Phytochemical Analysis and Cytotoxic Activities of Hantap Leaves (*Sterculia coccinea* Jack) Extract

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Abstract: *Hantap (Sterculia coccinea Jack) has been used traditionally for various health issues, including cancer treatment. The therapeutic effects of natural ingredients are often attributed to their chemical constituents. This study aimed to analyze the phytochemical contents and cytotoxic activities of* S. coccinea *leaves on HeLa and MCF-7 cancer cell lines. The quantitative phytochemical analysis was carried out following standard laboratory procedures. Phytochemical compounds were identified using LC-MS/MS QTOF. The MTT assay PrestoBlue™ Cell Viability Reagent test method was used to test cytotoxic activity in the cell culture. Extraction was carried out by the maceration method using 96% ethanol as solvent. The quantitative analysis revealed that tannins were the major phytochemical constituent in the highest percentage of 72.16%, followed by alkaloids, flavonoids, and steroids, with values of 30.80, 28.66, and 2.85%, respectively. Saponins were present in the lowest percentage of 1.15%. The ethanolic extract exhibited moderate cytotoxicity on HeLa and MCF-7 cells with IC$_{50}$ values of 591.00 and 578.10 µg/mL, respectively. Identification using LC-MS/MS showed the suspected compounds 5,7-dihydroxy-3-(4′-hydroxybenzyl)chromone as homoisoflavanones and kaempferide-3-O-α-L-rhamnosyl-7-O-α-L-rhamnoside from flavonol triglycosides. These results may contribute to the study on the use of leaves extract of* S. coccinea *for developing a chemoprevention agent.*

Keywords: *cytotoxicity; hantap leaves; HeLa; MTT assay; MCF-7*

■ INTRODUCTION

Cancer is one of the most prominent and still incurable diseases worldwide, including in Indonesia. A report has shown that approximately 10 million people die from cancer each year, with 70% occurring in developing or Low-Middle-Income Countries (LMICs), such as Indonesia [1-2]. The International Agency for Research on Cancer (IARC) predicts that by 2040, there will be around 29.5 million new cases and 16.3 million deaths from cancer in the world [3]. According to Basic Health Research, the prevalence of tumors/cancer in Indonesia increased from 1.4 per 1000 in 2013 to 1.79 per 1000 population in 2018 [4]. Based on GLOBOCAN 2020 data, the highest cancer cases in the country are breast and cervical cancers [5]. Several efforts have been made to cancer treatment, including surgery, radiation, monoclonal antibodies, and chemotherapy [6]. However, these treatments also have side effects such as cardiotoxicity, renal toxicity, and myelotoxicity [7-9]. Various problems in cancer therapy trigger the development of new drugs from natural compounds due to their advantages in therapeutic management, including relatively minor side effects. The existence of these side effects causes serious problems, leading people to the selection of other alternatives to treat cancer with herbal medicines. Therefore, there is a significant scientific and commercial interest in discovering new natural anticancer agents [10-11].

Medicinal plants or herbs can be used as complementary medicine alternatives. This approach combines conventional or medical services with traditional and only alternative treatment. Herbal medicine has been used as an alternative or complementary medicine for breast, cervical, and cervical cancers around the world [10,12]. In Indonesia, reports have shown that 61.8% of cancer patients with cervical cancer use herbal medicine in addition to conventional treatment [13]. Various studies have been conducted to discover new compounds that can serve as chemopreventive agents. Chemoprevention involves the use of synthetic or natural chemical compounds to prevent carcinogenesis, inhibit the development of cancer cells, reduce pain, and improve healing [14]. Therefore, chemotherapeutic agents are potential to be used as anticancer compounds in chemoprevention. Many natural compounds in plants, such as vincristine, vinblastine, taxol, and camptothecin, exhibit anticancer activity [15].

