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Phytochemical and Cytotoxic Evaluation of Krangean Fruits Extracts Against HeLa, MCF-7, and HepG2 Cancer Cell Line

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Cytotoxic; HeLa; Litsea cubeba (Lour.) Pers.; MCF-7; MTT assay

Abstract

Krangean [*Litsea cubeba* (Lour.) Pers.] is one of ancient aromatic plants in Indonesia which is used as traditional medicines such as for carminative, stimulant, stomach ache and expectorant. Otherwise, the anticancer activity of this plant has not been explored extensively. This research aimed to investigate phytochemical content and cytotoxic activity of krangean fruits extract on human cancer cell line *in vitro*. The research was an *in vitro* experimental design and the cytotoxic activity was carried out with MMT assay. The phytochemical compounds were characterized by TLC (Thin Layer Chromatography). MTT assay was done to observe morphology and viability of HeLa cervical cancer, MCF-7 breast cancer, and HepG2 liver cancer cell line. The results showed that TLC characterization of chloroform and methanolic extracts of *Litsea cubeba* revealed similar profile, with the major compound found were terpenoid and alkaloid. The MTT assay found that both extracts had strong inhibition on HeLa cell line. Chloroform extract exhibited stronger cytotoxic activities compared to methanol, with the IC_{50} values of 37.3 and 64.7 µg/mL respectively. While, the both extract have moderate cytotoxic activities to HepG2 and MCF-7 cancer cell line indicated by IC_{50} value more than 100 mg/mL. The benefit of this study is to provide the scientific information regarding the potency of krangean fruit as herbal natural medicine for cervical cancer therapy.

How to Cite

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INTRODUCTION

Litsea cubeba (Lour.) Pers. is medicinal plant belongs to Lauraceae family which known for its essensial oil content. This plant have several local name such as ki lemo or krangean (Sylviani & YS, 2010). The genus Litsea (Lauraceae) is composed of more than 622 species, distributed mainly in tropical and subtropical area from Australia, New Zealand, North America, South America, to Asia (Agrawal et al., 2011). China alone has 74 species (Li et al., 2008), among of this was *Litsea cubeba* (Lour.) Pers.

Litsea cubeba is trees or shrubs, evergreen or deciduous. The leaves are alternate, rarely opposite or verticillate, pinninerved, umbels, or umbellate cymes or panicles, and persistent at flowering. Leaves have pungent smell when squeezed. Flowers are unisexual, fruits are seated on perianth tube; berry, round, green when young and change to black when ripe, diameter of ± 5-6 mm (Bhuinya et al., 2010). It is native to China, Indonesia and some other parts of Southeast Asia, where it occurs mainly in mountainous regions. In the People's Republic of China, it occurs naturally in the south of the country but it has been successfully domesticated. Large cultivation area are found in central and eastern China, in south of the Yangtze River (Suwandhi et al., 2014). In Indonesia, the species grows wild in Java, Sumatra and Kalimantan from 700 m to 2,300 m above sea level (Heyne, 1987).

Like other plants of the genus Litsea, *L. cubeba* produces an essential oil (EOLC), which can be extracted from different parts of the plant, including the fruit, root and flower as well as stem and leaf, with significant diversity in composition and yield. The dried fruit were used for several medicinal properties such as for carminative (relieves flatulence), diuretic (aids urine passage), expectorant (aids secretion of sputum), stimulant, stomach ache, antiasthmatic, sedative, antidysentric, and antiseptic (Kamle et al., 2019; Zhao et al., 2010). *L. cubeba* oil is a flowing, pale yellow liquid, with an intensely lemonlike, and spicy aroma (Luo et al., 2004). It has been widely used in the food, chemical and medicinal industries (Si et al., 2012) and has been used as a crude material for the manufacture of citral, vitamins A, E, and K, ionone, methyl ionone, and perfumes (Jiang et al., 2009).

Extracts of *L. cubeba* have also been used in traditional Chinese medicine for the treatment of a variety of ailments (Mao et al., 2000). Recently, reports have demonstrated the bioactivities of *L. cubeba* essensial oils, which include antibacterial (Wang & Liu, 2010), antifungal (Luo et al., 2004; Yang et al., 2010), acaricidal (Pumnuan et al., 2010), insecticidal (Amer & Mehlhorn, 2006), insecticidal (Amer & Mehlhorn, 2006; Noosidum et al., 2008), antioxidant (Hwang et al., 2005), and anticancer properties (Chen-Lung et al., 2010).

