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Original Article

CYTOTOXIC EFFECT FROM ETHYL ACETATE-METHANOL SUBFRACTION OF *CARRISA CARANDAS* L TOWARD HELA CELLS BY *IN VITRO* TEST

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ABSTRACT

Objective: Cervical cancer is a malignant type of cancer, often affects women, particularly in developing countries. *Carrisa carandas* leaves contained many secondary metabolites that had potency as an anticancer. The purpose of this study was to understand the cytotoxic effect of a subfraction of *Carrisa carandas* leaves against HeLa cells.

Methods: Chloroform fraction was separated by VLC gradually with n-hexane–chloroform–ethyl acetate and methanol. The same profiles from eluent chloroform–ethyl acetate composed fraction 18-26 were categorised as Fr4 and ethyl acetate-methanol composed fraction 27-30 as Fr5. The cytotoxic effect was evaluated by MTT assay on HeLa cells

Results: The result showed that the cytotoxic effect of subfraction Fr4 and Fr5 had IC_{50} values of 177 mg/ml and 98 mg/ml, respectively. Colourless crystal of Subfraction Fr 5-3 had IC_{50} value of 333 mg/ml. Subfraction Fr 5 showed effective cytotoxic activity than the others. Conclusion: It had chemo-preventive effect against cancer cells

Conclusion: This study applied MTT (Microculture Tetrazolium) method by *in vitro* test. The advantages of this method are relatively rapid, sensitive and accurate

Keywords: Carandas leaves (*Carissa carandas* L), Cytotoxic, MTT assay,sub Fraction

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INTRODUCTION

Cancer is being the main health problem in the world and the biggest killer disease after cardiovascular. One of the most often cancers found in women is cervical cancer. It is the malignant type of cancer especially in developing countries [1]. The number of the sufferer is around 40 people per 100 thousand. In Indonesia, it is approximately between 25 and 40 in every 100 thousand people per year [2]. Carissa has been widely used for traditional medicine specifically as an anticancer. Carissa carandas is shrub plant of family Apocynaceae. Chemical compounds in this plant were alkaloids, anthraquinone, terpenoids, and flavonoid. Non polar compound found in leaves were triterpene carandinol, betulinic acid, β-cytosterol-3-O-β-d-glucopyranoside, ursolic acid, ursuline acid, and hydroxybenzoate acid [3]. Activities of Carissa fraction reported are antipyretic, anthelmintic, antitumor, insecticide, antioxidant, and antimicrobial [4]. Other reports, water extract of Carissa's leaves had activities as antioxidant and anticancer [5] while chloroform extract had activities as anti-cancer for Hela cell in ovarium with EC_{50} of 7.702 µg/ml [6]. This study aimed to determine the cytotoxic activity of subfraction of *Carrisa carandas* leaves against HeLa cells by *in vitro* test with MTT method (Microculture Tetrazolium Salt) which was indicated by the IC_{50} value.

MATERIALS AND METHODS

Materials

All chemical reagents and solvents were purchased from Bratachem and Sigma Aldrich. *Carissa carandas* was obtained from Solo, Central Java, particularly leaves of Carissa as the main material. The research was conducted at Pharmaceutical Biology Laboratorium of Universitas Sebelas Maret.

Procedure of fractionation

Methanolic extracts were prepared by maceration carried out for seven days with methanol. Obtained extract was then partitioned with 75 ml of distilled water and 75 ml chloroform (1: 1) three times. The chloroform phase was separated using vacuum coloumn chromatography. Silica gel 60 was used for the stationary phase. Thirty fractions were gained from the separation. Fractions that had the same amount of chromatogram and Rf value were compiled and was acquired 5 compiled fractions. The same profiles from chloroform-ethyl acetate eluent were composed as fraction 18-26 and were categorized as Fr4. Besides, ethyl acetate-methanol eluent was composed as fraction 27-30 and was categorized as Fr5.