One of the plants commonly used by the Donggala community as traditional medicine is hantap leaves (*Sterculia coccinea* Jack). Leaves are boiled and drunk to treat various cancers, such as breast, brain, blood (leukemia), uterine, cervical, and prostate cancers [16]. However, there is still limited information on its potential as an anticancer. In a previous study, coumarin compounds (stercularin) isolated from plants of the same genus, *Sterculia diversifolia*, showed a cytotoxic effect with an LD_{50} of 8.00 μg/mL and anticancer activity against PC-3 cell lines with IC_{50} 3.92 ± 0.20 μg/mL [17]. Other studies have also demonstrated an anticancer effect on three human cancer cell lines, namely MCF-7, HepG2, and HeLa, of the stercufoetin compound isolated from the leaves of *Sterculia foetida* L [18]. Based on the chemotaxonomic approach, plants from the same genus or family may also have compounds with similar structures and biological activity [19]. Many natural compounds in plants are known to have cytotoxic activity against cancer cells [20-21]. The chemical content in *S. coccinea* leaves is suspected of contributing to the anticancer effect. Therefore, this study aims to investigate the cytotoxic activity of *S. coccinea* leaf extract against breast (MCF-7) and cervical (HeLa) cancer cells and to determine the secondary metabolite levels contained in these extracts contributing to their cytotoxic effects. The results are expected to become scientific information for the development of *S. coccinea* plants as anticancer-standardized herbal medicinal ingredients.

■ EXPERIMENTAL SECTION

Materials

Chemicals and reagents required for cell culture and molecular biology grade in this study were purchased from Thermo Fisher Scientific. These included PrestoBlue™ Cell Viability Reagent, Roswell Park Memorial Institute Medium (RPMI), Fetal Bovine Serum (FBS), Trypsin-EDTA, and Trypan Blue. The pro-analyst solvents used for phytochemical analysis, including ethanol, methanol, chloroform, acetonitrile, and formic acid were purchased from Sigma Aldrich. Subsequently, HeLa and MCF-7 cells were obtained from the Central Laboratory of Padjadjaran University, Bandung. All other reagents were purchased from Merck.

Instrumentation

The tools used included analytical balance (Shimadzu), plate 96 wells (Thermo MicroWell), water bath, Erlenmeyer (Pyrex), oven (Memmert), beaker (Pyrex), blender (Miyako), rotary evaporator (Heidolph), micropipette (Thermo Fisher Scientific), biosafety cabinet, CO_2 incubator (Thermo Fisher Scientific), microscope (Olympus CX33), and Multimode reader (Thermo Fisher Scientific). LCMS/MS data were acquired using an Acquity UPLC I-Class/Xevo G2-XS, QT, USA) and column ACQUITY UPLC BEH C8 1.7 μm 2.1 × 50 mm.

Procedure

Sample preparation and extract

The used plant parts were leaves collected in Palu, Central Sulawesi, and identified at the Plant Biosystematics Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Tadulako University with No. 340/UN 28.1.28/BIO/2021. The leaf powder of *S. coccinea* plants, weighing 2.8 kg, was extracted using the maceration method using 96%

ethanol solvent with ± 28 L. Extract process was carried out for 1 × 24 h and occasionally stirred and filtered. The maceration in the form of the liquid extract was collected, and the dregs were maceration for 5 × 24 h with the same solvent. All the macerate was collected and evaporated with a vacuum vaporizer until a thick extract was obtained and weighed, followed by the calculation of the percent yield.

Phytochemical analysis

A quantitative phytochemical test of the ethanol extract of *S. coccinea* leaves was performed according to standard laboratory methods of the Integrated Research and Testing Laboratory Universitas Gadjah Mada (saponins and steroids) and the Pakuan University Service Laboratory (alkaloids, flavonoids, and tannins). The test procedures were conducted as follows:

Determination of total alkaloids. The 50 mg sample was dissolved with aqua distillate, and 2 mL of the solution was pipetted. This was followed by the addition of 2 mL of phosphate buffer solution pH 4.7 and 2 mL of bromocresol green (BCG), as well as 3 mL of chloroform for the extraction process, three times each. The chloroform layer formed was taken and put into a 10 mL test tube, and 10 mL of chloroform was added. The solution was tested with a spectrophotometer with a wavelength of 430 nm, with Atropine as the standard. Subsequently, concentration series of 50, 100, 150, 200, and 250 ppm were made. The standard solution was made by pipetting 2 mL of a certain concentration and adding 2 mL phosphate buffer pH 4.7, with 2 mL BCG solution, and extracting with 3 mL chloroform three times. The chloroform layer formed was put into a 10 mL test tube, and chloroform to 10 mL was added. The resulting solution was tested using a spectrophotometer with a wavelength of 430 nm [22].

Determination of total flavonoids. A total of 10 mg of the sample was dissolved in 10 mL aqua distillate. Approximately 0.5 mL of test solution was pipetted and mixed with 1.5 mL of 96% ethanol, 0.1 mL of 10% aluminum chloride, and 0.1 mL of sodium acetate in a total volume of 2.8 mL. The mixture was homogenized and incubated for 30 min at room temperature, followed by testing with a spectrophotometer at a wavelength of 430 nm. A total of 10 mg of quercetin was dissolved in methanol in a 10 mL volumetric flask to obtain a concentration of 1,000 ppm. Subsequently, 100, 200, 300, 400, and 500 μL of the solution was pipetted into a 5 mL scale test tube with methanol to obtain a concentration of 20, 40, 60, 80, and 100 ppm. The standard solution was made by mixing 0.5 mL of the solution with 1.5 mL of 96% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of sodium acetate, and 2.8 mL of distilled water. The mixture was homogenized and incubated for 30 min at room temperature, followed by testing using a spectrophotometer at a wavelength of 430 nm [23].

Determination of total tannins. A total of 10 mg of the sample was dissolved with the aqua distillate in a 10 mL volumetric flask. Approximately 1 mL of test solution was pipetted and added with 0.5 mL Folin Ciocalteu, 1 mL Na_2CO_3 20%, and distilled water up to 10 mL. The mixture was homogenized and incubated for 30 min at room temperature, followed by testing using a spectrophotometer at a wavelength of 430 nm. Subsequently, 50 mg of tannic acid was dissolved with the aqua distillate in a 50 mL volumetric flask to obtain a concentration of 1,000 ppm. A total of 100, 200, 300, 400, and 500 μL of the solution was pipetted into a 5 mL scale test tube and equilibrated with aqua distillate to obtain the concentrations of 20, 40, 60, 80, and 100 ppm. To prepare the standard solution, 1 mL of the diluted solution was pipetted and mixed with 0.5 mL Folin Ciocalteu, 1 mL of 20% Na_2CO_3, and 10 mL of aqua distillate 10 mL. The mixture was homogenized and incubated for 30 min at room temperature, followed by testing using a spectrophotometer at a wavelength of 740 nm [24].

Determination of total saponins. Approximately 50 mg of the sample was weighed and 2 mL of 25% H_2SO_4 was added. The mixture was autoclaved for 120 min at 110 °C, followed by extraction with ether. Subsequently, the filtrate was dried, and 1 mL of water was added. The solution was extracted by vortex for 5 min, and 50 μL of anisaldehyde was added, shaken, and allowed to stand for 10 min. A total of 2 mL of 50% sulfuric acid was added and heated in a water bath at

60 °C for 10 min. The volume was adjusted to 10 mL by adding water with a measuring flask and diluted 5 times. The absorption was read at a wavelength of 435 nm. For the preparation of the standard saponin curve from Quillaja bark, the standard of saponins was weighed at 10 mg and added to 5 mL of water. The solution was extracted by vortex for 5 min, added with 50 μL of anisaldehyde, shaken, and allowed to stand for 10 min before adding 2 mL of 50% sulfuric acid. The solution was heated in a water bath at 60 °C for 10 min, and a water volume of 10 mL was added using a measuring flask. The standard curve was prepared by diluting the solution to 200, 100, 50, 25, 12.5, and 6.25 μL. Finally, the absorption was read at a wavelength of 435 nm [25].

