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PROFILE OF ANTICANCER ACTIVITIES OF BROTOWALI (*Tinospora crispa L.*) PLANTS OFVARIOUS REGIONS IN EAST JAWA

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ABSTRACT

Brotowali (*Tinospora crispa L*) is a plant which has potential to be a chemopreventive agent. This study aims to determine the profile of anticancer activity of brotowali stem extracts (*Tinospora crispa L.*), from several regions in East Java. The extraction was carried out by maceration method using 80% ethanol solvent. Then, anticancer activity test was carried out on MCF-7 breast cancer cell model using the Microtetrazolium (MTT) Assay method. The results of the anticancer activity test showed that 15 brotowali stem extracts taken from 5 locations in East Java had significant differences in anticancer activity ($P < 0.05$). Brotowali extracts from Kanigoro, Blitar city, had the highest anticancer activity with an IC_{50} value of 30.64 µg / mL.

Keywords: Anticancer profile; Brotowali (*Tinospora crispa L.*); East Java; Microtetrazolium (MTT) Assay method

INTRODUCTION

Cancer is abnormal and uncontrolled cell growth. These cells can grow further and spread to other parts and can cause death. The type of cancer that mostly attacks women is breast cancer (Siegel *et al*., 2017). In 2013, breast cancer in East Java region ranked second in Indonesia after Central Java, with the incidence number of 61,230 patients (Ministry of Health of the Republic of Indonesia, 2015).

Cancer treatment in general is carried out by surgery, radiotherapy, and chemotherapy (Mutiah, 2017). However, some of the treatments still have limitations, including the occurrence of resistance and the side effects of drugs (Haryanti and Yuli, 2017). Therefore, it is necessary to develop a new drug that has relatively few side effects and is selective for cancer cells; one of which is by using natural materials (Isparning *et al*., 2015).

Brotowali (*Tinospora crispa L.*) is one of the plants that is potential as an anticancer. Previous research proved that the ethanol extract from brotowali plant (*Tinospora crispa L.*) has a cytotoxic effect on MCF-7 cells, HeLa cells, and Caov-3 cells (Adnan, 2016). Brotowali has been known to contain quaternary flavonoids and alkaloids, including apigenin, berberine, palmatine, borapetol a, borapetol b, borapetosid a, borapetosid b and pikroretin (Adnan, 2016). The largest active compound in brotowali (*Tinospora crispa L.*) plant is berberine compounds group. Berberine compounds have the most prominent pharmacological activity in various cancer cells (Rahmatullah, 2014). This compounds can inhibit the growth of MCF-7 and MDA-MB-23 breast cancer cell (Utami *et al*., 2015). This study can be used as a reference that brotowali plants (*Tinospora crispa L.*) are potential for anticancer

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treatment so that they can be developed as phytopharmaceutical drugs.

One obstacle in developing phytopharmaceutical is the variability of compounds content in the medicinal raw material (Verma and Shukla, 2015), that has different activities probably due to external factors and internal factors in each plant (Kim *et al*., 2011). The internal factors of plants are genetic factors and physiological variations, while the external factors (environment) are temperature, humidity, light intensity, water intake, mineral content, sun exposure, and rainfall (Verma and Shukla, 2015). The growing location can also be a possible reason of potential differences in the composition of the chemical content of plants that can influence pharmacological activity (Meisarani & Ramadhania, 2016).

To optimize the quality of raw materials, it is necessary to examine the potential of anticancer in brotowali plants for treatments. This study aims to profile the anticancer activity of brotowali (*Tinospora crispa L*.) plant extracts from several locations in East Java (Tulungagung, Blitar, Malang, Pasuruan and Jombang) against MCF-7 breast cancer cells. By doing so, it can be seen in which areas brotowali (*Tinospora crispa L*.) plants have the most potential anticancer activity against breast cancer cells. Moreover, this study can also be made as a reference for taking raw materials for breast cancer.