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 assosiated with other unbearable side effects coupled with their high cost which make this theraphy out of reach to most low income people (Ayinde et al., 2011). For these reasons, research related to the exploration of 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 effect. This study aimed to evaluate the cytotoxic activity of chloroform and methanolic extract of *Litsea cubeba* fruit on MCF-7, HeLa, and HepG2 cell lines. Furthermore, the results of this study were expected to provide the scientific information dealing with the potency of Indonesian medicinal plant as a new source for anticancer medicine.

METHODS

Preparation of sample *Litsea cubeba* fruits

The fruits of *L. cubeba* were collected from Tlogodlingo Research Station with the altitute of 1,768 m above sea level. Furthermore, 100 g of dried 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 krangean fruit was evaporated to dryness in a rotary evaporator at 50°C.

Qualitative phytochemical analysis

The chemical compounds of chloroform and methanolic extract were identified qualitatively with the following procedures (Thilagavathi et al., 2015). Test for alkaloid was carried out by mix the crude extract with 2 ml of 1% HCl and heated gently. Then 1 mL of Dragendorff's reagent was added. The appearance of orange to red precipitate indicated the presence of alkaloid. Flavonoid test was done with shinoda test, crude extract was mixed with few fragments of magnesium ribbon and concentrated HCl was added drop wisely. Pink scarlet colour appeared after few minutes which indicated the presence of flavonoids. Finally, the terpenoid test was carried out by dissolving the crude extract in 2 ml

of chloroform and evaporated to dryness. Then added 2 ml of concentrated H2SO4 and heated for about 2 minutes. A grayish colour indicated the presence of terpenoids.

Based on the previous result of chemical compounds, furthermore both extract and essensial oil were identified its chemical profile with thin layer chromatography analysis. The Thin Layer Chromatography were carried out with specific development and stationaire phase elution and the chromatogram were visualized with UV in 254 and 366 nm wave lenght, as well as vanilline acetic acid to perform the spot.

Cytotoxicity assay

The assay was performed at the Integrated Laboratory of Medicinal Plant and Traditional Medicine Research and Development Centre (MPTMRDC). The MCF-7 obtained from the ATCC, Manassas, VA, USA and cytotoxicity tests were carried out using the MTT assay. MCF-7 cancer cell line were maintained in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100U/ml penicillin and 100 µg/ml streptomycin) and cultured at 37°C in humidified atmosphere containing 5% CO_2. The cells were seeded at a density of 8 x 10^5 cells in 96-well plates with MEM 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 for 48 hours of exposure time. After 48 hours, 100 µl of a 1 mg/ml of solution of MTT in MEM was added to each well. The culture plates were incubated for 4 hours at 37°C in humidified atmosphere containing 5% CO_2. MTT was removed carefully and then stop solution (HCL in isopropanol) 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 (ELISA Reader, Biorad) and percentage of growth inhibition was calculated using the following standard formula.

IC_{50} was defined as the concentration of the plant extracts killing 50% of the cells. IC_{50} was determined for MCF-7 cell lines of both extracts (Anonim, 2009).

RESULTS AND DISCUSSION

Chemical compounds

The extraction of krangean fruit was performed by maceration with chloroform and methanol solvent that yielded the solid brown extract. The analysis of chemical compounds of krangean fruit were carried out for chloroform and methanolic extract that is presented in Table 1.

Table 1. Phytochemical evaluation of *Litsea cubeba* fruits

Chemical group content	Extracts	
	Chloroform	Methanol
Alkaloid	+	+
Flavonoid	-	-
Terpenoid	+	+

Note: (+) = detected; (-) = undetected

Qualitative determination of chemical group compound of krangean fruits extract showed that chloroform and methanolic extract contained the same compound i.e. alkaloid, and terpenoid, and both extract did not contain flavonoid. The differences of the polarity of solvent do not seem to affect the cytotoxic effect on HeLa, MCF7 and HepG2 cells line. Alkaloid and terpenoid compound are the chemical compound groups which responsible to cytotoxic activities, cell growth and to induce apoptosis in various cells line of cancer (Patel et al., 2011). The previous research has been shown that the main compound in the fruit oil of *Litsea cubeba* is citral and the fruit oil of *Litsea cubeba* exhibited cytotoxic activity against human lung, liver and oral cancer cells (Chen-Lung et al., 2010).

The chromatography profile of chloroform and methanolic extract of krangean fruit is presented in Figure 1.

