

Cytotoxic activities of Methanolic and Chloroform extract of *Cryptocarya massoy* (Oken) Kosterm. Bark on MCF-7 human breast cancer cell line

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Abstrak

Latar Belakang: Kanker merupakan penyakit penyebab kematian utama di dunia. Obat-obat yang digunakan untuk pengobatan kanker (kemoterapi), sebagian besar merupakan senyawa kimia yang berasal dari tumbuhan. *Cryptocarya massoy* (Oken) Kosterm. (Lauraceae) atau masoyi telah digunakan sejak lama untuk anti-inflamasi, anti mikroba dan aromaterapi. Dua senyawa aktif dari masoyi yaitu (-)-massoialactone dan α,β -unsaturated δ -lactone telah diteliti aktivitasnya terhadap beberapa jenis sel line kanker. Penelitian ini bertujuan untuk mengevaluasi aktivitas sitotoksik ekstrak metanol dan kloroform kulit batang masoyi terhadap sel MCF-7.

Metode: Pengaruh ekstrak metanol dan kloroform kulit batang masoyi terhadap viabilitas sel MCF-7 ditetapkan melalui uji sitotoksik dengan metode MTT assay. Ekstrak yang paling aktif selanjutnya diuji terhadap ekspresi protein Bcl-2 yang dilakukan dengan pengecatan imunositokimia.

Hasil: Hasil penelitian menunjukkan bahwa ekstrak kloroform memberikan daya penghambatan yang lebih tinggi dibandingkan ekstrak metanol dengan nilai IC50 sebesar 9,14 $\mu\text{g/ml}$ sedangkan metanol memberikan nilai penghambatan (IC50) sebesar 196,73 $\mu\text{g/ml}$. Hasil uji immunositokimia menunjukkan bahwa ekspresi Bcl-2 sel MCF-7 hampir sama pada sel yang diberi perlakuan maupun kontrol. Hasil analisis morfologi sel MCF-7 akibat perlakuan ekstrak kloroform menunjukkan adanya perubahan morfologi yang mengindikasikan terjadinya apoptosis.

Kesimpulan: ekstrak kloroform kulit batang masoyi mampu menghambat pertumbuhan sel MCF-7 dan memacu apoptosis yang tidak berpengaruh terhadap ekspresi protein Bcl-2. (*Health Science Journal of Indonesia* 2018;9(1):57-62)

Kata kunci: *Cryptocarya massoy*, sitotoksik, MTT assay, MCF-7

Abstract

Backgrounds: Cancer is the second leading cause of death worldwide. Most cancer drugs are derived from natural sources such as plants. *Cryptocarya massoy* (Oken) Kosterm. (Lauraceae) has been used in folk medicine for anti-inflammatory, antimicrobial, and aromatherapy. The (-)-massoialactone, an α,β -unsaturated δ -lactone, first isolated from *Cryptocarya massoy*, has been evaluated on tumor cell lines. This research aimed to evaluate the cytotoxic activities of methanol and chloroform extract of massoia bark on MCF-7 cell lines.

Methods: MTT assay was used to evaluate the viability inhibition of cell cultures in the presence and absence of the extracts for 48 hours. Furthermore, the effect of massoia extract to MCF-7 cells line viability with inhibition of protein expression antiapoptosis Bcl-2 was determined by immunocytochemistry assay.

Results: The result showed that the chloroform extract exhibited the most effective cytotoxic activities with the IC50 values of 9,14 $\mu\text{g/ml}$ while the methanolic extract showed medium cytotoxic activity with the IC50 values of 196,73 $\mu\text{g/ml}$. The expression of anti-apoptosis protein Bcl-2 was assessed by immunocytochemistry. The expression of Bcl-2 was the same level in treated and untreated cells. Morphological analysis of the treated cells represents features of apoptotic cells (reduction of cell volume, picnotic nucleus and chromatolysis).

Conclusions: This study indicated that chloroform extract of massoia bark affect MCF7 cell viability by inducing apoptosis, without any changes in the expression level of Bcl-2. The apoptosis pathway of the extract should be further investigated. (*Health Science Journal of Indonesia* 2018;9(1):57-62)

Keywords: *Cryptocarya massoy*, cytotoxic, MTT assay, MCF-7

Cancer is the second leading cause of death worldwide. In a decade years ago, cancer has been recognized as a group of diseases afflicting to more developed countries. Presently various cancer incidence is now growing rapidly worldwide. Refer to the WHO data, breast cancer is the number one cause of death both in develop and developing countries¹.

