

Phytochemical screening and cytotoxic evaluation of *Bauhinia scandens* leaf extracts using HeLa and T47D cell lines

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Abstract. Lianah L, Khasanah RAN, Pranatami DA, Krisantini K. 2021. Phytochemical screening and cytotoxic evaluation of *Bauhinia scandens* leaf extracts using HeLa and T47D cell lines. *Biodiversitas* 22: 913-919. The aim of the study was to analyze the phytochemical contents and to determine the cytotoxic activities of *Bauhinia scandens* leaf ethanol extracts against HeLa (human cervical cancer) and T47D (breast cancer) cell culture lines. Using a standard protocol, it was found that leaf extract of *B. scandens* contains phytochemicals that are widely known to have medicinal properties, such as phenols (17.57±0.098% w/w), flavonoids (5.91±0.098% w/w), saponins (19.42±0.091% w/w), tannins (1.24±0.035% w/w), alkaloids (1.31±0.001% w/w) and steroids (0.08±0.007% w/w). Results of 24-hr Microculture Tetrazolium Salt (MTT) assay show that leaf extracts of *B. scandens* can suppress cell growth at a half-maximal inhibitory concentration (IC₅₀) of 1.95 µg/mL for HeLa cells and 4.54 µg/mL for T47D cells. This indicates that *B. scandens* is a potentially strong candidate for anti-cancer agents. The cytotoxic activities of the leaf extracts of *B. scandens* can be attributed to the total effects of the phytochemical compounds found. Further studies are recommended in order to isolate and determine individual effects of bioactive compounds in *B. scandens*.

Keywords: *Bauhinia*, cytotoxicity, HeLa cells, phytochemicals, T47D cells

INTRODUCTION

Cervix and breast cancers are two of the topmost leading causes of death in the world (Indonesian Ministry of Health, 2019). Concerted efforts to manage cancers have led to various current treatments including chemotherapy, drugs, and radiotherapy, all of which adversely affect healthy cells to various extents. Another alternative way to manage cancer is through ethnomedicinal approaches. The use of alternative herbal medicine has been widely practiced by people worldwide to treat all kinds of diseases for a long time (Faisal et al. 2019). A diversity of plants around the world is now being studied and explored for their medicinal properties because of their phytochemical content.

Bauhinia is composed of 250 species of trees, shrubs, and climbers from the family *Leguminosae*. Several species of *Bauhinia* are used in traditional medicine (Hazra and Chatterjee 2008). Leaves of *B. monandra*, for example, contain phytochemicals with anti-nociceptive and anti-inflammatory activities (Campos et al. 2016) while leaves of *B. variegata* have anti-bacterial, antitumor, and anticancer properties (Pandey 2017; Mishra et al. 2013, Singh et al. 2019). Leaves of *B. purpurea* also have anti-inflammatory properties (Shreedhara et al. 2009), while the leaves of *B. variegata* were found to be effective for treating snakebites (Roy et al. 2008). Another study reported the bark of *B. racemosa* is known to provide analgesic remedies (Borikar et al. 2009).

Bauhinia scandens is a well-known legume species, which is well dispersed in Indonesia (Java, Madura, Sumatera, Sumba), Bangladesh, Bhutan, Cambodia, China (Hainan), India, Laos, Myanmar, Nepal, Thailand, and Vietnam (Bandyopadhyay and Lakshminarasimhan 2017). It is a woody climbing plant with height ranging from 30 to 50 m and possesses reverse tendrils (Lianah 2016). The young stems are green to brownish, while the old stems are darker with a variety of shapes, such as flattened, wavy, and resembling monkey staircases (Lianah et al. 2019). The young leaves are reddish, while the old leaves are green. The leaf laminae vary in shape from cordate, ovate to acute or emarginate, to deeply bilobed, sometimes almost to the base. The curved leaves have a heart-shaped base, flat edges, and tapered ends that split in half and they generally consist of 5-7 nerves. *Bauhinia scandens* grow well at an altitude of 500-800 m above sea level (Hou and Larsen 1996). Despite its wide distribution in Indonesia, there is limited information regarding the phytochemical properties and medicinal value of *B. scandens*. Previous studies showed that its leaves have non-toxic antitumor properties and antioxidant activities (Maitra et al. 1991; Hazra and Chatterjee 2008; Hossain et al. 2017). Therefore, the aim of the study was to analyze the phytochemical content of *B. scandens* leaves. Additionally, we would like to understand the medicinal properties of this species by investigating the cytotoxic activities of the leaf ethanolic extract against HeLa and T47D cell culture lines.

