 1 herpes simplex virus) and HSV-2 (type 2 Herpes Simplex Virus).[19]

The phytochemical of *A. inulaefolium* has Diterpene contents such as Kaurene, Labdane, Austroinulin.[20-22] Flavonoid groups such as Flavon,[23] Flavonol,[24] Inulifolinone,[25] Austroeupatol,[26] 4'-tetrahydroxy-6-methoxy-flavone (Eupafolin),[27] 5, 6,3'-trihydroxy-7,4'-dimethoxyflavone and Pedalitin.[23] The present study attempted to screening the cytotoxicity activity of *A. inulaefolium* extract from leaves and stalks in a Brine Shrimp Lethality Bioassay and an *in vitro* MTT assay.

Cite this article: Riskianto, Soemardji AA, Tan MI. Cytotoxic Effects of Kirinyuh Herb (*Austroeupatorium inulaefolium* (Kunth) R. d. King & H. Robinson) Extracts and Fractions on BSLT, MCF-7 Cells and T-47D Cells. Pharmacogn J. 2022;14(1): 374-378.

MATERIALS AND METHODS

Plant material and identification

The samples used are dry herb (leaves and stalk) of *A. inulaefolium* obtained from Bandung, West Java, Indonesia. Plant determination at Herbarium School of Science and Technology, Institut Teknologi Bandung.

Extraction

The dry leaves (levels 1-3 and 4-6 from bud) and dry stalks of *A. inulaefolium*, 200 g refluxed using ethanol 96% at the temperature of 80°C for 4 hours. The results were obtained, filtered, then treated using a rotary evaporator and dried in a water bath at 60°C until obtained a condensed extract of the leaves 1-3 (EA) and 4-6 (EB) and stalks (EC). Extracts tested through Brine Shrimp Lethality Test (BSLT), an extract which was actively in BSLT fractionated and obtained Water Fraction (WF), Ethyl Acetate Fraction (EAF) and N-Hexane Fraction (NHF). Cytotoxicity tested of extracts and fractions was carried out using MTT assay.

Fractionation

EB is the active extract in the BSLT fractionated by the liquid-liquid extraction method. A total of 50 g of EB extract dissolved using hot water, filtered and put into a separating funnel after being separated with N-Hexane and Ethyl Acetate solvents so that there are three large fractions of which N-Hexane (FNH), Ethyl Acetate (FEA) and Water (FW).

Animal

Shrimp samples were determined at the Zoology Museum, School of Biological Sciences and Technology, Institut Teknologi Bandung.

Phytochemical screening

Screening of phytochemical contents in the *A. inulaefolium*, including alkaloid, flavonoids, saponin, steroids, tannins and terpenoids compounds.[28]

Screening of cytotoxicity by using BSLT

The test practiced *Artemia salina* as test animals in BSLT method. *A. Salina's* eggs are hatched in artificial seawater (38 g of salts in 1000 mL of water) at room temperature. After 48 hours, hatch grew a nauplii instar III/IV and ready to use. Ten numbers of nauplii were inserted into the vial, that has contained a solution of extracts with a series of concentrations 10, 100, 500 and 1000 ppm with six times repeated. Then vials incubated at room temperature for 24 hours. After 24 hours and observation was performed by looking at the number of nauplii that died at each concentration. The determination of LC_{50} in µg/mL by using probit analysis with linear regression.[29]

Cytotoxicity assay

Samples were made in several variations of concentration. The number of cells suspension taken and the amount of medium added to calculated and obtain the cell concentration amount 104 Cells/100 µL. Cells are distributed to the microplate 96 wells, with a concentration of 104 cells/well in 100 µL, and incubated for 24 hours. The sample solution was inserted into each well of the 100 µL. The microplate was incubated for 24 hours at a temperature of 37°C with a CO_2 5 mL/min. After 24 hours, washed and rinse with PBS. Then, each of the MTT added by 100 µl, incubated again at the incubator of CO_2 for 4 hours, then the reaction of MTT stopped by adding 100 µL of sodium dodecyl sulfate (SDS) 10%. Microplate-incubated for 12 hours at room temperature. After 12 hours, each well-read with a Spectrophotometer Plate Reader at a wavelength of 595 nm.[30]

The percentage of living cells is calculated through cell absorbance and then made a curve that is the relationship between concentration and percent value of living cells. The percentage of living cells is calculated using the formula % Living cells.

% Living cells =

$$\frac{Absorbance\ of\ treatment - Absorbance\ of\ media\ control}{Absorbance\ of\ cell\ control - Absorbance\ of\ media\ control} \times 100\%$$

The IC_{50} value determined from the graph with a linear regression equation, the "y" value is inserted 50% in the linear regression equation (y = bx + a), then the "x" value is calculated as IC_{50} value.