Determination of total steroids. A total of 50 mg of the sample was weighed and placed in a microtube. Subsequently, 1 mL of ethanol was added, and the mixture was vortexed for 30 s and sonicated for 60 min. The sample was macerated for 24 h at room temperature and centrifuged. This was followed by the application of 1 and 2 μL of the supernatant on a silica gel 60 F254 plate, including a beta-sitosterol comparator. The plate was entered into the saturated chamber with the mobile phase toluene: ethyl acetate (80:20) and expanded to limit, removed, and dried. Spraying was carried out using Lieberman Burchard reagent, and the mixture was heated at 110 °C for 2 min. The sample spotting was 1 and 2 μL, with a propagation distance of 8 cm. The Rf beta-sitosterol was found to be 0.55, and a 1,000 g/mL (60% purity) solution was used as the standard [22]. Different concentrations of standard beta-sitosterol (0.12, 0.24, 0.48, 0.72, and 0.96 μg/mL) solution were prepared identically as that of the sample to construct a standard curve. The interpretation of the TLC plate with a densitometer was carried out by scanning the maximum wavelength and measuring the area under the curve (AUC). The steroid content in the sample extract was calculated by linear regression between AUC and the content of the standard beta-sitosterol curve [26].

Identification of phytochemical compounds ethanoicl extract by LC-MS/MS

A sample of 1 μL was injected into the column using a gradient elution method with water-formic acid 0.1% (v/v) as solvent A, acetonitrile-formic acid 0.1% (v/v) as solvent B, and a flowing rate of 0.3 mL/min with a 17 min gradient elution. All parameters, namely column, mobile phase, and MS detection, were optimized to obtain the best separation conditions with a suitable run time. The optimal conditions of analysis were as follows: column temperature 40 °C, sample temperature 20 °C, acquisition start time, 0.00–17.00 min start mass, full scan 100.00–1,200.00 m/z, scan time 0.100 s; acquisition mode, ESI (+). The fragmentor capillary was set at 2 kV, cone 30 V, source temperature 120 °C, desolvation temperature 500 °C, cone gas flow 50 L/h, and desolvation gas flow 1000 L/h. The UNIFI software was used to process the LC-MS/MS data files.

Cytotoxic activity assay

Cytotoxic preparation and testing followed the Central Laboratory, Padjajaran University test protocol. The cytotoxic activity of *S. coccinea* leaves extract was evaluated against HeLa and MCF-7 cells using Presto Blue Cell Viability reagent. Cisplatin was used as the standard drug [27] and extracts were dissolved with 2% DMSO in PBS and serially diluted to obtain different concentrations of 7.81, 15.6, 31.25, 62.5, 125, 250, 500, and 1,000 μg/mL. In 96 well plates, 10 μL of suspension cells with a density of 170,000 cells/mL were added and incubated at 37 °C in a 5% (v/v) CO_2 incubator for 24 h. The cells were treated with 100 μL various concentrations of extract as well as positive control and incubated for 48 h. After incubation, the culture medium was removed, and the cells were washed with PBS. The medium was immediately replaced by 10 μL Presto Blue reagent in a 90 μL RPMI medium and was incubated for 1–2 h until a color change was observed. Subsequently, the absorbance was measured at a wavelength of 570 nm. The experiment was performed in triplicate using a 19 μM concentration of cisplatin for the test. The results of the cytotoxic test on HeLa and MCF-7 cancer cells showed that the absorption data was searched for a linear regression relationship between the concentration log and the percent of living cells leading to the equation $y = bx + a$. The IC_{50} was calculated by substituting the value 50 for Y to obtain the value of x, and the value of IC_{50} was taken as the antilog of x.

Data analysis

The quantitative phytochemical results were presented in a descriptive method, while the cytotoxicity examination was analyzed and graphed using Graph Pad.

