Evaluation of *Tinospora cordifolia* stem extract bioactive content and anticancer activity against breast cancer cells

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Abstract. Breast cancer remains a major public health challenge, highlighting the importance of finding new therapeutic approaches, including the use of traditional plants with unstudied medicinal properties. This study aims to explore the bioactive potential of *Tinospora cordifolia* stem extract by investigating the content of flavonoids, phenols, terpenoids and alkaloids as well as the cytotoxic and apoptotic effects on T47D breast cancer cells. Analysis of bioactive compound content revealed a total flavonoid content of 29.7 ± 0.30 mg QE/g extract, a total phenol content of 120.4 ± 4.25 mg GAE/g extract, a total terpenoid content of 2.41 ± 2.18 mg LE/g extract and a total alkaloid content of 2.55 ± 0.27 mg LE/g extract. Cytotoxicity tests on T47D cells using the WST-1 assay showed a dose-dependent decrease in cell viability with an IC_{50} of 571.3 ± 33.41 µg/ml. Furthermore, at a concentration of $2IC_{50}$, *T. cordifolia* stem extract can induce apoptosis by 36.7 ± 4.19%. These results suggest that *T. cordifolia* has considerable anticancer activity, although further studies are needed to understand its mechanism of action and evaluate its potential in breast cancer therapy.

1 Introduction

Breast cancer is the most common cancer among women, with a prevalence of 16% of all cancers in women [1]. In 2020, 684,996 deaths from breast cancer were reported. There will be an estimated 2.2 million new cases of cancer, of which 1 in 10 will be breast cancer [2]. Breast cancer is often potentially curable if treated in early stages, but metastases are almost always fatal due to the development of therapeutic resistance during the treatment process. [3]. In most cancers, single-target drug development does not provide optimal therapeutic outcomes or tends to develop resistance even in the initial response to treatment [4].

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Currently, the utilisation of herbs is one of the promising alternatives for the development of cancer therapy and shows real advantages in dealing with the complexity of cancer treatment due to its multi-target mechanism [4–6]. One of the herbs that has been utilised empirically for the prevention and treatment of various diseases is *Tinospora cordifolia* [7]. *T. cordifolia*, belonging to the Menispermaceae family, is an important medicinal plant in Ayurvedic medicine and is included in the Indian System of Medicine (ISM) due to its bioactive constituents and various therapeutic properties. In Hindu mythology, the plant is called '*Guduchi*', which means a celestial herb that saved a celestial being from old age and kept him young [8–10]. In Indonesia, this plant is known by the local name *'Brotowali'*, which has traditionally been used by the community as *Jamu Pahitan* [11]. *T. cordifolia* has various potentials as antioxidant, antiallergic, anti-inflammatory, antimicrobial, antiviral, antidote, antitumor, antileprotic, antispasmodic, and antidiabetic, and has been used extensively in traditional medicine, especially the stem [12,13].

Bioactive compound content in *T. cordifolia* is dominated by glycosides, terpenoid steroids, phenolics, polysaccharides, aliphatic compounds, and alkaloids [14–16]. These compounds are known to have anticancer activity through various apoptotic pathway mechanisms, including reactive oxygen species (ROS) enhancement, cell cycle inhibition, caspase pathway activation, and cell proliferation inhibition [10,16,17]. *T. cordifolia* shows great potential to be developed as an anti-breast cancer herbal candidate. The objectives of this study were to evaluate the content of bioactive compounds in the ethanol extract of *T. cordifolia* stem and test its effectiveness on Breast cancer cell line T47D in vitro.

2 Materials and methods

2.1 Extraction of *T. cordifolia* stems

T. cordifolia stem simplisia was obtained from UPT Materia Medica Herbal Laboratory, Batu city, East Java, Indonesia, with batch number 220215.BRT.B.MMB.001. Simplisia and 96% ethanol in a proportion of 1:10 (w:v) were placed in a microwave-assisted extraction (MAE) vessel (Anton Paar, Austria). The following settings were used for the MAE: holding temperature, 50°C; 5 min heat-up, 50°C; holding time, 15 min; 5 min cool-down; power, 1500 W. The filtrate was filtered with filter paper, and the solvent was evaporated with a Rotary Evaporator Hei-VAP Expert (Heidolph, Korea) at 50 rpm and 50°C. The storage temperature of the extract was 4°C [18].