Up to presently, the underlying cause of cancer was attribute to the acceleration or deregulation of genes that lead to cellular expansion and accumulation of tissue mass². Furthermore, cancer is one of the disease that have received the attention of many researchers in the world. In some cases, the use of drug is associated with other unbearable side effects coupled with their high cost which make this therapy out of reach to most low income people³. For these reasons, research related exploring plants which have anti-tumor property is still encouraged in order to discover any new compound or chemicals entity with less toxic but more potent effect⁴.

Cancer indeed is very specific disease which is still poorly defined in term of folklore or traditional medicine. It was contrast to other plant-based therapies use in traditional medicine for the treatment of common illness such as fever or pain. Therefore, exploring of plants that contain several type of phytochemicals such as carotenoids, flavonoid and anti-oxidant have been studied as potential chemopreventive agents⁵.

Mesoyi or massoia (*Cryptocarya massoy* (Oken) Kosterm.) is a type of tree reach to 25 m high that belong to Lauraceae family. This plant in Papua known as aikor or aikori tree, with a large trunk (\pm 30 cm in diameter), and the bark can reach 0.5 cm and smelling fragrant. The fragrant aroma comes from volatile oil content known as massoia lactone. The bark and hardwood of massoia contains C-10 lactone with golden colored oil. Massoia lactone has an odour that is described as sweet, coconut meat, creamy, milky and waxy⁶.

The wood of massoia is used as building material and the bark used for red dye mixture, food aroma and traditional medicine. It is also an essential ingredient in some herbal medicines in Java, where it has many uses, including preventing cramps during pregnancy, being used to stimulate recovery and restore vitality after childbirth, for woman after childbirth, to improve odor, as a tonic, and antispasmodic⁷.

Taxonomically, Lauraceae famili close to the Annonaceae family, and it abounds with aporphinoid alkaloid. Woo et al. (1999) has found a remarkable

advance in the search for topoisomerase inhibitors from Lauraceae. Dicentrine one of the apropine alkaloid found in several species within Lauraceae family has shown potent topoisomerase I activity and has cytotoxicity for a number of cancer cell line in vitro⁸. This study aim to evaluate the cytotoxic activity of chloroform and methanolic extract of *Cryptocarya massoy* bark on MCF-7 cell line.

METHODS

Preparation of sample *Cryptocarya massoy* (Oken) Kosterm

The bark of *Cryptocarya massoy* were collected from Pasar Gede the herbal market at Surakarta. One hundred gram of dried powdered of material was pulverized, then macerated in chloroform for three days and then filtered. The solvent was removed using a rotary evaporator at 50° C. The residue was further macerated in methanol for three days and then filtered. The entire extracts of massoi were evaporated to dryness in a rotary evaporator at 50° C.

Cytotoxicity assay

The assay was performed at the Integrated Laboratory of Medicinal Plant and Traditional Medicine Research and Development Centre (MPTMRDC). The MCF-7 cell line from ATCC-USA were cultured and cytotoxicity tests were carried out using the MTT assay. MCF-7 cancer cell line were maintained in Eagle's Minimum Essential Medium (EMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco 16000044) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) and cultured at 37° C in humidified atmosphere containing 5% CO₂. The cells were seeded at a density 8 x 10⁵ cells in 96-well plates with EMEM medium and incubated for 24 hours. The cells were treated with extracts of various concentration such as 5:10;20;40;80 and 160 µg/ml in 0,1% DMSO (Sigma Aldrich) for 48 hours exposure time. After 48 hours, 100 µl of a 1 mg/ml of solution of MTT in EMEM was added to each well. The culture plates were incubated for 4 hours at 37° C in humidified atmosphere containing 5% CO₂ (Incusafe). MTT was removed carefully and then stop solution (HCL in isopropanol) (EMerck) was added to each well and the plate was vigorously shaken to ensure that the blue formazan was completely dissolved. The absorbance was measured at 595 nm in automated plate reader (Biorad) and percentage of growth inhibition was calculated using the following standard formula⁹.

$$\text{Inhibitory rate} = \frac{((\text{control absorbance} - \text{test absorbance}) \times 100\%)}{(\text{control absorbance})}$$

IC₅₀ was defined as the concentration of the plant extracts killing 50% of the cells. IC₅₀ was determined for MCF-7 cell lines of both extracts.