MATERIALS AND METHODS

Research design and sample preparation

Two kilograms of young and mature leaves of *B. scandens* were collected in May 2018 and July 2019 from trees growing in the cemetery of Tugerejo, Ngaliyan Regency, Semarang, Central Java, Indonesia. The leaves were cleaned using running water and dried for 2 weeks at room temperature. Leaves were crushed and dissolved in 96% ethanol for 2 days to obtain about 1.8 L solution with a pH of 6.3, dark green coloration, and rancid smell. The solution was transferred to a rotary evaporator (60°C, 60 rpm) and evaporated for 7 hours, obtaining a viscous black solution, which was then air-dried.

Qualitative phytochemical analysis

Bauhinia scandens extract (hereafter referred to as sample extract) was analyzed qualitatively by standard procedures of metabolic identification as described by Sofowora (1993), Trease and Evans (1989), and Harborne (1973).

Detection of flavonoids

Three ml of 1% aluminum chloride solution was added into 1 ml of sample extract, followed by 5 mL of dilute ammonia and concentrated H₂SO₄. Yellow coloration confirmed the existence of flavonoids.

Detection of saponins

One ml of sample extract was boiled in 20 ml of distilled water and filtered, then 10mL of the filtrate was mixed with 5 mL of distilled water, and shaken vigorously. The formation of a stable froth at least for 10 min indicated the existence of saponins.

Detection of tannins

One ml of sample extract was added to 20 ml distilled water, and then filtered. A little drop of 0.1% ferric chloride (FeCl₃) was added. The formation of blackish-blue color confirmed the existence of tannins.

Detection of alkaloids

One ml of sample extract was stirred with 5 drops of 1% aqueous HCl on a steam bath, and filtered when it was in hot condition. Afterward, 1 ml of the filtrate was tested with a few drops of Hager's reagent (picric acid). The formation of yellow precipitate confirmed the existence of alkaloids.

Detection of terpenoids

One mL of sample extract was mixed with 2 mL of chloroform. Subsequently, 3 mL of concentrated H₂SO₄ was carefully added to form a layer. The formation of a reddish-brown coloration confirmed the existence of terpenoids.

Detection of steroids (Salkowski test)

The acetic anhydride (2 mL) was added to 1 mL of sample extract, followed by the addition of 2 mL of H₂SO₄.

The yellow fluorescent coloration confirmed the existence of steroids.

Detection of glycosides (Keller-Kiliani test)

One mL of sample extract was mixed with 2 mL of acetic acid containing 1-2 drops of 2% FeCl₃, followed by the addition of 2 mL concentrated H₂SO₄. The formation of a reddish-brown color confirmed the existence of glycosides.

Quantitative phytochemical analysis

Determination of flavonoid content

One hundred mg of sample extract was mixed with 0.3 mL of sodium nitrite, and 0.6 mL of aluminum chloride. Two mL of 1 M sodium hydroxide was added to the mixed solution obtained. The volume was made up to 10 mL using distilled water. The resulting solution was diluted (10 times) before the absorbance was read by UV-Vis spectrophotometer (Shimadzu UV-1800) at a wavelength of 510 nm (Baba and Malik 2014). A standard curve was obtained from diluted concentrations of quercetin under the same conditions.