METHODS

Materials

The subjects of this research were brotowali(*Tinospora crispa L*.) stems, aged around 8-12 months old which were taken from 5 different regions in East Java, namely Tulungagung (Ngunut, called TcTN; Besuki, called TcTB; Sendang, called TcTS), Blitar (Garum, called TcBG; Kanigoro, called TcBK; Selopuro, called TcBS), Malang (Kedungkandang, called TcMK; Lowokwaru, called TcML; Singosari, called TcMS), Pasuruan (Purwodadi, called TcPPW; Pandaan, called TcPPD; Sukorejo, called TcPS), Jombang (Kabuh, called TcJK, Megaluh, called TcJM; Ploso, called TcJP) . The plant determination was carried out at Materia Medika Batu, East Java, Indonesia with the number of 074 / 354A / 102.7 / 2018.

The solvents used in this study were 80% ethanol (Merck, Germany), chloroform (Merck, Germany), methanol (Merck, Germany), 10% sulfuric acid (Merck, Germany), aquadest, silica gel GF254 (Sigma Aldrich Chemie GmbH, Germany), pure doxorubicin (Sanbe Farma 2 mg/ml, Indonesia). MCF-7 breast cancer cells were obtained from the Parasitology Laboratory of the Faculty of Medicine of Gajah Mada University, Yogyakarta. The cell culture media used were Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA), Dimethyl sulfoxide (DMSO) (Sigma Aldrich Chemie GmbH, Germany), Phosphate buffer saline (PBS) (Gibco, USA), Mikrotetrazolium (Sigma Chemical, USA), Sodium Dodecyl Sulfate (Sigma Co, USA), 10% in 0,1 N HCl and Incubator (Heraeus) at 5% CO_2, 95% O_2 at 37 ° C.

Sample Preparation

A hundred grams of brotowali stem from each region was washed, sorted, and dried. The drying process wascarried out by aerating the stem sunder the sun indirectly, by covering the stems with black cloth for 4-5 days. After the steams were dried, they were grinded.

Moisture Analysis

The moisture analysis was carried out by weighing 0.5 g of simplicia. Then,

the moisture content was measured using the moisture content analyzer (Ohaus, Indonesia). The working principle of this tool is by evaporating the water contained in the sample. The result of water evaporation was then measured as a percentage of moisture content.

Extraction

The extraction of brotowali stem simplicia was carried out using maceration method with Ultrasonic Assisted Extraction(UAE) (Sonica, USA), using 80% ethanol solvent. The ratio between simplicia and solvent was 1:10. ± 10 grams of simplicia was dissolved in 100 ml of 80% ethanol solvent which was divided into 3 macerations. The first maceration was 10 grams of simplicia, dissolved in 35 ml of 80% ethanol in the UAE for 3x2 minutes, and filtered. The filtrate was collected and the residue was macerated again using 35 ml of 80% ethanol in the UAE for 3x2 minutes, then filtered and accommodated. The residue was re-macerated with 30 ml of 80% ethanol. After the residue was re-macerated, it was extracted again with the UAE for 3x2 minutes. Then the filtrate was collected and evaporated using a rotary evaporator (IKA RV10 DIGITAL V, Germany) to produce a thick extract.

Identification of compounds using thin-layer chromatography (TLC)

The identification of compounds was carried out to detect the content of compounds in 80% ethanol extracts of brotowali (Tinospora crispa L.) stems. The identification of compounds was determined using the thin layer chromatography (TLC) and the spots were visualized using TLC Visualizer (CAMAG, Switzerland). The stationary phase used was silica gel F_{254} which was polar. However, the mobile phase used was chloroform: methanol with a ratio of 9.5: 0.5.

The ethanol extract of 10 mg brotowali (*Tinospora crispa L.*) stems was weighed and dissolved in 1 ml 80% ethanol. Then, the solution was bottled in the stationary phase (silica gel F_{254}) as much as 2µm. Then, it was eluted in the chamber which contained the saturated mobile phase (eluant). The plate was sprayed using a 10% H_2SO_4 stain in the fume hood for visualization. Then, it was heated on TLC plate heater (CAMAG, Switzerland) at 105 ° C for 5 minutes then observed under UV lamps with wavelengths of 254 and 366 nm.