Immunocytochemistry evaluation

The MCF-7 cells (density of 5×10^4 cells /wells) were grown on a coverslip in a 24-well plate until 80% confluent. Well plate were incubated with chloroform extract of massoia bark for 18 hours. Medium was taken from the well plate, than washed with PBS (Thermo). Cover slips containing the cells removed and placed in a 6-well plate. Preparations were fixed with cold methanol. Preparations were washed with PBS (Thermo) and then sprinkled with hydrogen peroxidase solution for 10 min at room temperature, and then discarded. Preparations were incubated with prediluted blocking serum for 10 minutes at room temperature, and then discarded. Furthermore, preparations was sprinkled with Primary Monoclonal Antibodies anti Bcl-2 (Santa Cruz) by 1:50 dilution for 24 hours at a temperature of 4 C. At the end of the incubation, preparations were washed with PBS for 5 minutes, then incubated with biotinylated universal secondary antibody for 10 minutes and washed with PBS for 5 minutes. Preparations in streptavidin - peroxidase was incubated with complex reagent for 10 minutes and washed with PBS for 5 minutes. Preparations were incubated in DAB substrate solution for 10 minutes and then washed with distilled water. Finally, preparations immersed in Mayer's Haematoxylin and Eosin (HE) for 3 minutes and washed with distilled water, then dipped in alcohol, cleaned, dipped in xylol and then sprinkled with mounting medium and the cover slips closed. Protein expression was observed under a light microscope 10 - 100x magnification¹⁰.

Ethical clearance

Regarding to this research of in vitro study, ethical clearance is no need to be issued.

RESULT

Inhibitory effect of chloroform and methanolic extract of massoi bark on MCF-7 cell growth

To determine the potency of chloroform and methanolic extract of massoi bark as co-chemopreventive agents, the cytotoxicity properties were examined in MCF-7 cell line. Cell viability was examined using MTT assay method with 48 hours incubation. The treatment of chloroform extract of massoi bark on MCF-7 cells resulted the decreasing number of viable cell in dose dependent manner as showed in Table 1.

Table 1. Effect of chloroform and methanolic extract of massoi bark to the MCF-7 cell viability (treatments were replicate 3 times with the 48 hours incubation)

No	Concentration	Extract	
		Chloroform	Methanol
1.	5	83,75 ± 5,736	88,33 ± 2,033
2.	10	60,94 ± 8,196	69,48 ± 4,467
3.	20	16,46 ± 2,080	82,08 ± 1,603
4.	40	0,04 ± 0,954	95,73 ± 5,469
5.	80	0,937 ± 0,826	74,27 ± 5,513
6.	160	0,43 ± 1,126	74,27 ± 5,513
IC ₅₀		9,18 mg/ml	196,73 mg/ml

Linier regression between concentration versus viability in percent gave the IC₅₀ value of 9,14 mg/ml. The value showed that chloroform extract of massoia bark has a very potent cytotoxic activity against MCF-7. The treatment of chloroform extract of massoia bark in low dose has showed cytotoxic activities. On the other hand, the treatment of methanolic extract of massoia bark on MCF-7 cells resulted the medium citotoxicity with IC₅₀ value of 196,73 mg/ml but the treatment of methanolic extract yielded non dose dependent response as seen in Figure 1.

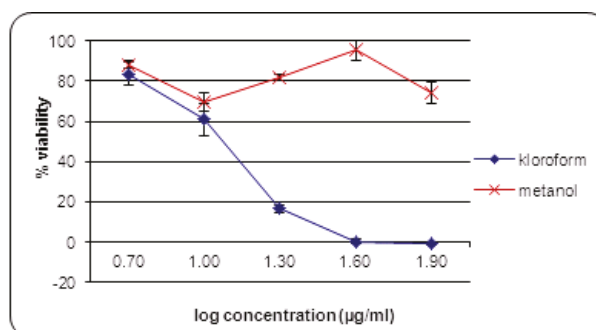


Figure 1. Chloroform and methanolic extract effects on MCF-7 cell viability.

Furthermore, the influence of chloroform and methanolic extract of the massoia stem bark on the morphological changes of MCF-7 cells can be seen in Figure 2.