Statistical data analysis

The data obtained from the observation results are processed by linear regression using Microsoft Excel's version 2016 and SPSS version 22. The data were evaluated statistically.

RESULTS AND DISCUSSION

Extraction and fractionation of *A. inulaefolium*

The results of the extraction process and the percentage yield of leaves and stalk with the reflux method using ethanol 96% solvent shown in Table 1. Based on the yield data in Table 1 can be concluded that the largest yield is extracted from leaves levels 1-3 (EA) by 25%. EB was active in BSLT then fractionated. Data on EB fractionation results can be seen in Table 2. The highest yield of fractionation results is the water fraction of 21%. This indicates that the largest component of chemical compounds in the EB fraction of *A. inulaefolium* is polar compounds.

Phytochemical screening

Phytochemical screening was carried out to determine the content of secondary metabolites in the sample. The results in Table 3, dried

Table 1: The percentage (%) of yield of *A. inulaefolium* extract.

Plant Parts	Weight Dry (g)	Weight Extract (g)	Yield (%)
Leaves			
EA	1000	251	25.1
EB	1000	202	20.2
Stalk EC	650	79	12.15

Table 2: The percentage (%) of yield of *A. inulaefolium* fraction.

EB (g)	Fraction	Weight Fraction (g)	Yield (%)
50	N-Hexsan	6.21	12.42
	Ethyl acetate	2	4
	Water	10.5	21

Table 3: Phytochemical screening.

Compound	Dried			Extract		
	A	B	C	A	B	C
Alkaloids	-	-	-	-	-	-
Tannins	+	+	+	+	+	+
Steroid/Triterpenoid	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+
Saponins	-	-	-	-	-	-
Kuinon	-	-	-	-	-	-
Phenol	+	+	+	+	+	+

(A) leaves levels 1-3 from the bud, (B) leaves levels 4-6 of the bud, (C) stalk, (-) undetectable, (+) detected.

samples and extracts of *A. inulaefolium* contains tannin, steroid/triterpenoid, flavonoid and phenol.

Screening of cytotoxicity by using BSLT

The criteria of toxicity on BSLT according to Meyer that the value of Lethal Concentration 50% (LC_{50}) <1000 µg/mL is toxic and LC_{50} >1000 µg/mL is nontoxic[29] and criteria toxicity according to Clarkson on the plant extracts in the following classify: Extract with LC_{50} from 500-1000 µg/mL low toxicity, extracts with LC_{50} from 100-500 µg/mL toxicity and extracts with LC_{50} from 0-100 µg/mL very toxic.[31] The results of the BSLT conducted from the test samples, LC_{50} values obtained from each test sample can be seen in Figure 1.

The LC_{50} of the sample test obtained for EA, EB, EC, and Doxorubicin are 79.2 µg/mL, 12.86 µg/mL, 53,02 µg/mL, and 3,012 µg/mL. Based on the criteria of toxicity according to Meyer (1982),[29] all test samples belong to toxic because it has LC_{50} <1000 µg/mL and based on Clarkson toxicity criteria is highly toxic because it has an LC_{50} 0-100 µg/mL and based statistical analysis on EA, EB, EC and significantly different from Doxorubicin ($P < 0.05$). Therefore, the EB is the candidate compounds that have the potential as cytotoxic with LC_{50} 12.86 µg/mL.

Activity test of cytotoxicity to MCF-7 cells and T-47d cells

Cytotoxicity studies were performed with MTT to further verify the cytotoxic function of the cancer cell lines. Cytotoxicity tests were conducted to determine the potential of cytotoxic extracts and EB fractions on MCF-7 cells and T-47D cells. Cytotoxicity activity of test samples on MCF-7 cells and T-47D cells is presented in Figure 2 and Figure 3. Cytotoxic activity in MCF-7 cells with the smallest IC_{50} values from the samples on MCF-7 cells was from EA 204.96 µg/mL and FEA 205.43 µg/mL and on T-47D cells were EB 217.03 µg/mL and FEA 192.36 µg/mL. Based on statistical analysis was significantly different ($p > 0.05$).

A natural compound that has the potential as an anticancer has an IC_{50} value of ≤ 30 µg/mL.[32] Therefore, based on the results of a cytotoxic test of the MCF-7 cells and the T-47D can be concluded that the extracts and fraction of *A. inulaefolium* is a compound that lacks the potential of cytotoxicity to the MCF-7 cells and T-47D cells. Data on the ability of cytotoxic extracts and fraction of the leaves and stalk of the *A. inulaefolium* on BSLT and breast cancer cells (MCF-7 and T-47D). LC_{50} and IC_{50} values are summarized in Table 4.