Cytotoxic activity test

Approximately 10 mg of sample was diluted into 50 ml of DMSO (dimethyl sulfoxide) in Eppendorf as test agent and stock solution. To make series of solutions for the treatment levels was conducted aseptically in the LAF (Laminar Air Flow) cabinet. Each of stock solutions was taken 10 µl then added into 90 µl of culture medium. Afterwards, it was made for some various concentrations for each test. Each subsequent concentration was taken about 100 µl into each of hole/well with three times repetition for each concentration.

Cytotoxic MTT test (Microculture tetrazolium salt)

HeLa cells were suspended at a density of 3 x 104 cells/hole. About 100 µl of the cell was inserted in microplate 96 holes and incubated in 5% of CO_2 condition at 37 °C for 24 h. The holes contained cell suspension was added into 100 µl of test solution in each hole in order to obtain particular various concentration (100; 50; 25; 12.5; 6.25) µg/ml per hole. Control medium was the cell without an additional solution. At the end of incubation process, the medium was removed and washed using FBS (Fetal Bovine Serum) then was added by 100 µl of new media and 10 µl MTT 5 mg/ml in FBS. Microplates were incubated again for 4 h at 5% CO_2 at 37 °C. Living cell would react with MTT to form purple formazan. To stop the

reaction between cells and MTT and to dissolve formazan, it was added by 100 ml of SDS (Sodium Dodecyl Sulphate) 10% in 0.01 N HCl, incubated for 24 h at room temperature. Finally, absorbance was observed using ELISA reader at 595 nm.

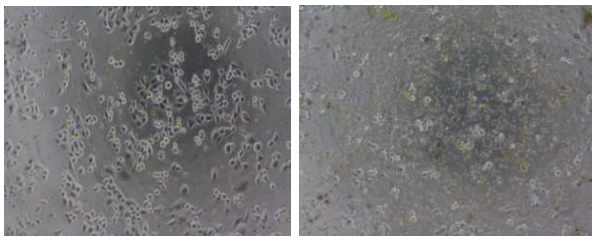
Analysis

The percentage of living cell was determined by this equation: IC_{50} is value that induces 50 % live Hela cell and is measured by linier regression with probity percentage as ordinat and log value as absis [7]

$$\% \text{ live cells} = \frac{\text{treated absorbance} - \text{control media absorbance}}{\text{control absorbance} - \text{control media absorbance}} x100\%$$

RESULTS

Cytotoxic test of Fr4 and Fr6 from Carissa's leaves toward Hela cells culture can be seen by morphological observation of cells themselves [8] The differences between living cells and dead cells will be clearly visible after exposuring MTT salt. This was caused the living cells can reduce MTT to become formazan purplish crystal. Living cells morphology was round, clear and vivid slightly. The dead cells would be muddy because they were lost of cytoplasm as a consequent of broken cells membrane. This presented that subfraction of Carissa's leaves could inhibit Hela cells proliferation



A B

Fig. 1: The morphology of Hela cells of Fr5 (500 µg/ml) from Carissa's leaves a) before MTT administering; b) after administering

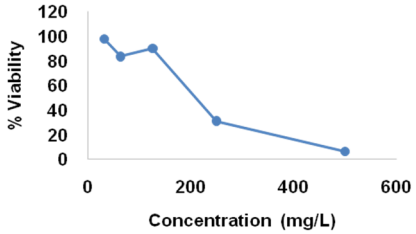


Fig. 2: Cytotoxic activity of subfraction from Fr 4 of leaves *Carissa carandas* in HeLa cell lines

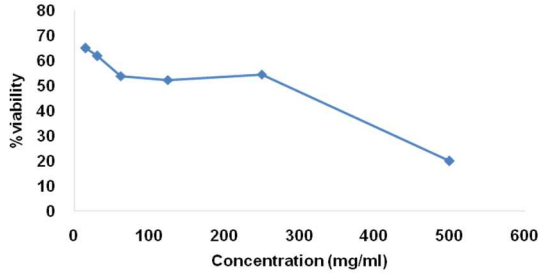


Fig. 3: Cytotoxic activity of subfraction Fr 5 of leaves *C. carandas* in HeLa cell lines