Determination of saponin content

Fifty mg of sample extract was added to 2 mL concentrated H₂SO₄, and autoclaved (110°C) for 120 minutes. Sample extract was re-extracted with ether, filtered, and dried. One mL water was added to the extraction, vortexed for 5 min before 50 µl anisic aldehyde was added to the solution, then shaken for 10 min, filtered, 2 mL 50% H₂SO₄ was added before heating (water bath; 60°C; 10 min), and diluted 10 times with water. The absorbance was read with the spectrophotometer at 435 nm wavelength (Vador et al. 2012). A standard curve was obtained from some diluted concentrations of saponins isolated from *Quillaja* bark under the same conditions.

Determination of tannin content

Fifty mg of sample extract was diluted in 10 mL methanol for 20 hr, then filtered. Distilled water was added to make the volume 10 mL before 0.1 mL of Folin-Ciocalteu reagent was added. The mixture was vortexed for 5 min; 2 mL of 20% sodium carbonate solution was added and vortexed for 5 minutes. Ten mL of distilled water was added to each sample to make the total volume of 10 mL, then the solution was kept for 30 min at room temperature. Absorbance was measured using spectrophotometer at a wavelength of 760 nm (Galvao et al. 2018). A standard curve was obtained from some diluted tannic acid concentrations under the same conditions.

Determination of alkaloid content

One hundred mg of sample extract was diluted in 2N HCl, shaken vigorously, and washed (3 times) with 10 mL of chloroform in a funnel separator. The chloroform phase was discarded, and the solution was neutralized by adding 0.1N NaOH before adding BCG (bromocresol green) solution, and 5 mL of phosphate buffer. The mixture was further treated with 5 mL of chloroform to bring the volume to 10 mL, and stirred with a magnetic stirrer (500

rpm, 15 min). The extraction was conducted twice and the chloroform was evaporated with nitrogen gas. The absorbance was measured at a wavelength of 470 nm using the spectrophotometer (Ajanal et al. 2012). A standard calibration curve was obtained from various diluted concentrations of quinine.

Determination of phenol content

Fifty mg of sample extract was weighed and added to 0.5 mL of Folin-Ciocalteu reagent and 7.5 mL of distilled water. The mixture was kept at room temperature for 10 min. Later, 1.5 mL of 20% Na₂CO₃ solution was added and ultimately the volume was made up to 10 mL with distilled water. This was followed by vigorous shaking and then solution was allowed to stand for 2 hours. The resulting solution was diluted (25 times) then the absorbance was measured at a wavelength of 760 nm using the spectrophotometer (Vador et al. 2012). A standard calibration curve was obtained from various diluted concentrations of gallic acid.

Determination of steroid content

In order to Determine steroid as β -sitosterol equivalents, thin layer chromatography (TLC) method (Bhawani et al 2010) was used. One hundred mg of sample extract was dissolved in 1 mL ethanol, after which the mixture was homogenized. The 5 μ L sample was spotted on silica gel 60 F254, including the standard beta sitosterol. Silica gel 60 F254 was put in the chamber containing the mobile phase of toluene: ethyl acetate (80:20). The mobile phase was eluted and traveled upwards with the sample component. The TLC plate was lifted and dried then sprayed with Lieberman Bucard reagent, then dried in the oven at 110°C for 2 min. The silica gel plate was visualized under UV light at a wavelength of 340 nm.

Cytotoxicity examination

Investigation of the cytotoxicity of the ethanolic extract of *B. scandens* leaves was conducted at LPPT University of Gadjah Mada, Central Java, from May to August 2019. The sample extract was tested for *in vitro* cytotoxicity against HeLa (human cervical cancer) and T47D (breast cancer) cell culture lines using Microculture Tetrazolium Salt (MTT) assay. The cell viability can be detected by measuring the activity of the mitochondrial dehydrogenase enzymes, whereby the MTT salt was reduced to water-soluble purple formazan (Mas et al. 2010). Aliquots of various concentrations (800 μ g/mL, 400 μ g/mL, 200 μ g/mL, 100 μ g/mL, 50 μ g/mL, 25 μ g/mL, 10 μ g/mL, 5 μ g/mL, 2.5 μ g/mL and 1.25 μ g/mL) were prepared from the concentrated sample extract by dissolving in 0.25% DMSO. The HeLa and T47D cell culture lines were washed and treated with 0.01 mL MMT per well. Plates were incubated in an incubator set at 37°C with 5% CO₂ for 4 hours and 100 μ L of the extraction buffer (10% sodium dodecyl sulfate in 0.01% HCl) was added. After overnight incubation, the absorbance was measured at wavelength of 550 nm using the microplate ELISA reader (Bio-Rad). The cytotoxicity against the HeLa and T47D cells of the