CONCLUSION

The conclusion in this preliminary study based on *in vitro* and *in vivo* studies showed that the toxicity on BSLT of the ethanol 96% extract of leaves level 4-6 from shoots (EB) has an LC_{50} value of 12.86 µg/mL classified as very toxic. The cytotoxicity of the leaves extract levels 1-3 (EA) and Ethyl Acetate Fraction (FEA) from leaves extract levels 4-6 (EB) had the smallest IC_{50} values of 204.96 µg/mL and 205.43 µg/mL on MCF-7 cells. The cytotoxicity of the ethanol 96% extract of leaves level 4-6 (EB) and Ethyl Acetate Fraction (FEA) from leaves extract level 4-6 (EB) had the smallest IC_{50} values of 217.03 µg/mL and 193.42 µg/mL on T-47D cells. The toxicity and cytotoxicity of the herb (leaves and stalks) ethanol 96% extracts of *A. inulaefolium* had a very toxic effect on BSLT however had less potential for cytotoxicity on MCF-7 cells and T-47D cells.

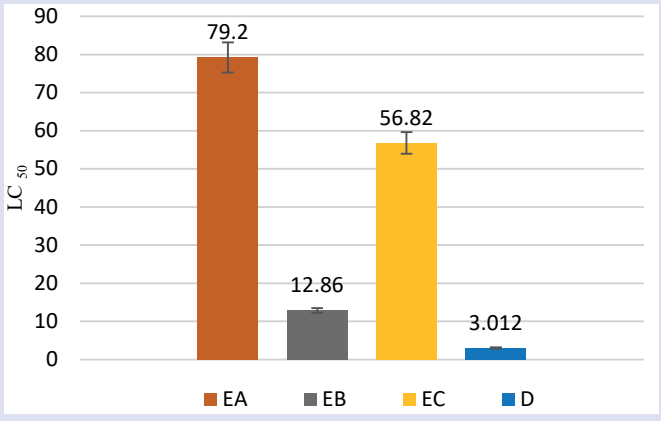

Figure 1: Diagram LC_{50} value on BSLT.

(EA) leaves extract levels 1-3 from the bud, (EB) leaves extract levels 4-6 from the bud, (EC) extract of the stalk, (D) doxorubicin

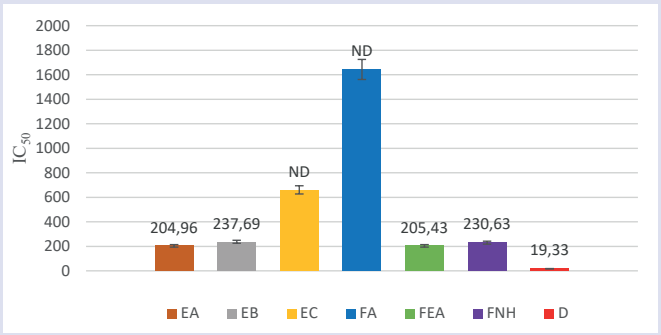

Figure 2: Diagram IC_{50} value in MCF-7 cells

(EA) leaves extract levels 1-3 from the bud, (EB) leaves extract levels 4-6 from the bud, (EC) extract of the stalk, (FA) fraction of water from EB, (FEA) fraction of ethyl acetate and (FNH) fraction of n-hexane from EB, (D) doxorubicin, (ND) not determined.

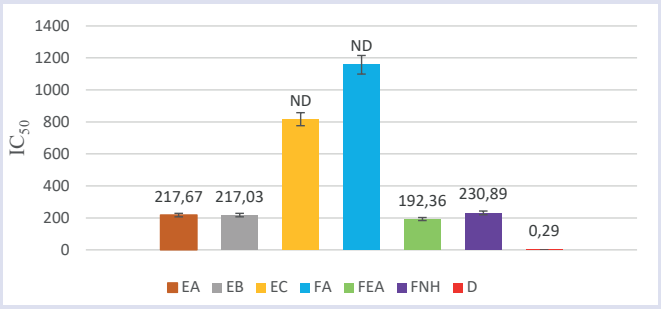

Figure 3: Diagram IC_{50} value T-47d cells

(EA) leaves extract levels 1-3 from the bud, (EB) leaves extract levels 4-6 from the bud, (EC) extract of the stalk, (FA) fraction of water from EB, (FEA) fraction of ethyl acetate and (FNH) fraction of n-hexane from EB, (D) doxorubicin, (ND) not determined.