Figure 2 shows that the changes in cell morphology seen in MCF-7 chloroform extract of the massoia stem bark which started at a dose of 10 mg/ml. MCF-7 cells have been shown to change shape into smaller, more rounded and follows by occurring of cell death. However, the methanol extract at the same dose have not showed any morphological changes that led to the death of MCF-7 cells. The effects of chloroform extract of massoia bark on MCF-7 cell death may associate with the appoptosis mechanism. Lactone

and other derivatives are more soluble in nonpolar solvent, that's why the chloroform extract of massoia bark have more cytotoxic effect on MCF-7 compare to methanolic extract.

Effect of chloroform extract of massoia on the expression of Bcl-2

Anti-apoptosis protein Bcl-2 is expressed at high levels by MCF-7 cells, therefore the response of MCF-

7 cell against chemotherapeutic agents is very weak. Under these conditions, exploring the mechanisms of chloroform extract of the massoia bark induces apoptosis in MCF-7 cells are directed at inhibiting the expression of Bcl-2.

Immunocytochemistry using antibody staining of Bcl-2 in MCF-7 cells treated by chloroform extract of massoia bark and control (untreated) can be seen in Figure 3.

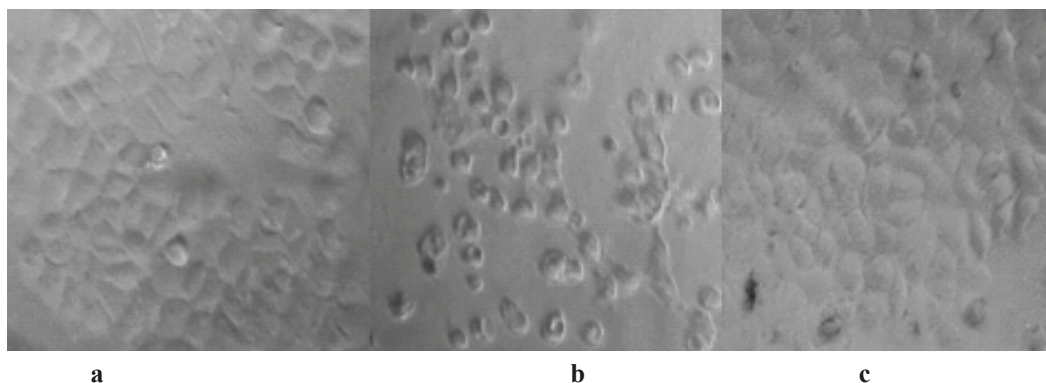


Figure 2. MCF-7 cells after incubated with 10µg/ml massoia chloroform extract (b) and massoia methanol extracts 20µg/ml (c) for 48 hours. Morphological changes occur in the chloroform extract treatment compared to control cells (a) control without treatment.

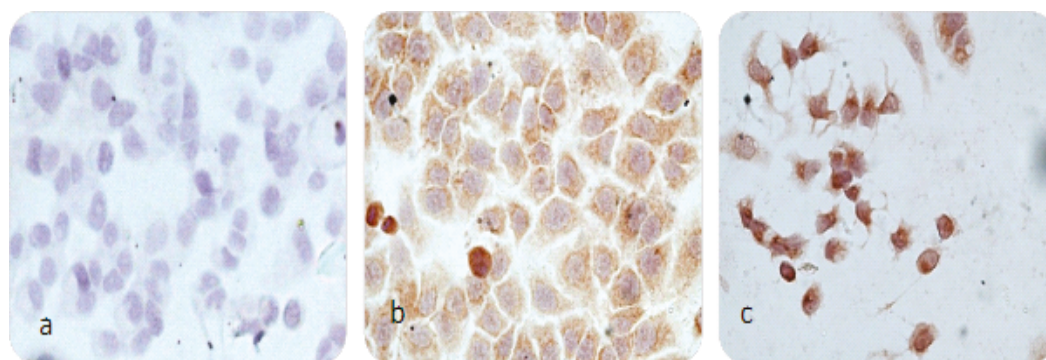


Figure 3. Immunocytochemistry of MCF-7 cells using Bcl-2 antibody. a) cells without antibody, b) control cells with antibody, c) treatment cells with chloroform extract of massoia. Magnification 400x