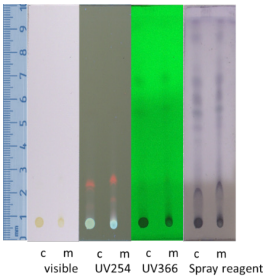

Figure 1. Chromatogram of chloroform and methanolic extract of krangean fruit, developed in toluene:ethyl acetate (93:7), visualized by UV at 254 and 366 nm, and vaniline acetic acid as spray reagen (c=chloroform; m=methanol).

Figure 1 showed the chromatography profile of chloroform and methanolic extract of *Litsea cubeba* which have similar profile of the number and color of the spot. The red spot visualized by 254 and 366 nm of UV wave length indicated of the presence of terpenoid compound within the two extract. Furthermore, to examine

qualitatively of the present of flavonoid and alkaloid compound, the spot profile of chloroform and methanolic extract of krangean fruit were observed with TLC method. The result showed the absence of blue/green/yellow spot on the chromatogran profiles that indicated the absence of flavonoid compound, however, the presence of blue spot on the chromatogram indicated the presence of alkaloid.

Litsea cubeba (Lour.) Pers. (Lauraceae) is one of the oldest herbs known with its pleasant aroma, which is distributed in southern China, Japan and Southeast Asian. Futhermore, the *Litsea cubeba* fruits are used in pharmacy, perfumery and food. The dried fruits of *L. cubeba* are used in traditional Chinese medicine and other folk medicines, since it is considered a carminative, diuretic, expectorant, stimulant, stomachic, anti-asthmatic, sedative, anti-dysenteric and antiseptic (T Bhuinya et al., 2010). Essential oil containing by *Litsea cubeba* fruits are demonstrated to have antioxidant and cytotoxic activity as well as being anticancer properties (Wang et al., 2012).

Inhibitory effect of cloroform and methanolic extract of *L. cubeba* fruit on MCF-7, HeLa, and HepG2 cells growth

To determine the potency of chloroform and methanolic extract of *L. cubeba* fruit as co-chemopreventive agents, the cytotoxicity properties were examined in MCF-7, HeLa and HepG2 cells line. Cell viability was examined using MTT assay method with 48 hours of incubation. The treatment of chlorofom and methanolic extract of *L. cubeba* fruit on all cells line resulted the decreasing number of viable cell in dose dependent manner.

Krangean fruit extract againts HeLa cancer cell line

The cytotoxic evaluation of chloroform and methanolic extract of Litsea fruits to HeLa cancer cell lines showed that chloroform extract have stronger activity compared to methanolic extract by dose dependent manner as presented on Figure 2.

Based on the morphological examination of HeLa cancer cell line treated by chloroform and methanolic extract of *Litsea cubeba*, it was obviously shown the change in shape and size of HeLa cells compared with controls as shown in Figure 3. Increasing the concentration of extract will lead to the increasing of the number of cell apoptosis. Research with the same method has also been done by Hikam that could prove that chloroform extract of *Auricularia auricular* effect strongly on the HeLa cancer cell line viability by dose dependent manner (Hikam et al., 2019).

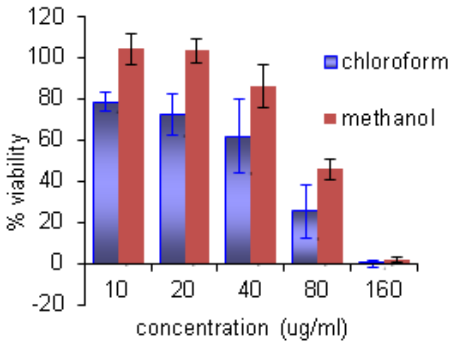

Figure 2. Cell viability affected by chloroform and methanolic extract of krangean fruits on HeLa cancer cell line in dose dependent manner. Test were carried out by incubating $5x10^3$ HeLa cells with chloroform and methanolic extract of krangean fruits (0-200 µg/ml) for 48 hours.

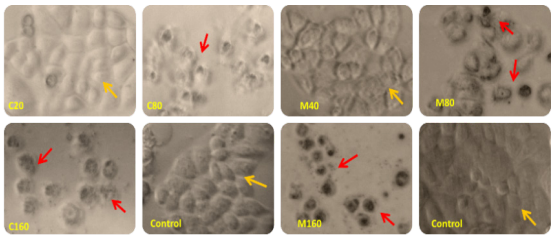

Figure 3. Morphological change of HeLa cells seen at concentration of 40, 80 and 160 µg/ml for both chloroform (C) and methanolic (M) extract, compared to control cells. Magnification at 400x.