Anticancer activity test using MTT method

Anticancer activity tests were conducted in the laboratory of Parasitology, Faculty of Medicine, Gadjah Mada University, Yogyakarta. The MCF-7 cells were cultured with DMEM media and then harvested. Before the cells were planted on 96-well plat, they were counted first with the hermocytometer and the result was $160x10^4$/ml. Based on that, the calculation of how many cells will be planted per well obtained the result of $5x10^4$ cells/well. Then, the MCF-7 cells were distributed to the well plate 96.After that, 100 µl of DMSO solution was added to the extract and doxorubicin (positive control) was made with a certain series of concentrations. Moreover, cell control group was given culture media, the incubation was carried out for 24 hours in an incubator with a flow of 5% CO_2 and 95% O_2. At the end of the incubation, the culture media containing the sample was discarded and then washed with 100 µl of PBS.Then, each well was added by 100 µl of DMEM culture media containing MTT 0.5 mg / ml (dilution 10 x MTT stock 5mg / ml). It was incubated for 4 hours at 37 °C and CO_2 5% flow. Then, the cell condition was observed with a microscope. The observation showed that the living cells reacted with MTT by

forming purple formazan crystals. After 4 hours, the MTT reaction was stopped by adding 100 µl of SDS stopper. After that, the microplate was wrapped and incubated overnight at the darkroom with normal temperature. The test results were read by ELISA reader (Benchmark, Lithonia;Georgia) at a wavelength of 595 nm (Mutiah, 2014).

Data Analysis

The data obtained in the form of absorbance of each well was converted into cell viability percentage by using this formula:

Living cell percentage (%):

$$\frac{(\text{Abs treatment} - \text{Abs media control})}{(\text{Abs cell control} - \text{Abs control media control})} \text{ x } 100\%$$

Notes: Abs: absorbance

The percentage of living cells was calculated to obtain the IC_{50} value of concentration. This value of concentration could cause a growth inhibition of 50% of the cell population this is cytotoxic potential could be seen. IC_{50} values are determined by probit analysis using Statistical Product and Service Solution (SPSS) 24.0 for Windows statistics (Mutiah, 2017).

IC_{50} values are divided into three categories: if the IC_{50} values <50 µg / ml, they are categorized as having a strong cytotoxic effect; if IC_{50} values 50 µg / ml - <200 µg / ml, they are categorized as having a moderate cytotoxic effect; if IC_{50} 200 µg / ml - <1000 µg / ml, they are categorized as having a weak cytotoxic effect, and;if IC_{50} values>1000 µg / ml, they are categorized as having no cytotoxic effect (Kuete, 2017).

RESULTS AND DISCUSSION

Moisture Analysis

Moisture content analysis aims to determine the quality and stability of the material. The high water content in simplicia can be a medium for the growth of molds and fungi. In addition, the high water content can also cause an enzymatic reaction that is able to decipher the active substances in simplicia (Salamah and Widyasari, 2015). The results of the analysis of water content in this study showed that the water content of brotowali (*Tinospora crispa L.*) stem powders which were obtained from the average brotowali stem powder was below 10%. Therefore, the results of the analysis of the water content of 15 brotowali (*Tinospora crispa L.*) stem powder can be said to have fulfilled the requirements set by BPOM. It is stated that water content requirements for solid preparations for natural medicine must have a moisture content of ≤10%, except for efferent moisture content, that is ≤5% (Ministry of Health Republic of Indonesia, 2013). The results of the water content analysis can be seen in Table I.

Extraction

The results obtained from maceration extraction were in the form of concentrated dark brown extracts. The yield value of each extract is a parameter to find out the size of the product resulted from the extraction process. However, the calculation was done by dividing the number of products by the number of materials used (Warsono *et al*., 2013). The results of the rendement calculation can be seen in Table I.

Based on the results of the calculations as shownin Table I, the extract of TcPPW (Purwodadi) has the highest yield value compared to other extracts.It can be seen that the extract of TcPPW (Purwodadi) has many compounds. It is because the higher the yield, the more compound producted (Warsono *et al*., 2013).