different extracts was shown in a log dose series, and IC₅₀ or 50% cell death was calculated. Cytotoxicity is calculated using the following formula:

$$\% \text{ Cytotoxicity} = [\text{Control cells OD} - \text{Sample OD}] / \text{OD control cells} \times 100\%$$

Data analysis

The quantitative and qualitative phytochemical results were presented in a descriptive method. The quantitative data were analyzed by SPSS version 19. Cytotoxicity examination results were analyzed and graphed using Microsoft Excel.

RESULTS AND DISCUSSION

Phytochemical properties of *Bauhinia scandens* leaf extract

The screening of phytochemicals of *B. scandens* showed several phytochemicals, including flavonoids, saponins, tannins, alkaloids, terpenoids, and steroids, and glycosides (Table 1). The leaves of *B. scandens* had the highest concentration for total phenols (17.57 \pm 0.098% w/w) and saponins (19.42 \pm 0.091% w/w) among these phytochemicals (Table 1).

Cytotoxic effect of *Bauhinia scandens* extract in vitro

In determining the cytotoxic effects of *B. scandens in vitro*, we started by using the highest leaf extract concentration range of 3000 μ g/mL to 5.85 μ g/mL. We obtained the IC₅₀ values of 4.60 μ g/mL for HeLa cells (Figure 2.A) and 5.87 μ g/mL for T47D cells (Figure 2.Cc). When the leaf extract concentration was lowered to a range of 2000 μ g/mL to 3.906 μ g/mL, we obtained the IC₅₀ values of 5.18 μ g/mL for HeLa cells (Figure 2.B) and 5.86 μ g/mL for T47D cells (Figure 2.D). As leaf extract concentration was further lowered, from 800 μ g/mL to 1.56 μ g/mL it had an IC₅₀ at 1.95 μ g/mL against the HeLa cells (Figure 3.A) and 4.54 μ g/mL against the T47D cells (Figure 3.B). It means that the higher concentration of the extract may lead to a lower percentage of live cells.

Table 1. Qualitative (presence or absence of active compounds) and quantitative (amount of compounds) test results of the phytochemicals in *Bauhinia scandens* leaves.

Compound	Results	Content (% w/w)
Flavonoids	(+)	5.91 \pm 0.098
Saponins	(+)	19.42 \pm 0.091
Tannins	(+)	1.24 \pm 0.035
Alkaloids	(+)	1.31 \pm 0.001
Steroids	(+)	0.08 \pm 0.007
Total Phenols	(+)	17.57 \pm 0.098
Terpenoids	(+)	Not quantified
Glycosides	(+)	Not quantified