■ RESULTS AND DISCUSSION

Medicinal plants were found useful in managing several diseases, including cancer [28]. *S. coccinea* leaves were one of the medicinal plants used empirically by a tribe in Donggala, Central Sulawesi, for the treatment of breast and cervical cancers [29]. In this study, ethanolic extract from the maceration process resulted in a yield of 15.7%, which was an important value in extract manufacture. The yield value showed the effectiveness of the extract process, which was influenced by the sample particle size, extract method, extract time, and the type of solvent used [30].

Phytochemical Analysis

A quantitative phytochemical analysis was carried out on the ethanolic extract of *S. coccinea* leaves using the standard methods for alkaloids, flavonoids, saponins, tannins, and steroids. The results of the phytochemical analysis obtained from ethanolic extract were presented in Table 1. Phytochemicals are important for human health and can potentially treat various diseases. According to Effendi [16], the results of the chemical identification of *S. coccinea* leaves powder showed the presence of tannins, steroids, and alkaloids. Several active compounds, such as flavonoids, alkaloids, saponins, tannins, and steroids, were shown to possess anticancer effects [31]. Therefore, a quantitative analysis of the phytochemical of the extract was conducted to determine the levels of secondary metabolites contributing to the anticancer effect.

Table 1. Total alkaloids, flavonoids, saponins, tannins, and steroids on *S. coccinea* leaves extract

No	Type of analysis	Result (%)
1	Alkaloids	30.80
2	Flavonoids	28.66
3	Saponins	1.15
4	Tannins	72.16
5	Steroids	2.85

The ethanol extract was found to contain total alkaloids, which were quantified using the linear regression equation $y = 0.0012x + 0.1982$ ($R^2 = 0.9990$) of the atropine standard curve. Total flavonoids were quantified using linear regression equation $y = 0.0074x + 0.0055$ ($R^2 = 0.9992$) of the standard quercetin curve. Moreover, total tannins and saponins were determined, respectively, by applying the linear regression equation $y = 0.0086x + 0.0132$ ($R^2 = 0.9993$) of the tannic acid standard curve and $y = 9.4416 \times 10^{-4}x - 0.0038$ ($R^2 = 0.9996$) of the Quillaja bark.

The results of the quantitative analysis in Table 1 showed that tannins had the highest levels, followed by alkaloids and flavonoids. Steroids and saponins were present in small amounts. The determination of the levels of secondary metabolites in the leaf extract showed that the most numerous groups of phenolic compounds were tannins and flavonoids, with values of 72.16 and 28.66% w/w. Previous studies showed that phenolics and flavonoids were the most important secondary metabolites in the genus *Sterculia* [32].

Identification of Phytochemical Compounds in Ethanol Extract of Hantap Leaves by LC-MS/MS

Analysis of the secondary metabolite profile of *S. coccinea* leaves extract was performed using LC-MS/MS. The results showed that the extract contained at least five compounds in Table 2, but only two compounds belonging to the flavonoid group. The LC-MS/MS spectrum is shown in Fig. 1. This study supported the results of quantitative phytochemical analysis of the leaves extract that phenolic compounds, including flavonoids, were present in large enough quantities. This was also confirmed by the study by El-Sherei et al. [33] on the genus *Sterculia,* indicating the presence of various flavonoid compounds, mostly as flavones and flavonol glycosides.

