Anticancer properties of methanolic extract of *Piper crocatum* leaf using BST and cytotoxicity on HeLa cell lines

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Abstract.
Context: Cancer is one of the leading causes of death throughout the world. Treatment failure in cancer can be caused due to drug resistance and toxicity. Therefore, it is necessary to develop new drugs that have relatively smaller side effects, one of which is by screening natural ingredients. One of the plants that have the potential for anticancer therapy is red betel (*Piper crocatum*). **Aims**: The purpose of this study was to determine the potential of methanolic extract of *P. crocatum* leaf (MEPcL) as a cytotoxic agent and phytochemical screening of MEPcL. **Settings and Design**: This research was conducted at Faculty of Pharmacy University of Jember and Faculty of Medicine Gadjah Mada University Indonesia in March to May 2018 using The Post Test Only Control Group Design. **Methods and Material**: Phytochemical screening was carried out by tube test and TLC, while the Brine Shrimp Lethality Test and cytotoxicity tests were carried out by direct counting method. **Statistical analysis used**: Pearson correlation test was used to analyze the cytotoxic effects and IC_{50} with probit analysis. **Results**: Based on the results of phytochemical screening, MEPcL contained flavonoids, tannins, alkaloids, saponins, and terpenoids. The results of the study showed that the LC_{50} value of MEPcL was (70.013 ± 3.874 2) µg mL^{-1} on BST and IC_{50} value was (34.20 ± 1.480 6) µg mL^{-1} on HeLa cell lines. **Conclusions**: Based on explanation above, it can be concluded that MEPcL has potency to be developed as anticancer agent.

Keywords: Cytotoxic, phytochemical screening, red betel

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Introduction

Cancer is one of the leading causes of death throughout the world. In 2012, around 82 000 000 deaths were caused by cancer. Today, therapies that are often used for cancer are chemotherapy and radiation which are relatively expensive. Treatment failure in cancer can be caused by drug resistance and toxicity. Therefore, it is necessary to develop new drugs that have relatively small side effects, one of which is by screening natural ingredients. One of the plants having the potential to be developed into anticancer drugs is red betel [*Piper crocatum* (Ruiz and Pav. 1798)]. Red betel leaves contain alkaloids, saponins, tannins, and flavonoids [1]. Flavonoids, polyphenols, and tannins are antioxidants, anti–tumor [2]. Alkaloid compounds have antineoplastic properties that can inhibit the growth of cancer cells[3]. Empirically, red betel leaf is widely used by the community to treat diabetes mellitus, breast cancer, cervical cancer, acute ulcer, kidney stones, haemorrhoid, heart attacks, and strokes[4].

In the search for bioactive ingredients that have anticancer activities, several methods of screening for biological activity were used. These methods included the testing of sea shrimp larvae, tumor resistance testing on potato plates, lemna bud proliferation tests, cytotoxic test in vitro and in vivo[5]. In this study an anticancer screening test of methanolic extract of *P. crocatum* leaf (MEPcL) was carried out through the Brine Shrimp Lethality Test (BST) test and in vitro cytotoxicity test on HeLa cell culture using the direct calculation method.

The purpose of this study was to determine the anticancer properties of MEPcL using BST and cytotoxicity against HeLa cells and to confirm any classes of compounds contained in MEPcL.

Materials and Methods

Collection and identification of *P. crocatum* sample

The research material included red betel leaves obtained from Jember, East Java, Indonesia and identified by Herbarium Jemberiense–Jember, HeLa cell culture was the collection of Tropical Medicine Laboratory UGM**.** This research was conducted in several stages, namely: (i) making red betel leaf powder, (ii) making methanol extract, (iii) anticancer screening test (BST test) (iv) cytotoxicity test on HeLa cell culture, and (v) data analysis.

Preparation of MEPcL

The prepared red betel leaf was sorted first with the aim of ensuring the correctness of the ingredients and eliminating foreign organic matter, then, weighed, air–dried, and grinded. The powder obtained was weighed for extraction. Weighed 100 g of red betel leaf simplicia then macerated in vessel (maserator) with redestylated methanol for 3 × 24 h while occasionally shaken out, filter with a buchner filter. The obtained maserate was dried using rotavapour until reduced volume. Furthermore, the solvent was evaporated in an oven temperature of 40 °C until thick extract.

BST test

Preparation of larvae

The eggs of *Artemia salina* (Linnaeus, 1758) Leach were weighed as much as 50 mg then put in a jar containing 500 mL of filtered sea water. After being aerated, the eggs were left for 48 h so they hatch perfectly. Hatched larvae can be taken and used in toxicity tests by piping them.

Sample preparation

The dry extract was weighed 106.6 mg of methanol and then added 1 mL DMSO (1 %) and sea water up to 100 mL, in order to obtain an insoluble parent with concentration of 1 066 µg mL^{-1}, then used for treatment to *A. salina* Leach already hatch.