Note: (+): present

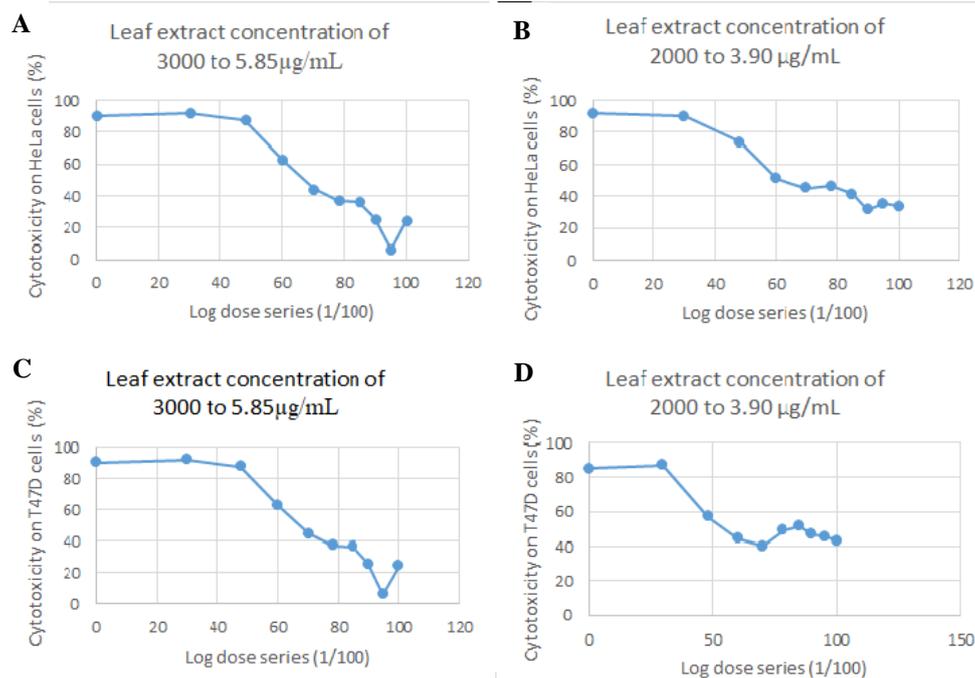


Figure 2. Cytotoxicity of *Bauhinia scandens* leaf extracts: A-B. Against HeLa Cells at concentration of (A) 3000µg/mL to 5.85 µg/mL and (B) 2000µg/mL to 3.90 µg/mL; C-D. Against T47D cells at concentration of (C) 3000µg/mL to 5.85 µg/mL and (D) 2000µg/mL to 3.906 µg/mL.

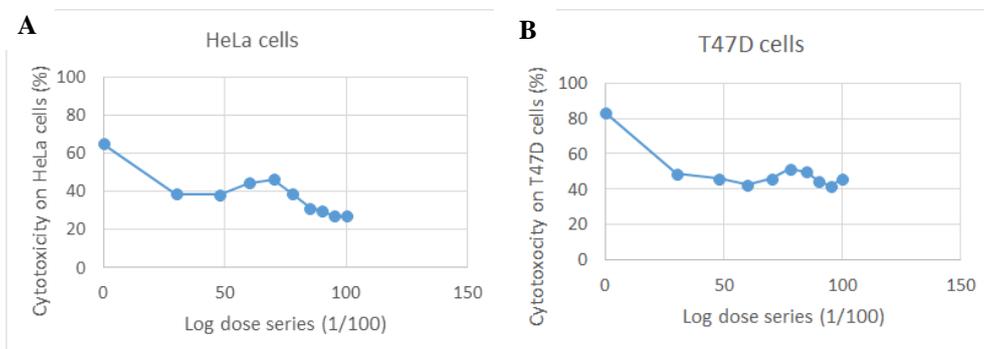


Figure 3. Cytotoxicity of *Bauhinia scandens* leaf extracts against: A. HeLa cells, B. T47D cells

Discussion

Phytochemicals are important for human health and have potential to treat various diseases (Pandiagan et al. 2019). Our study confirmed that the leaves of *B. scandens* contain significant amount of phytochemicals, such as flavonoids, saponins, alkaloids, steroids, and phenols. The leaves of *B. scandens* have more flavonoids ($5.91 \pm 0.098\%$ w/w) (Table 1), than those reported for the leaves of *B. racemosa* ($0.04 \pm 0.09\%$ w/w) and *B. purpurea* ($0.09 \pm 0.11\%$ w/w) (Sharanabasappa et al. 2007). Flavonoids are involved in anticancer activities, such as inhibition of cell growths, protein kinase activities, angiogenesis, and induction of apoptosis by activating p53 protein and their target genes (Plaumann et al. 1996). Several studies reported that single compound of flavonoids has been