Cytotoxic Activity Assay

Previous studies showed that *S. coccinea* leaves ethanolic extract had a very strong antioxidant activity, with an IC_{50} of 6.48 μg/mL and moderate cytotoxic activity (LC_{50} 591.56 μg/mL), as demonstrated by the
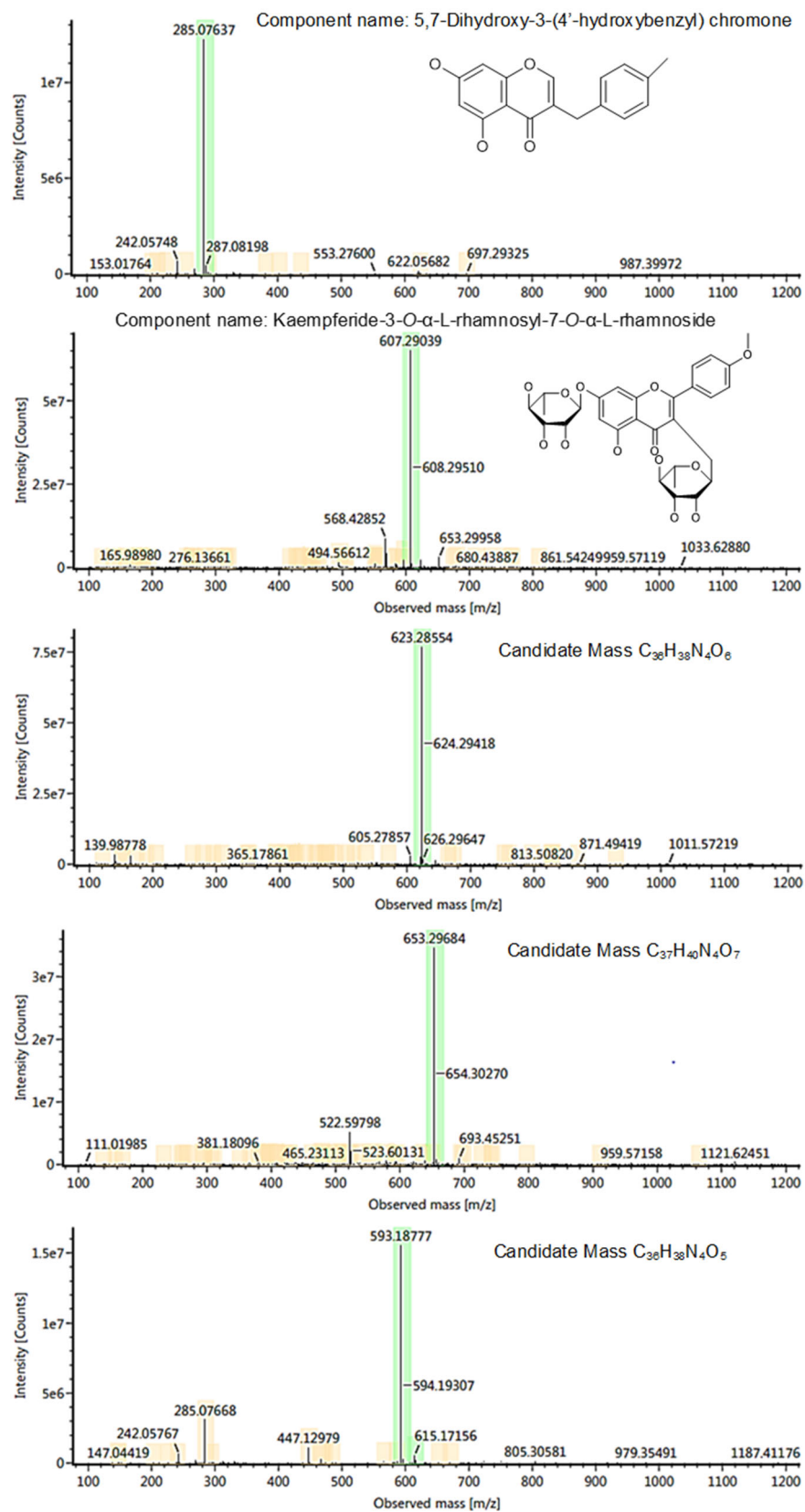
Fig 1. Mass spectrum of *S. coccinea* leaves extract