2.2 Total flavonoid determination

The aluminium chloride ($AlCl_3$) colourimetric method was used to measure the total flavonoid content of *T. cordifolia* stem extract according to the method of Nurcholis et al. with modification [19]. A total of 50 µL of extract (1 mg/mL) and quercetin standard solution (1.5625 - 400 µg/mL) was mixed with 150 µL of 96% ethanol and 10 µL of 10% $AlCl_3$ in a 96-well plate. The mixture was then incubated for 40 minutes in the dark at room temperature with the addition of 10 µL of 1M sodium acetate (CH_3COONa). Absorbance was measured at a wavelength of 405 nm using a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific, USA). The total amount of flavonoids in the extracts was expressed as milligrams of quercetin equivalent per gram of extract (mg QE/g) [19].

2.3 Total phenolic determination

The Folin-Ciocalteu method was used to determine the total phenolic content with a modified protocol adapted from Molole et al. [20]. In a 96-well plate, 10 µL of extract (1 mg/mL) and gallic acid standard solution (1.5625 - 400 µg/mL) were mixed with 100 µL of Folin-Ciocalteu reagent in dH_2O (1:10, v/v). Then, 1 µL of 7.5% sodium carbonate solution (Na_2CO_3) was added. The mixture was incubated for 90 minutes in the dark at room temperature. Sample absorbance was measured at a wavelength of 725 nm using a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific, USA). The total phenolic content of *T. cordifolia* stem extract is expressed as milligrams of gallic acid equivalents per gram extract (mg GE/g) [20].

2.4 Total terpenoid determination

The total terpenoid content of the extracts was determined according to the modified method of Łukowski et al. (2022). Total terpenoid content was expressed as mg linalool equivalent per gram of extract (mg LE/g). Standard solutions (0 - 63.66 µg/mL) and *T. cordifolia* stem extract (3 mg/mL) of 1 mL were put in a test tube, then 3 mL of chloroform was added and homogenised. Then 500 µL of concentrated sulphuric acid (H_2SO_4) was slowly added to the wall of the test tube and the mixture was incubated for 4 hours in the dark at room temperature. A reddish-brown precipitate formed at the bottom of the tube after incubation. The precipitate formed was retained, and added with 500 µL of 96% methanol and mixed until homogeneous. A total of 150 µL of the sample mixture was transferred to a 96-well plate. The absorbance was measured at 538 nm using a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific, USA) [21].

2.5 Total alkaloid determination

The total alkaloid content of *T. cordifolia* stem extract was determined using a modification of Tan's method (2018). A total of 69.8 g of bromocresol green (BCG) was dissolved in 3 mL of 2N NaOH and 5 mL of distilled water. The solution was then heated to complete dissolution. The extract (2 mg/mL) was dissolved in 2N HCl, filtered, and pH adjusted to neutral using 0.1N NaOH. 1 mL of extract solution and atropine standard solution (0-16,670 µg/mL) were added to 5 mL of BCG solution and 5 mL of phosphate buffer. The mixture was homogenised. An equal volume of chloroform was added to the mixture, followed by vigorous shaking until a yellow layer was formed at the bottom of the solution. A total of 150 µL of the yellow layer was transferred to a 96-well plate. The absorbance was measured at 470 nm using a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific, USA). Total alkaloids were expressed as milligrams of atropine equivalent per gram extract (mg AE/g) [22].

2.6 Cell culture preparation and cell viability assay

Breast cancer cell line T47D was obtained from the Laboratory of Animal Physiology, Structure, and Development, Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya. T47D cells were cultivated in RPMI 1640 medium (Gibco, USA) with an additional 10% fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA). The cells were grown in 96-well plates at a density of 7,500 cells per well and incubated for 24 hours at 37°C in a 5% CO_2. Cells were then treated with various graded concentrations of *T. cordifolia* stem extract (0, 160, 320, 640, and 1280 µg/mL) for 24 hours. The treated medium was replaced with fresh medium containing 5%

WST-1 reagent (Sigma-Aldrich, USA), and incubated for 30 minutes. This was followed by a measurement of the absorbance at 450 nm using a Multiskan SkyHigh microplate spectrophotometer (Thermo Fisher Scientific, USA) [23]. Percentage cell viability for T47D was calculated, and IC_{50} was determined using a linear regression equation between percentage cell viability and extract concentration.