proven to possess strong cytotoxicity such as diosmetin and diosmetin 7-O-β-D-glucopyranoside isolated from the flowers of *Chrysanthemum morifolium* Ramat. 'huaiju' cv. nov. (family Asteraceae) against Colon205, with their IC50 values being 96.9 and 82.9µM, respectively (Xie et al. 2009); apigenin, chrysin, and luteolin compounds against cervical cancer (Sak 2014); and 3,3',4'-trihydroxy-(3'',4''-dihydro-3'',4''-dihydroxy)-2'',2''-dimethylpyrano-(5'',6'':7,8)-flavone isolated from *Centaurea scoparia* (family Asteraceae) agent against HeLa cells, with an IC50 of 0.079 µM (Ahmed and Kamel 2014). Because of its high flavonoid content, *B. scandens* leaves could potentially be developed as an anticancer agent. This study, however, did not include isolation of the individual active compound of the flavonoids.

The leaves of *B. scandens* contain more saponins (19.42±0.091 % w/w) (Table 1) than those reported in *Citrus* leaves, such as *C. paradisi* (0.89±0.01% w/w), *C. grandis* (0.87±0.06% w/w), *C. reticulata* (0.81±0.01% w/w), *C. aurantifolia* (0.64±0.01% w/w), *C. sinensis* (0.43±0.01% w/w) and *C. limon* (0.34±0.02% w/w) (Ezeabara et al. 2014). Several studies reported that saponins have been proven to possess strong cytotoxicities. Saponins can activate the intrinsic or extrinsic apoptotic pathways, hold the cell cycle, autophagic dynamics, and inhibit angiogenesis and metastasis (Plaumann et al. 1996; Sobolewska et al. 2020). Podolak et al. (2010) reported that the cytotoxic effects of saponins may be due to either apoptosis inducement or nonapoptotic cell death stimulation. Cytotoxic effects of saponins were also studied by Alam et al. (2017) who reported that saponins from fruit, bark, and leaves of traditional medicinal plant *Zanthoxylum armatum* (family Rutaceae) have potential cytotoxic effect on breast and colorectal cancer cell lines through a mechanism involving apoptosis.

Tannin concentration in *B. scandens* leaves (1.24 ± 0.035 % w/w) (Table 1) is lower than those reported in Turkish wine, beer and black tea (2.76-6.54% w/w, 8.03-6.59% w/w and 6.20-8.33% w/w, respectively) (Tinkilic and Uyanik 2001). Tannins are known to induce death of cancer cells in various types of cancer. Several studies reported that single compound of tannins has been proven to possess strong cytotoxicities. Hong et al. (2011) reported that the extracted hydrolyzable tannin from the bark of *Rhizophora apiculata* (family Rhizophoraceae) has cytotoxicity effects on HepG2 cancer cells at IC50 of 12.26 µg/mLs. Tannic acid is involved in the induction of apoptosis in human glioma Hs 683 cells by increasing reactive oxygen species (ROS) molecules, which are responsible for inducing apoptosis in cancer cells by generating transitional pore opening in mitochondria (Zhang et al. 2018). Tannins have also been applied as anti-diarrhea, hemostatic, anti-bacterial, antioxidant, anti-inflammatory, anti-hemorrhoidal and antiseptic compounds (Ashok and Upadhyaya 2012; Khanbabaee and Van Reeb 2001). In this study, however, we did not isolate tannin from *B. scandens* leaves and we did not evaluate cytotoxic activity from the isolated tannins.