Testing against larvae

Solution parent extract methanol 1 066 µg mL^{-1} was then pippetted to obtain solution test with concentration of 640 µg mL^{-1}, 480 µg mL^{-1}, 320 µg mL^{-1}, 160 µg mL^{-1}, 80 µg mL^{-1}, and 16 µg mL^{-1} in the vial that has been calibrated, then put 10 larvae of A. salina plus sea water up to 10 mL with three replication for each concentration, then left alone for 24 h. Dead larvae were then counted.

Cytotoxicity test on Hela cells

Cytotoxicity test of direct counting method.

Ammoutt 100 µL sample in culture media was added in the 100.0 µL of each cell suspension of different wells (cell density of 2×10^4 cells/ wells) to achieve the final concentration in wells series of 500 µg mL^{-1}; 250 µg mL^{-1}; 125 µg mL^{-1}; 62.5 µg mL^{-1}; 31.25 µg mL^{-1}; and 5.63 µg mL^{-1}. In the media controls, 100.0 µL test compound was replaced with 100.0 µL of culture media, being in control of the solvent, was added to 100 µL DMSO in the culture medium to achieve the final concentration in wells series of 0.5 %; 0.25 %; 0.125 %; 0.06 %; 0.03 %; and 0.015 %. Then the plate was incubated in an incubator of 5 % CO_2 for 24 h at 37 °C, then added 50.0 µL of 0.5 % trypan blue solution in aquadestilata. After that the solution in each well was resuspended strongly and taken 10.0 µL for direct calculation under a light microscope with 40 × magnification.

Test screening phytochemical

Phytochemical screening on MEPcL was done by tube test and thin layer chromatography (TLC) for confirm tannin, alkaloid, saponin, steroid and flavonoid compound.

Statistic analysis

Statistic analysis for this research using Pearson Correlation test with a level of trust 95 % to analyze the cytotoxic effects of MEPcL to HeLa cell culture with BST method and to determaine IC_{50} value using probit analysis.

Result

Brine shrimp test (BST)

Toxicity test was carried out by the method of direct calculation of the number of dead *A. salina* larvae. The shrimp larvae used in this study were 48 h old larvae. The results of observations of MEPcL at various test concentrations of *A. salina* within 24 h of observation had toxic power. Results of LC_{50} value of MEPcL were showed in Table 1.

Table 1. LC_{50} value of MEPcL

Replication	LC_{50} (µg mL^{-1})
DMSO control	-
MEPcL Replication 1	68.442 08
MEPcL Replication 2	67.170 54
MEPcL Replication 3	74.42 558
Mean ± SD	70.013 ± 3.8 742

Table 1 showed that the greater the concentration value of the extract, the greater the mortality of *A. salina*. This is in accordance with Harborne[3], which states that the higher the concentration of extracts the higher the toxic properties. The extract is said to be active or has anticancer activity according to the BST method if it has an LC_{50} < 1 000 µg mL^{-1}[6]. From the data from the BST test results of MEPcL (Table 1) it has an average LC_{50} value of < 1 000 µg mL^{-1} which is equal to 70.013 µg mL^{1}. So that it can be said that the MEPcL has anticancer activity.

Cytotoxic test on HeLa cell lines

Cytotoxic tests were performed on HeLa cells to determine the potential inhibition of cell growth due to the treatment of MEPcL. This test was conducted to determine the level of test samples that can inhibit HeLa cell growth by up to 50 % (IC_{50}) and confirm the cytotoxic ability of test extracts against HeLa cells as the initial screening for natural drugs that can be used in cancer treatment especially cervical cancer.

Results test cytotoxicity stated in percentage cell dead. The percentage of deaths was calculated by the number of control living cells minus the number of treated living cells both in the DMSO control and with the sample as the test compound divided by the number of live control cells (without treatment) multiplied by 100 %. MEPcL inhibition was expressed in Table 2. The negative control showed no inhibition against cell growth.

Table 2. HeLa cells inhibition by MEPcL.

Treatment	IC_{50} (µg mL^{-1})
Media Control	-
DMSO Control	-
MEPcL Replication 1	33.34 873
MEPcL Replication 2	35.91 377
MEPcL Replication 3	33.34 978
Mean ± SD	34.20 ± 1.4 806

MEPcL has cytotoxic activity which is indicated by IC_{50} values averaging 34.20 µg mL^{-1}. This value indicates that the methanol extract is quite potential because the IC_{50} value is below 100 µg mL^{-1}[7]. The results of cytotoxicity assay are used as a reference for the development of mechanisms of MEPcL in inhibiting cell growth. Therefore, further research is needed to show the potential of MEPcL as a natural chemotherapy agent in the treatment of cancer, especially cervical cancer.

Phytochemical screening

Phytochemical screening of MEPcL was carried out qualitatively by chemical reaction and Thin Layer Chromatography (TLC). The results of MEPcL are listed in Table 3.

Table 3. Phytochemical screening of MEPcL.