DISCUSSION

From that early examination for massoia bark potency as anti-cancer, it is known that the chloroform extract show more cytotoxic effect to MCF-7 viability than methanol extract. The chemical content of massoia is volatile oil known as massoia lactone which more soluble in non-polar solvent. Zhang *et al.* (2005) stated that sesquiterpene lactones (SLs) are the

active constituents of a variety of medicinal plants used in traditional medicine for the treatment of inflammatory diseases. Various SLs has attracted researchers worldwide and extensive research work has been carried out to characterize the anti-cancer activity, the molecular mechanisms, and the potential chemo preventive and chemotherapeutic application. In recent years, SLs has been assessed the experimental evidence for the anti-cancer

function obtained from both in vitro cell culture and in vivo animal models. Various SLs have been demonstrated to execute their anti-cancer capability via inhibition of inflammatory responses, prevention of metastasis and induction of apoptosis¹¹.

The present of massoia lactone in the massoia bark may be the main compound which is responsible for anticancer activity. Lactones are a large group of chemical compounds. Besides Asteraceae, this compound could also be derived from other primitive plant families such as Lauraceae and Magnoliaceae¹². Massoia belongs to the Lauraceae family which has massoia lactone as active principles. Massoia lactone is one of the SLs group of compounds which indicated the cytotoxic activity of massoia bark. From the previous research revealed that SLs contain in the root of *Lindera strychnifolia* (Lauraceae) has cytotoxic activity against SBC-3 (human small lung cancer cell lines) by using MTT assay¹³. Bcl-2 expression was observed with immunocytochemistry method using Bcl-2 antibody. Immunocytochemistry is a method commonly used in biomedical research to identify other proteins or macromolecules in a tissue or cell¹⁴. MCF-7 cells are one of the models of breast cancer cells that are widely used in research in vitro. MCF-7 cells have characteristics such as having a weak response to several chemotherapeutic agents, expressing estrogen receptors (ER +), Bcl-2 overexpression and not expressing caspase-3 due to deletion in the caspase gene 3. Bcl-2 and caspase 3 are proteins important regulators in apoptosis events; so the MCF-7 cell is a very interesting cell type used as an in vitro model to study the mechanism of chemoresistance associated with apoptosis¹⁵. Antiapoptosis Bcl-2 proteins in mammalian cells localized in the endoplasmic reticulum, the outer membrane of the cell nucleus and in the outer mitochondrial membrane¹⁶.

In the untreated control cells showed a fairly intense brown color in the cytoplasm and the membrane of the cell nucleus showed expression of Bcl-2 with a fairly high level. Control cells also showed a fairly good contrast between the cytoplasm and the cell nucleus. In contrast to cells treated with chloroform extract of massoia, counterstain with HE (Haematoxylin & Eosin) showed no cell nuclei stained blue. Possibility telophase cells at the stage of formation of the nucleus, but there is a failure or damage to daughter cells and the parent cell nucleus. This is confirmed by the occurrence of cell size reduction and the majority of cells are seen in the meiotic phase. Intensity of the brown color in the cytoplasm was more intense than the control cells,

so that the cytotoxic mechanism of chloroform extracts of massoia is not through suppression of Bcl-2 expression. Cytotoxic mechanism was likely through inhibition of the cell cycle phase of meiosis. Functionally, the Bcl-2-related proteins either inhibit or promote apoptosis, and interaction(s) between proteins belonging to opposing factions determines whether a cell lives or dies¹⁷. Kroemer (1997) stated that the family of Bcl-2-related proteins constitutes one of the most biologically relevant classes of apoptosis regulators which act at the effect stage of apoptosis and others as promoters of cell death¹⁸.

In most cases Bcl-2 overexpression related to cell survival. Presently, there are increasing reports indicating that Bcl-2 overexpression is associated with a better prognosis in many solid tumors¹⁹. So, it is interesting to further investigate the mechanism of action of cytotoxic to prove the existence of cell cycle arrest.

In conclusion, Chloroform extract of massoia has the most effective cytotoxic activities with the IC₅₀ values of 9,14 µg/ml while the methanolic extract showed medium cytotoxic activity with the IC₅₀ values of 196,1 µg/ml. The expression of Bcl-2 was the same level in treated and untreated cells. Chloroform extract of massoia bark affects MCF-7 cell viability by inducing apoptosis, without any changes in the expression level of Bcl-2.

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