The concentration of alkaloids from *B. scandens* (1.31± 0.001 % w/w) (Table 1) is found to be higher than the alkaloids in *Actinidia arguta* (family Actinidiaceae) leaves (0.95 mg/g or 0.095% w/w) (Liu and Liu 2015). Several studies also reported that single compound of alkaloids has been proven to possess strong cytotoxicities, such as aporphine isolated from *Alphonsea sclerocarpa* (family Annonaceae) showed antioxidant and anti-cancer activities upon testing with K562 (CML-chronic myeloid Leukaemia blood cancer) cells (Joshi DSD et al. 2018). The total and individual alkaloids from *Broussonetia papyrifera* (family Moraceae) fruits have higher activities on BEL-7402 and HeLa cell lines with low IC50 values (6.61-47.41 and 5.97-40.17 µg/mL, respectively) (Pang et al. 2014). The total alkaloids (400 µg/mL) of *Eucalyptus camaldulensis* (family Myrtaceae) could reduce the cell viability of both breast cancer cell line Michigan Cancer Foundation-7 (MCF-7)

and nontumorigenic fetal hepatic cell line (WRL-68) to 45.25±2.20% and 92.00±1.55%, respectively, and the IC50 was 375.50 µg/mL for MCF-7 (Al-Marzook and Omran 2017). The total alkaloids isolated from different parts of *Solanum pseudocapsicum* (family Solanaceae) mainly at its leaves contain the most potent against human adenocarcinoma (HT-29) cells compared to the other plant organs (Vijayan et al. 2004). Therefore, we think that the alkaloids of phytochemicals in *B. scandens* leaves also contribute to cytotoxic activity against HeLa and T47D cells.

The leaves of *B. scandens* contain steroids at a concentration of 0.08± 0.007 % w/w (Table 1). These compounds seem to play an important role in treating cancer. Several studies also reported that isolated steroids has been proven to possess strong cytotoxicities. Guan et al. (2016) found that the two steroids such as 'compound 4' showed potential antitumor activities against HeLa, KB cell lines (IC50 = 3.65–6.90 µM) and 'compound 6' showed potential antitumor activities against HeLa, BGC-823, KB, A549, and HCT-8 cell lines (IC50 = 2.40–9.05 µM). Katja et al. (2011) reported that three cytotoxic steroids such as stigmasterol (1), stigmast-5-en-3β-ol (2) and β-sitosterol-3-O-acetate (3) isolated from the stem bark of *Chisocheton cumingianus* showed cytotoxicity activity against P-388 murine leukemia cells with IC50 values of 12.4, 60.8, and > 100 µg/mL, respectively. In this study, we also did not isolate steroids from *B. scandens* leaves and we did not evaluate cytotoxic activity from the isolated steroids.

The total phenol that was detected and quantified in *B. scandens* leaves (17.57±0.098 % w/w) (Table 1) is higher than those found in the leaves of *B. racemosa* (0.55±0.36% w/w) and *B. purpurea* (0.48±0.57% w/w) (Sharanabasappa et al. 2007). The leaves of *B. scandens* was also found to have terpenoids and glycosides, but their concentrations were not quantified (Table 1). Macwan et al. (2016) reported that glycosides extracted from leaves of *Corchorus trilocularis* and seeds of *Corchorus aestuans* (family Malvaceae) have cytotoxic activity against K-562 with IC50 of 67.44 µg/mL and 37.09 µg/mL, respectively. Terpenoids were also shown in a previous study to have anticancer activity (Prakash 2018). The presence of terpenoids and glycosides in *B. scandens* leaves makes it a good anticancer source candidate.

We have determined that phytochemicals detected in *B. scandens* leaves have cytotoxic activity although we did not evaluate every single compound. The extract of *B. scandens* leaves showed cytotoxic activity with a low IC50 value of 1.95 µg/mL against HeLa cells and 4.54 µg/mL against T47D cells (Fig 3a. and 3b). Compounds in leaf extracts could have synergistic and antagonistic activities (Yin et al. 2014). Compared to having a single component, the presence of several interacting compounds could have a cumulative effect. In this study, the phytochemicals in *B. scandens* leaves could have synergistic interactions, making it a potentially good candidate for anticancer agent screening. However, it must be emphasized that we have not isolated the individual active compounds in this study and as expected, active components are generally more toxic, as the extracts may contain a variety of secondary

compounds that can reduce or modulate the activities (Wink 2015). Further research should be pursued to isolate bioactive compounds in *B. scandens* leaves, determine the activities of these compounds, and investigate further the anticancer potential of this species.

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