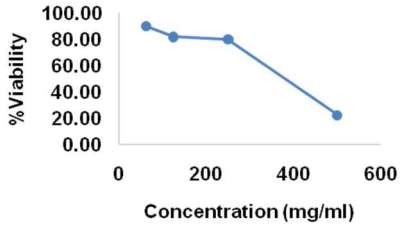


Fig. 4: Cytotoxic activity of subfraction from Fr 5-3 of leaves *C. carandas* in HeLa cell lines

Table 1: IC50 value of sub fraction from Fr 4, Fr 5 and Fr 5-3 of C. carandas in HeLa cell lines

Subfraction	Linear regression	IC_{50}
Fr 4	y =-23.476x+96.881	177
Fr 5	y =-77.832x+225.07	98
Fr 5-3	y =-68.054x+221.7	333
FR 5-4	y=-10.814x+105.02	>333

Identification and spectra analysis of subfraction Fr5-3 and Fr5-4

Fr5-3 was the unpured compound resulted from recrystallization process using chloroform of subfraction 5. Identification using KLT with a mobile phase of chloroform: methanol (9:0.5) with Liebermann Burchard showed dark purplish in RF 0.7. According to FTIR spectra, the compound consisted of the hydroxyl group at 3728 cm^{-1}, methylene group at 823 cm^{-1} and carbonyl group at 1017 cm^{-1}. Strong intensity peak at less 1000 cm^{-1} was probably bonding between carbon and halogen element. Fr5-4 was white crystal. According to FTIR spectra of Fr5-4, their C-O carbonyl presents at 1650 cm^{-1} and also stretching OH from alcohol group at 3450 cm^{-1}, separately with CH (sp3) at 2960 cm^{-1}. Alcohol group is supported by carbonyl group at 1100 cm^{-1}. Another sharp peak presents at 2500-2000 cm^{-1} that indicates CN group amplified with twin peaks at 3700 cm^{-1}

Obtained compound was analysis using LC-MS Spectro-photometer via ESI (Electron Spray Ionization). The chromatogram shows a single peak at time retention of 2.67 min that indicated the main compound. Other peaks that appeared were pointed out the contaminants. Continued with MS spectrophotometer, MS spectra showed base peak m/z with value of 361.19. It was the most stable ion that indicated the weight molecule. The fragmentation patterns were still complicated because there were only methyl, ethyl, and hydroxyls group that released as the fragments.