Table I. Results of Maceration Extract of Brotowali Stem (*Tinospora crispa L.*)

Samples	Water content (%) b/b)	Simplicia (grams)	The color of the concentrated extract	The weight of the concentrated extract (gram)	rendement (%) (b / b)
TcTN	9.24	9.059	Deep brown	0.711	7.848
TcTB	9.45	7.000	Deep brown	1.025	14.642
TcTS	7.19	3.316	Deep brown	0.411	12.394
Tc BG	8.40	9.002	Deep brown	1.267	14.075
TcBK	7.69	5.027	Deep brown	0.729	14.502
TcBS	8.09	4.000	Deep brown	0.445	11.125
TcMK	7.85	7.097	Deep brown	0.493	6.946
TcML	8.73	6.402	Deep brown	0.551	8.606
TcMS	7.95	10.226	Deep brown	0.803	7.852
TcPPW	8.76	10.254	Deep brown	1.632	15.916
TcPPD	7.22	4.976	Deep brown	0.383	7.696
TcPS	8.17	3.040	Deep brown	0.043	1.414
TcJK	8.64	10.705	Deep brown	0.320	2.989
TcJM	9.17	10.364	Deep brown	0.381	3.675
TcJP	7.39	6.331	Deep brown	0.415	6.555

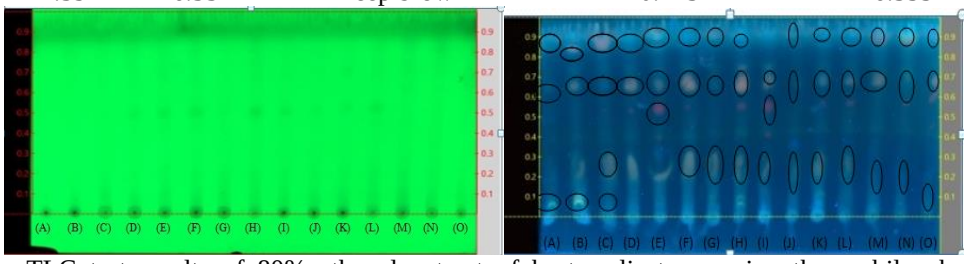

Figure 1. The TLC test results of 80% ethanol extract of brotowali stems using the mobile phase of chloroform: methanol (9.5: 0.5) in the observation of TLC visualizer with wavelengths of 254 nm and 366 nm. (A)TcTN (Ngunut), (B) TcTB(Besuki), (C)TcTS(Sendang), (D)TcBG(Garum), (E)TcBK(Kanigoro), (F)TcBS(Selopuro), (G)TcMK(Kedungkandang), (H)TcML(Lowokwaru), (I)TcMS(Singosari), (J)TcPPW(Purwodadi), (K)TcPPD(Pandaan), (L)TcPS(Sukorejo), (M)TcJK (Kabuh), (N)TcJM(Megaluh), (O)TcJP(Ploso).

The Identification of compounds using thin layer chromatography (TLC)

The results of the observations on compound identification tests showed that there were yellow stains on all brotowali plant extracts from several locations in East Java. It shows the presence of alkaloid compounds in brotowali plants taken from several regions in East Java (Wagner & Bladt, 2001). The results of the observations are shown in Figure 1.

Anticancer activity of Brotowali (*Tinospora crispa. L*) from several regions in East Java

Cytotoxic tests in this study were carried out by using the MTT method. The purpose of the cytotoxic test was to determine the potential of the toxicity of brotowali ethanol extracts on the MCF-7 breast cancer cells. The results of the observations carried out under an inverted microscope showed that there were differences in the morphology of MCF-7 breast cancer cells after the administration of brotowali stem ethanol extracts. The shape of living cells appeared like leaves and sticked to the bottom of the well, while the shape of the dead cells appeared to be round and floated on the surface of the well (Machana *et al*. 2011).