2.7 Apoptosis assay

T47D cells were grown at a density of 50,000 cells in 500 µL of culture medium per well in a 24-well plate. The cells were incubated for 24 hours. Cells were exposed to *T. cordifolia* stem extract at concentrations of ½IC_{50}, IC_{50}, and 2IC_{50} for 24 hours at 37°C with 5% CO_2. Treated T47D cells were harvested by the trypsinisation method and stained with FITC-Annexin V/propidium iodide (PI) (BioLegend, USA) according to the method of Worsley et al. with modifications. The cells were incubated for 20 minutes at 4°C in the dark. Stained cell suspensions were analysed using FACS-Calibur flow cytometry (BD FACS Calibur, USA) and data were analysed using Cell Quest software (BF Bioscience, USA) [24].

2.8 Statistical analysis

One-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) for post hoc comparisons was used for statistical analysis of apoptosis assay results. Statistical significance was set at a probability level of $p < 0.05$. All data are presented as mean ± standard deviation (SD), with each value representing the average of three replicates.

3 Results and discussions

3.1 Content of flavonoids, phenolics, terpenoids, and alkaloids in the *T. Cordifolia* stem extract

T. cordifolia possesses a wide range of important bioactive chemical constituents such as steroids, alkaloids, glycosides, tannins, sesquiterpenoids, flavonoids, phenols, polysaccharides, essential oils, aliphatic compounds and fatty acid combinations that have been previously investigated [10,25]. *T. cordifolia* extraction process and solvent selection are important steps in the concentration and targeting of plant bioactive compounds [26]. The existence of bioactive compounds in the stem extract of *T. cordifolia* was determined by measuring the total content of flavonoids, phenols, terpenoids and alkaloids. The most abundant among these compounds was found to be phenolic, with a concentration of 120.4 ± 4.25 mg GAE/g (Table 1). This indicates that phenolics are the dominant class of bioactive compounds in the extract.

Table 1. Total flavonoids, phenolics, terpenoids, and alkaloids content in *T. cordifolia* stem extract.

Total Bioactive Content	*T. cordifolia* stem extract
Total flavonoid content (mgQE/g extract)	29.7 ± 0.30
Total phenolic content (mgGAE/g extract)	120.4 ± 4.25
Total terpenoid content (mgLE/g extract)	2.41 ± 2.18
Total alkaloid content (mgAE/g extract)	2.55 ± 0.27

Note: Data are presented as mean ± SD.

Phenolic compounds in *T. cordifolia* play an important role in its bioactivity in free radical scavenging due to the availability of hydroxyl groups and can act as reducing agents, metal chelators, and hydrogen donors [27,28]. Previous studies have shown that the ethanolic extract of *T. cordifolia* stem contains a large amount of phenolics, which play a role in antioxidant activity, as well as significant proliferation inhibition on the HeLa cervical cancer cell line [27]. This study evaluated the anticancer potential of the bioactive compounds of *T. cordifolia* stem extract on the breast cancer cell line T47D based on cytotoxicity and apoptosis induction.

3.2 Cytotoxicity of *T. cordifolia* stem extract on T47D breast cancer cells

The toxicity of medicinal plants is closely linked to the presence of bioactive compounds in the plant material and the toxic potential of these compounds [29]. The cytotoxicity test of *T. cordifolia* stem extract showed toxicity to T47D cells that increased with increasing extract concentration with an IC_{50} value of 571.3 ± 33.41 µg/mL (Figure 1). *T. cordifolia* has shown anticancer activity in several types of hepatocellular carcinoma, lymphoma and glioblastoma [30–32].

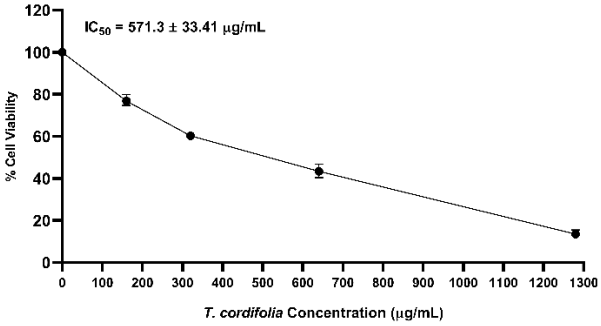

Fig. 1. Cytotoxicity of the stem extract of *T. cordifolia* on T47D cells and its IC_{50} value. Data are expressed as mean ± SD.