Table 2. LC-MS/MS profile of *S. coccinea* leaves extract

Component name	Formula	Observed m/z	Observed RT (min)	Mass error (mDA)	Class
5,7-Dihydroxy-3-(4'-hydroxybenzyl)chromone	$C_{16}H_{12}O_5$	285.0764 [M+H]	5.73	0.60	homoisoflavanones
Kaempferide-3-O-α-L-rhamnosyl-7-O-α-L-rhamnoside	$C_{28}H_{32}O_{14}$	593.1878 [M+Na]	4.18	1.30	flavonol triglycosides
Candidate mass $C_{36}H_{38}N_4O_6$	$C_{36}H_{38}N_4O_6$	623.2855 [M+H]	10.74	-0.90	nd
Candidate mass $C_{37}H_{40}N_4O_7$	$C_{37}H_{40}N_4O_7$	653.2968 [M+H]	11.20	-0.10	nd
Candidate mass $C_{36}H_{38}N_4O_5$	$C_{36}H_{38}N_4O_5$	607.2904 [M+H]	11.00	-1.10	nd

Note: nd = not determined

Brine Shrimp Lethality Test (BSLT) method [29]. The cytotoxic potential was further examined using the MTT assay method with ELISA at a wavelength of 570 nm. Cytotoxic potential in cancer cells was calculated based on the percentage of living cells through the absorbance data of the cells. Subsequently, a correlation curve of the log concentration versus the percentage value of live cells was made, and the IC_{50} value was calculated. The examination was carried out on MCF-7 and HeLa cancer cells, and the magnitude of cytotoxic potential was described by the lower IC_{50} values. In this study, the IC_{50} value was used as a cytotoxicity parameter, indicating the cytotoxicity of a compound to cells. The natural material was considered very toxic when the IC_{50} value was 10–100 µg/mL, moderate 100–1,000 µg/mL, and non-toxic when the IC_{50} value was > 1,000 µg/mL [34].

According to the results in Fig. 2, the leaves extract had IC_{50} < 1,000 µg/mL in MCF-7 and HeLa cancer cells, which was classified as moderate cytotoxicity.

Based on the results, the IC_{50} criteria for *S. coccinea* leaves extract exhibited moderate cytotoxicity and had chemopreventive potential. This showed that *S. coccinea* leaf extract could not be used as an anticancer but to prevent the growth or further development of cancer cells. The moderate cytotoxic of *S. coccinea* leaves extract was found to inhibit the growth of cancer cells and increase the percentage of cell death at graded concentrations. This indicated the positive effect of *S. coccinea* leaves on inhibiting cancer cell growth.

The results of the identification of LCMS/MS indicated the presence of a class of flavonoids, which mostly contributed to the chemopreventive activity.
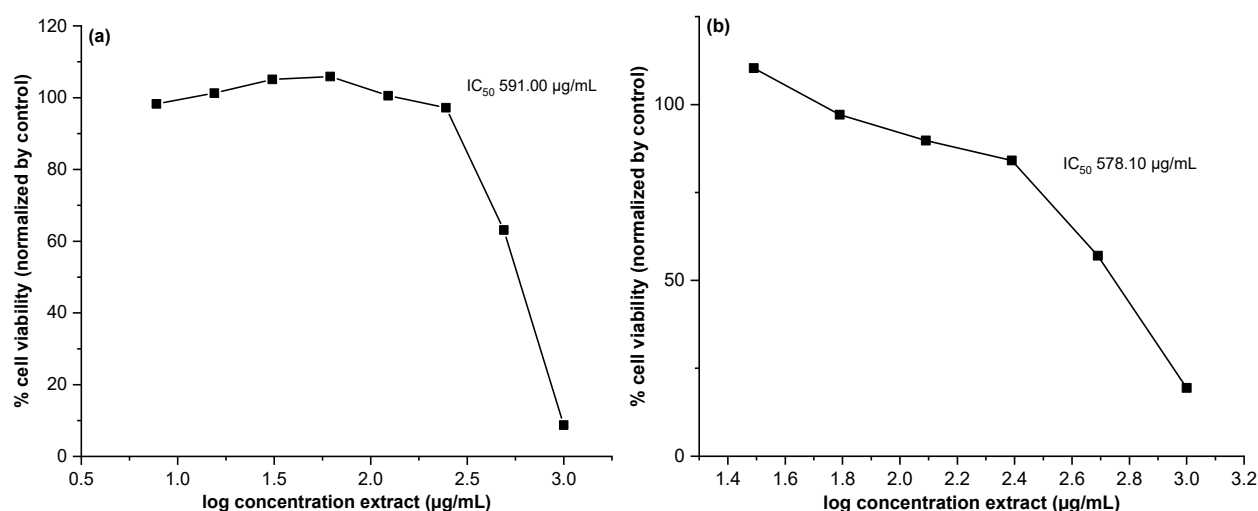