No.	Group Test	Result	Information	Conclusion
1.	Alkaloids			
	I. Reaction Deposition	I. Turbidity was available	Positive	Contained alkaloid
	II. TLC Test Toluene elu ent: ethylic etate (7 : 3)	II. With viewer stain dragendrof on Rf 0.375, no looked stain orange color	Negative	
2.	Saponon and steroids			Contained saponin, steroids or terpenoids
	I. Froth Test	I. Happen froth stable for more from 30 min with 3 cm high	Positive	
	II. Salkowski Test	II. Arised ring colored red	Positive	
	III. TLC Test Toluence elu ent: ethilac etate (7:3)	III. Available stain Purple with viewer stain anisaldehyde acid sulfate on Rf 0.75 and Rf. 0.875	Positive	

(continued on next page)

Table 3. Continued

No.	Group Test	Result	Information	Conclusion
3.	Falvonoid			
	i. Wilstater Test	i. Arised color red old	Positive	Contained Flavonoid
	ii. TLS Test Wluent chloroform : methanol: water (9.7 : 0.2 : 0.1)	ii. Available stain tallow intensive on Rf. 0.5 with viewer stain steam ammonia	Positive	
4.	Polyphenols and tannin			
	i. Ferricloride test	i. Happen color green black	Positive	Contained phenol, no tannin
	ii. Gelatin test	ii. Not happen sediment white	Negative	
	TLC Test eluent chloro form: methanol : water (7 : 3 : 0.4)	iii. Available stain black on Rf. 0.2 with $FeCl_3$ sprayers	Positive	

Based on the results of phytochemical screening it is suspected that MEPcL contains compounds flavonoids, saponins, alkaloids, and steroids or terpenoids.

Discussion

Toxicity tests using the BST method are not specific to anticancer, but can provide an initial description of the extracted therapeutic activity tested. This method was chosen because *A. salina* is easily found and obtained less than 24 h since it was hatched from its dormant phase[8]. Based on Meyer's study on the type of Euphorbiaceae, from 24 species active in 9 PS (leukemia cells in vitro), 14 of them were toxic to sea shrimp larvae[6]. This proves that there is a positive correlation between the BST method and the cytotoxic power of anticancer compounds, so this BST method is often used for screening the initial search for antitumor and anticancer active compounds[5]. To get a more realistic picture of therapeutic activity, it is better to test the MEPcL using a more specific method.

Cytotoxicity test was carried out by direct counting method by calculating directly the number of living cells and dead cells with a haemocytmeter under a microscope. The calculation of the number of living cells and dead cells was carried out based on cell morphology with the help of trypan blue dyes. The trypan blue coloring method is based on the principle that living cells do not bind trypan blue dyes, while dead cells can bind trypan blue. This is due to a decrease in membrane integrity in dead cells, so that trypan blue can enter and dye cells, on the contrary it does not occur in living cells because the cell membrane is intact so it looks brilliant because proteins in cells do not bind to trypan blue[9]. The morphology of HeLa cells is alive after incubation for 24 h and has not been given trypan blue dyes shaped like leaves, oval, clear and membrane boundaries with the media clearly visible. The morphology of HeLa cells after being given trypan blue dyes for living cells will be

clear, brilliant, not cloudy in the cell nucleus. While the structure of the dead HeLa cell is cloudy, not luminous and the cell membrane looks broken or rather faint.

Groups of chemical compounds in plants related to anticancer and antioxidant activities include alkaloids, terpenoids, polyphenols, resin and flavonoid compounds[5]. The mechanism of anticancer alkaloid compounds is to bind tubules and inhibit the formation of components of microtubuli on mitotic coils so that metaphase stops[11]. The mechanism of terpenoid compounds as anticancer compounds is terpenoid compounds can block the cell cycle in the phase G2/M by stabilizing spindle threads in the mitotic phase, causing the mitosis process to be inhibited. In the next stage, there will be inhibition of cell proliferation and pacification of apoptosis. Terpenoid compounds are also able to inhibit topoisomerase enzymes in mammalian cells[12]. Flavonoid compounds can inhibit the carcinogenesis process both in vitro and in vivo. Inhibition occurs at the stage of initiation, promotion and progression through molecular mechanisms including inactivation of carcinogenic compounds, antiproliferatives, inhibition of angiogenesis and cell cycle, induction of apoptosis, and antioxidant activity[13]. This shows the similarity with the statement from Sarah, which recommends the BST test as the initial test of screening for the acquisition of bioactive compounds[14].

Based on the description that has been submitted, the conclusions from this study are as follows, MEPcL has a toxic effect on *A. salina* Leach with an LC_{50} value averaging 70.013 µg mL^{-1} ± 3.874 2 µg mL^{-1}; MEPcL has a cytotoxic effect on HeLa cell culture with IC_{50} values averaging 34.20 µg mL^{-1} ± 1.480 6 µg mL^{-1}; the groups compound contained in the MEPcL contains flavonoid, alkaloid, saponin and terpenoids or steroids compounds. It is concluded that MEPcL has potency to be developed as anticancer agent.

Conclusion

Based on explanation above, it can be concluded that MEPcL has potency to be developed as anticancer agent.

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