The treatment of chloroform and methanolic extract of krangean fruits on HeLa cells growth resulted in the decreasing number of viable cell in dose dependent manner as showed in Figure 2. Linier regression between concentration of chloroform and methanolic extract of krangean fruits versus viability in percent gave the IC_{50} value of 37.3 µg/ml and 64.7 µg/ml respectively. The value showed that chloroform and methanolic extract of krangean fruit has a very potent cytotoxic activity against HeLa. The treatment of chloroform and methanolixc extract of krangean fruit in low dose has showed cytotoxic activities.

At the concentration of 40 mg/ml, the HeLa cells line began to shrink, and at the concentration of 80 and 160 mg/ml, cell became dead. This result was in line with the previous research that have found the new diterpene compound isolated from the methanolic extract of *Litsea cubeba* fruit named cubelin. This compound exhibited activity againts HeLa cell viability and proliferation (Trisonthi et al., 2014). Based on

the morphological examination of HeLa cancer cell line treated by chloroform and methanolic extract of *Litsea cubeba*, it obviously showed the change in shape and size of HeLa cells compared with controls. Increasing the concentration of extract will lead to the increasing of the number of cell apoptosis.

Krangean fruit extract against MCF7 cancer cell line

The cytotoxic evaluation of chloroform and methanolic extract of Litsea fruits to MCF-7 cancer cell lines showed that chloroform and methanolic extract have moderate activity as proved by regression analysis that have IC_{50} value of more than 100 mg/mL. The effect of chloroform and methanolic extract of Litsea fruit on MCF-7 cell viability is showed on Figure 4.

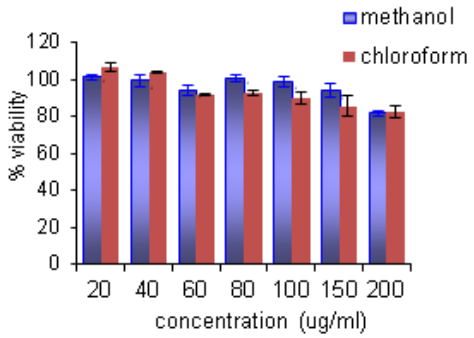

Figure 4. Cell viability affected by chloroform and methanol extract of krangean fruits on MCF-7 cancer cell line. Test weres carried out by incubating 5x10³ MCF7 cells with chloroform and methanolic extract of krangean fruits (0-200 µg/ml) for 48 hours.

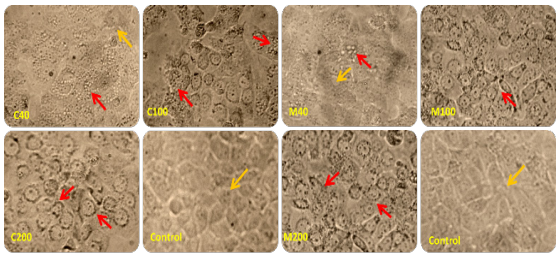

Figure 5. Morphological change of MCF7 cells seen at concentration of 40, 80 and 160 mg/ml for both chloroform (C) and methanolic (M) extract, compare to control cells (CS). Magnification at 400x.

The treatment of chloroform and methanolic extract of Litsea fruit to MCF-7 resulted in moderate cytotoxic activity since the IC_{50} value was more than 100 mg/mL. The treatment of chloroform and methanolic extract of krangean fruits on MCF-7 cells showed the moderate cytotoxic activity with IC_{50} value of more than 100 mg/ml, but the treatment of both extract yielded dose dependent response as seen in Figure 4. In contrast, Figure 5 clearly shows that morphological characters of MCF-7 cancer cell line were affected by chloroform and methanolic extract of Litsea fruit. MCF-7 is one of breast cancer cell line which already resistant to some cancer drug. Therefore, finding the novel and effective phytochemical against MCF-7 cancer cell is an important challenge. Previous study revealed that methanolic extract of *Litsea cubeba* bark has anti-inflammatory activity (Choi & Hwang, 2004). The anti-inflammatory properties may be initiated by the chemicals compound contained in the methanolic extract of *Litsea cubeba* bark. It was recognized that infections and inflammation are related to cancer, and it has correlations between the presence of inflammation and the development of pre-cancerous lesions. The effect of anti-inflammation of *Litsea cubeba* bark was promising to be further investigated for its potency as anti-cancer agent.