Cytotoxic test of brotowali stem ethanol extracts on MCF-7 breast cancer cells resulted in a reduction of cell

viability (number of living cells) along with increased concentration levels. The higher the concentration, the lower the cellviability. The results of viability of MCF-7 breast cancer cells are shown in Figure 2.

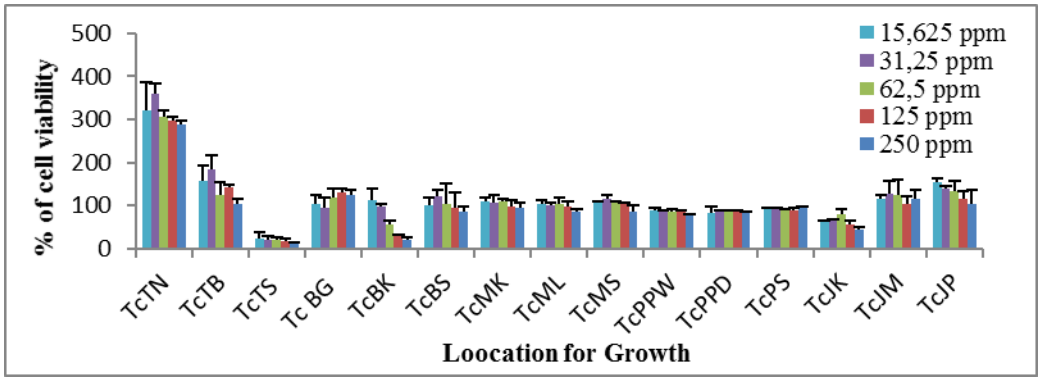

Figure 2. Cell viability of MCF-7 breast cancer cells after administration of brotowali extracts taken from several regions in East Java

Table II. The results of IC_{50} Value of Ethanol Extracts of Brotowali stems from 5 Regions in East Java

Plants	**IC_{50} (µg/ml)±SD**
TcTN	131,48±4,64
TcTB	126,80±9,66
TcTS	140,32±12,28
Tc BG	197,95±12,65
TcBK	30,64±2,18
TcBS	177,12±35,13
TcMK	243,08±63,60
TcML	198,33±9,40
TcMS	254,15±30,77
TcPPW	172,37±16,17
TcPPD	234,89±16,03
TcPS	187,96±19,76
TcJK	177,58±12,25
TcJM	178,95±12,51
TcJP	245,43±25,12
Doxo	21,11±3,047

The results of the data analysis using SPSS 24.0 which show the IC_{50} values of 15 samples of brotowali stem ethanol extracts are presented in Table II.

Based on the data from Table II, the results of IC_{50} values can be categorized as follows: the extracts that are from Kanigoro, Blitar have the IC_{50} value of 30.64 µg/ml and are categorized as having strong cytotoxic potential. Moreover, extracts which are categorized as having moderate cytotoxic potential come from the following regions. Tulungagung: Besuki (IC_{50}126.80µg/ml), Ngunut (IC_{50}131,48µg/ml), Sendang (IC_{50}140.32 µg/ml); Blitar city: Garum (IC_{50}197.95 µg/ml), Selopuro (IC_{50} 177.12 µg/ml); Malang city: Lowokwaru (IC_{50}198.33 µg/ml); Pasuruan city: Purwodadi (IC_{50}172.37 µg/ml) and Sukorejo (IC_{50}187.96 µg / ml); Jombang city: Kabuh (IC_{50}177.58µg/ml), Megaluh (IC_{50}178.95 µg/ml) and Ploso (IC_{50}245.43 µg/ml). The 4 other extracts, namely extracts from Malang city: Kedung Kandang (IC_{50}243.08 µg/ml) and Singosari (IC_{50}254.15 µg/ml); Pasuruan city: Pandaan (IC_{50}234.89 µg/ml); Jombang: Ploso (IC_{50}245.43 µg/ml) are categorized as having weak cytotoxic potential.