The results of another study showed that the hexane fraction of *T. cordifolia* has potent anticancer activity on breast cancer cells MCF-7 and MDA-MB-231 with IC50 values of 37.2 ± 2.77 and 47.5 ± 2.53 µg/mL, respectively [33]. While the results of a related study using 50% ethanol solvent showed anticancer activity on U87MG, HeLa and C6 cell lines with higher IC50 values at a concentration of 200 µg/mL [32]. Differences in the bioactivity of *T. cordifolia* are due to differences in extraction methods and types of solvents used, which allow differences in the content of bioactive compounds contained in the extracts.

3.3 *T. cordifolia* stem extract induced apoptosis of T47D cells

The induction of apoptosis in T47D cells was evaluated using propidium iodide (PI) and annexin V to detect the relative number of live, apoptotic and necrotic cells. Annexin V can bind to phosphatidylserine, which translocates to the outside of the plasma membrane during apoptosis, whereas PI binds to DNA in necrotic cells [33, 34]. *T. cordifolia* stem extract was potent in inducing apoptosis in T47D cells, which increased significantly with increasing extract concentration (Figure 2B). The highest increase in apoptosis was 36.7 ± 4.12% at an extract concentration of 1142.6 µg/mL (Figure 2A-2B).

T. cordifolia has been reported to contain a variety of phytochemical components capable of inducing anticancer effects through mitochondrial-mediated apoptosis, triggering reactive

oxygen species, mutagenic activity, and decreased expression of genes that regulate the cell cycle [10,14,17,33]. The mechanism of anticancer action of *T. cordifolia* depends on its phytochemical components. Ethanolic extract of *T. cordifolia* has been reported to be capable of inducing apoptosis by enhancing the sub-G0 phase without altering the cell cycle [14].

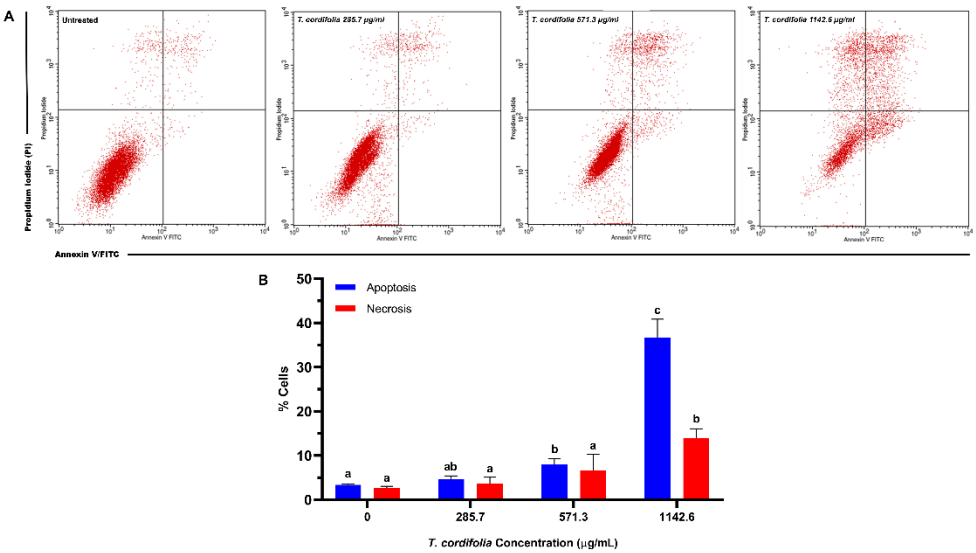

Fig. 2. Apoptosis induction in T47D cells by *T. cordifolia* stem extract. (A-B) Annexin V/FITC-PI assay showing the induction of apoptosis in T47D cells. Different letters on the graph indicate statistically significant differences between groups ($p < 0.05$). Data are expressed as mean ± SD.

The alkaloid content of *T. cordifolia* extract plays a role in the anticancer activity in addition to the phenolic compounds. Alkaloid compounds in the extract have been widely reported to have potent anticancer activity against various cancers such as prostate cancer, liver cancer and leukaemia due to their antioxidant activity. They reduce cancer-causing ROS, induce apoptosis by increasing the expression of caspase-8, caspase-9 and caspase-3, and arrest the cancer cell cycle in the G1 phase [12,32,36].

4 Conclusion

T. cordifolia stem extract contains bioactive compounds with the highest concentration of phenolics, followed by flavonoids, alkaloids and terpenoids. *T. cordifolia* stem extract has toxicity to T47D cells and can induce apoptosis, which increases with increasing extract concentration. *T. cordifolia* has considerable potential as an anticancer agent, but further research is necessary to understand the mechanism of action and potential compounds in the anti-breast cancer pathway.

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