Krangean fruit extract againts HepG2 cancer cell line

The cytotoxic evaluation of chloroform and methanolic extract of Litsea fruits to HepG2 cancer cell lines showed that chloroform and methanolic extract have moderate activity as proved by regression analysis that have IC_{50} of more than 100 mg/mL. The effect of methanol and chloroform extract of Litsea fruit on HepG2 cell viability is shown on Figure 6 and 7.

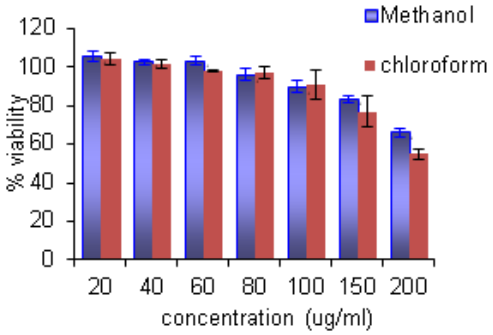

Figure 6. Cell viability affected by chloroform and methanolic extract of krangean fruits on viability of HepG2 cancer cell line in dose dependent manner. Test were carried out by incubating 5x10³ HepG2 cells with chloroform and methanolic of krangean fruits (0-200 µg/ml) for 48 hours.

Chloroform and methanolic extract against HepG2 cancer cell lines have been shown

in Figure 6 and 7. Morphological evaluation of HepG2 cancer cell lines showed that chloroform and methanolic extract of Litsea fruit cause the morphological change and tend to undergo apoptosis by dose dependent manner.

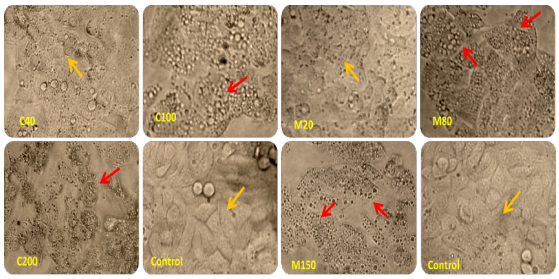

Figure 7. Morphological change of HepG2 cells be seen at concentration of 40, 80 and 160 mg/ml for both chloroform (C) and methanolic (M) extract, compared to control cells (CS).Magnification at 400×.

The treatment of chloroform and methanolic extract of krangean fruits on HepG2 cells resulted in the moderate cytotoxicity with IC_{50} value of more than 100 µg/ml but the treatment of both extract yielded dose dependent response as seen in Figure 6. An extract derived from natural product is considered potential to be developed as an anticancer therapy if it has IC_{50} less than 100 µg/ml (Mans et al., 2000). Figure 4 and 6 showed that the higher the concentration of chloroform and methanolic extract of krangean fruits resulted in greater numbers of cell undergo morphological change and death.

The characteristic features observed in MCF-7 and HepG2 cells with the treatment of both extract, were the presence of numerous cytoplasmic vacuoles with the cells that still reacted with MTT to form formazan (Figure 5 and 7). The previous study revealed that a new diterpene isolated from the fruit of *Litsea cubeba* induced apoptosis on HeLa cancer cell lines (Trisonthi et al., 2014). This study indicated that the chloroform and methanolic extract of *Litsea cubeba* fruit which containts of terpenoid compound also effect to MCF-7 viabitily. Cells can recover from vacuolation or undergo cell death with apoptosis mechanism. Unfortunatelly, in this research, cells were not tested longer to ensure the final destiny, dead or survive.

Since the information regarding the anticancer activity of krangean fruit is still limited, the result of this research could provide scientific information about the potential of Indonesian medicinal plant as chemopreventive agent. This research will contribute to the development of natural herbal medicine especially for cervical cancer therapy since the krangean fruit extract has strong cytotoxic activity against HeLa cancer cell lines.

CONCLUSION

Chloroform and methanolic extract of krangean fruit contain terpenoid and alkaloid compound but did not contain flavonoid. Chloroform and methanolic extract of krangean fruits have strongest cytotoxic activity against HeLa cell line with the IC_{50} value of 37.3 µg/ml and 64.7 µg/ml. Both extract have moderate cytotoxic activity to MCF-7 and HepG2 cancer cell line with the IC_{50} value of more than 100 µg/ml.

This research could be continued to evaluate the cytotoxic activity of Krangean fruit extracts through molecular anticancer activity studies, whether the extract modulates the cell cycle or induces apoptosis on HeLa cancer cell lines.

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