The results of the data analysis showed that 15 extracts from 15 sub-districts had significant differences in

their anticancer activity ($p < 0.05$). The variance in anticancer activity was caused by growth location differences, which differ in their altitude, temperature, rainfall, climate, and soil type. The growth location difference is a factor that can influence the content of secondary metabolites of brotowali plants. Furthermore, it also influences their pharmacological activity (Kim *et al*., 2011). The location characteristics of where the samples were taken can be seen in Table III.

Table III. Location Characteristics Where the Samples were Taken

No.	Samples	Altitude (MDPL)	Average Temperature (°C)	Rainfall (mm)	Climate	Soil Type
1.	TcTN	106	25,3	1735	Aw	Brownish alluvial gray and alluvial grayish brown; Brownish Regosol
2.	TcTB	146	25,2	1897	Am	Alluvial; Association of Brownish alluvial gray and alluvial; Litosol
3.	TcTS	102	25,4	1742	Aw	Association of Brownish alluvial gray and alluvial; Mediterranean litosol and resina
4.	TcBG	236	24,2	2085	Am	Regosol; Litosol
5.	TcBK	174	24,5	1954	Am	Regosol; Litosol
6.	TcBS	184	24,3	2040	Am	Regosol; Litosol
7.	TcMK	454	23,7	2090	Am	Alluvial; Mediteran; Association of reddish-brown or grayish latosol; Gray chocolate and humus association
8.	TcML	459	23,8	2101	Am	Andosol
9.	TcMS	486	23,8	2203	Am	Alluvial; Mediteran; Association of reddish-brown or grayish latosol; Gray chocolate and humus association
10.	TcPPW	255	25,1	1844	Aw	Alluvial; Mediteran; Labosal; Grumasol; Andosol
11.	TcPPD	345	24,6	2003	Am	Alluvial; Mediteran; Labosal; Grumasol; Andosol
12.	TcPS	254	26,2	1740	Af	Regosol, Gumusol
13.	TcJK	36	26,5	1727	Aw	Grumasol taupe
14.	TcJM	35	26,4	1686	Aw	Mediterranean brown and latosol complex
15.	TcJP	35	26,4	1723	Aw	Association of Gray alluvial and grayish alluvial

Based on the data of the location characteristics where the samples were taken in table 3, there are differences from each location. The results showed that the extract from in Kanigoro sub-district, Blitar city, had the highest anticancer activity (IC_{50}30.64µg / ml) compared to the extracts from 14 other cities. This is due to differences in the content of the compounds that are influenced by internal factors and external factors of each plant (Heuberger *et al*., 2014). From the results obtained, when it is associated with the characteristics of the location of sampling, altitude can affect the content of compounds contained in plants (Nurnasari, 2010). However, in this study, it has not been proven that altitude can affect the content of plant compounds. This is possible because microorganisms help the process of the plant growth. Therefore, extract from kanigoro had the highest anticancer activity compared to other location (Meisarani & Ramadhania, 2016).

Meanwhile, if it is seen from the type of soil, there is also a difference. Nevertheless, the difference is not quite significant. The differences of soil type result in the different characteristics of one species. Besides, the temperature, rainfall, and also heat correspond to the location. Therefore, these factors can be various (Shukla, 2015). The anticancer activity that plays a role in brotowali plants is the composition of alkaloids that can inhibit DNA topoisomerase II activity (Zuhair and Subchan, 2010). DNA topoisomerase II is an enzyme that removes positive DNA supercoiling which occurs during DNA replication. The mechanism of alkaloid compounds as anticancer also plays a role in activating caspase (Macabeo *et al*., 2008). The activation of caspase is an alternative to kill cancer cells (Prescott, 2006). Berberine compounds in brotowali also play a role specifically in binding nucleic acids (DNA or RNA) and inducing DNA damage in cancer cells through regulation of DNA topoisomerase activity.Therefore, it can cause cell of cancer to die(Wang *et al*., 2016).

CONCLUSION

Based on the results of the study, it was found that the ethanol extracts of brotowali (Tinospora crispa L.) stems obtained from several locations in East Java had different anticancer activities. The extract which hadthe highest anticancer activity wasthe extract obtained from Kanigoro, Blitar with an IC_{50} value of 30.64 µg / ml.

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