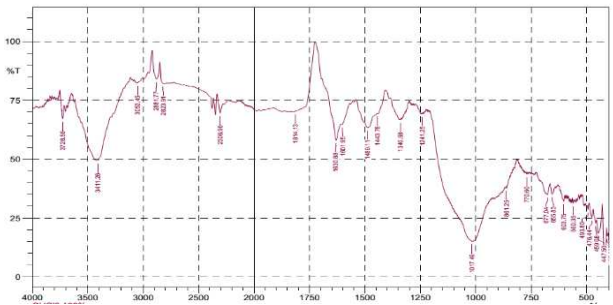


Fig. 5: IR spectrum of subfraction Fr5-3

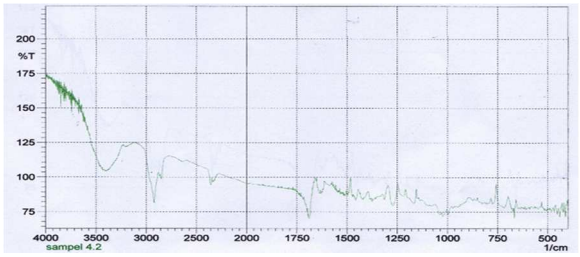


Fig. 6: IR spectrum of subfraction Fr5-4

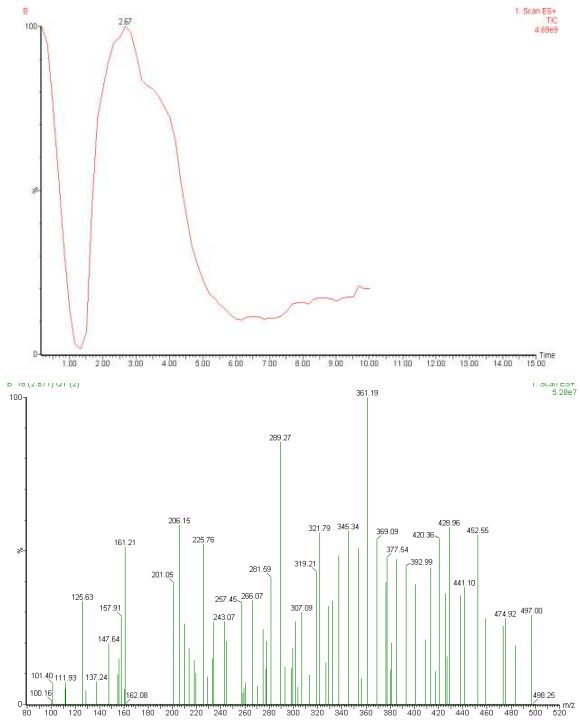


Fig. 7: Chromatogram and mass spectrum of subfraction Fr5-4

DISCUSSION

This study applied MTT (Microculture Tetrazolium) method by *in vitro* test. The advantages of this method are relatively rapid, sensitive and accurate [9]. MTT is a colorimetric method which MTT reagent (Tetrazolium 3-(4,5-Dimethylthiazol2-yl)-2,5 Diphenyl-tetrazolium Bromide salt) can be disparted into formazan salt by succinate tetrazolium in respiration track of cell (in mitochondria) in any living cell. Formazan salt is soluble salt at SDS (Sodium Dodecyl Sulphate) 10% in 0,1 N HCl but not soluble in water. It is a purple salt [10]. Viability cells method is one of anticancer activity test methods regarding to the ability of cells to survive after administering toxic compound. Calculation of viability percentage can find the number of living cells after administering. From the calculation, it can be seen about reducing of viability percentage from the lower concentration to higher concentration. The higher concentration of the sample, the lower viability percentage obtained. IC50 for the cytotoxic test can be called as very active if the value is<10 µg/ml, active if the value between 10 and 20 µg/ml and less active if more than 20 µg/ml. But if the value of IC50 is<100 µg/ml, it can still be called as a chemopreventive agent against cancer cells[11]. Chemopreventive is retardment of cancer cells growth which the retarder is given at before or simultaneously with induction agent. Chemopreventice can use natural pharmacological agents that typically inhibit cancer cells growth. The agents have to comply standard of the security levels and efficacy for therapeutic [12]. Based on The American National Cancer Institute, an extract that can be called having cytotoxic activity if the value of IC50 is<20 µg/ml [13].

IC50 value of Fr5 is bigger than Fr4. Carrisa carandas contained carandinol that had cytotoxic activity toward Hela cells [6]. The result of phytochemical screening of Fr4 was not found out any terpenoid compound. Triterpene can induce calcium (Ca^{2+}) which can stimulate cell death in tumor cells or cancer by blocking the cell cycle at the G2/M phase with stabilizing threads Spindle in the phase of mitosis so that the process of mitosis can be inhibited [14]. One class of flavonoids that alleged role in the cytotoxic effect was flavon or flavonol which can induce discontinuation phase G1 by damaging the DNA of cells [15]. The alkaloids can inhibit the enzyme activity of DNA topoisomerase II; an enzyme that plays an important role in the process of replication transcription, DNA recombination, and the proliferation of cancer cells that causes death by apoptosis [16].

CONCLUSION

Subfraction Fr 4 and 5 showed effective cytotoxic activities. Carissa carandas leaves had a cytotoxic effect on HeLa cells with IC_{50} values of 98 mg/ml and 177 mg/ml, respectively. Fr 5-3 and Fr5-4 subfraction did not have cytotoxic effect on HeLa cells with IC_{50} value of 333 mg/ml and>333 mg/ml.

CONFLICTS OF INTERESTS

Declared none

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