Fig 2. Cytotoxicity against HeLa and MCF-7 cell proliferation (a) HeLa treated with *S. coccinea* leaves extract showing an IC_{50} value of 591.00 µg/mL (b). MCF-7 treated with *S. coccinea* leaves extract an IC_{50} value of 578.10 µg/mL

Flavonoids, especially 5,7-dihydroxy-3-(4'-hydroxybenzyl) chromone and kaempferide-3-*O*-α-L-rhamnosyl-7-*O*-α-L-rhamnoside, have the potential as anticancer by in vivo testing and clinical trials up to phase II with various mechanisms of action, namely carcinogen inactivation, antiproliferative, stop the cell cycle, induce apoptosis, promotion of differentiation, inhibiting angiogenesis, antioxidant, and modulating multidrug resistance [35-37]. The results of the phytochemical analysis test showed the presence of other classes of compounds in the leaf extract, such as tannins, alkaloids, saponins, and steroids which contributed to the chemopreventive effect. Tannin can inhibit cancer promotion, progression, proliferation, and angiogenesis, induction of cell cycle arrest and apoptosis, and inhibition of cancer cell migration, invasion, and colony formation [38]. Alkaloids were also found to possess potential chemopreventive properties due to induction cell cycle arrest, increased cell apoptosis, disruption of redox homeostasis, inhibition of angiogenesis, inhibition of PI-3K/Akt signaling pathways and modulation of ER stress, and autophagy [39-40]. Several studies have reported the role of saponins and steroids in cancer and the mechanism of actions, including apoptosis stimulation, mainly on its intrinsic pathway, cell-cycle arrest, antioxidant activity, and cellular invasion inhibition [41-42].

This study suggests that *S. coccinea* leaves extract has the potential as a natural source of chemopreventive. However, further study is needed to understand the underlying mechanisms of chemoprevention action and identify the compounds that are responsible for such activity. The limitation of this study is that LC-MS/MS analysis did not detect all the components of the chemical content contained in *S. coccinea* leaves extract. This shows the need for further investigation to determine all the components of secondary metabolites and isolate bioactive compounds in the leaf extract. The evaluation of the cytotoxicity of this compound against normal cell lines will also be carried out to obtain the selectivity index.

■ CONCLUSION

This study showed that *S. coccinea* leaves extract contained important phytochemicals, such as tannins (72.16%), alkaloids (30.80%), flavonoids (28.66%), steroids (2.85%), and saponins (1.15%). The results of identification with LCMS/MS revealed that there were at least 5 compounds with 2 flavonoid compounds. *S. coccinea* leaves extract also exhibited moderate cytotoxicity on HeLa and MCF-7 cancer cells. However, further studies were recommended to investigate the active compound for potential anticancer activity and confirm the mechanism of action.

■ ACKNOWLEDGMENTS

■ AUTHOR CONTRIBUTIONS

Yuliet: study design, data interpretation, data collection, data analysis, original manuscript writing, revision, manuscript finalization and administration. Khildah Khaerati: data acquisition, data analysis, and data collection. Agustinus Widodo and Joni Tandi: data analysis and interpretation. All the authors have contributed equally.

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