Table 4: LC_{50} and IC_{50} values.

Sample	BSLT	MTT (IC_{50})	
Test	(LC_{50})	MCF-7	T-47D
	(µg/mL)	(µg/mL)	(µg/mL)
EA	79.2	204.96	217.67
EB	12.86	237.69	217.03
EC	53.02	ND	ND
FW	-	ND	ND
FEA	-	205.43	192.36
FNH	-	230.63	230.89
D	3.012	19.33	0.29

(EA) leaves extract levels 1-3 from the bud, (EB) leaves extract levels 4-6 from the bud, (EC) extract of the stalk, (FW) fraction of water from EB, (FEA) fraction of ethyl acetate from EB and (FNH) fraction of n-hexane from EB, (D) doxorubicin, (ND) not determined.

DISCLOSURES STATEMENT

No potential conflicts of interest are available to declare by the authors.

ACKNOWLEDGMENTS

The authors would like to thank the director and laboratory assistants of the Pharmacology and Toxicology Laboratory of the School of Pharmacy, Institut Teknologi Bandung and the Department of Parasitology, Faculty of Medicine, Universitas Gadjah Mada. The author would also like to thank the LPPM of Universitas Pelita Harapan and the Faculty of Health Sciences of Universitas Pelita Harapan.

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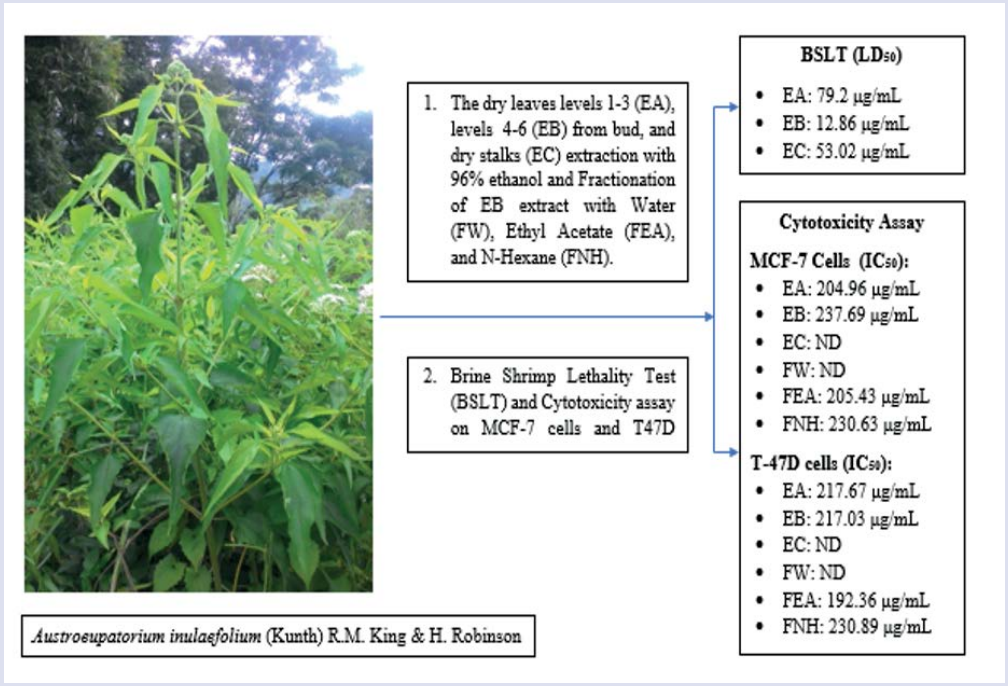
GRAPHICAL ABSTRACT



SUMMARY

Kirinyuh (*Austroeupatorium inulaefolium* (Kunth) R. D. King & H. Robinson) contained tannin, steroids/triterpenoids, flavonoids, and phenols. The BSLT results showed that the EB has the smallest LC50. MTT assay results showed that EA and FEA have the smallest IC50 on MCF-7 cells, and EB and EAF have the smallest IC50 on T-47D cells. Toxicity and cytotoxicity of the herb (leaves and stalks) ethanol 96% extracts of *A. inulaefolium* had a very toxic effect on BSLT and had less potential cytotoxicity on MCF-7 cells and T-47D cells.

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Cite this article: Riskianto, Soemardji AA, Tan MI. Cytotoxic Effects of Kirinyuh Herb (*Austroeupatorium inulaefolium* (Kunth) R. d. King & H. Robinson) Extracts and Fractions on BSLT, MCF-7 Cells and T-47D Cells. Pharmacogn J. 2022;14(1): 374-378.