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CYTOTOXIC ANALYSIS OF CAPSAICINOID COMPOUND FROM ROTTEN AND FRESH *Capsicum frutescens* L. ON T47D CELLS

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Abstract

Cayenne pepper has a distinctive spicy and hot taste that indicates the presence of *capsaicinoid* compounds. The content of *capsaicinoid* compounds is thought to increase along with the level of maturity. This study aims to distinguish between the *capsaicinoid* content in Rotten *Capsicum Frutescens* (RCF) and fresh *Capsicum Frutescens* (FCF) samples and their use as anticancer agents. *Capsaicinoid* extraction was performed by using reflux extraction and the identification processes using *Thin Layer Chromatography* (TLC) with a chloroform:methanol eluent. Identification was conducted by using *Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy* (ATR-FTIR) while a cytotoxicity test on T47D breast cancer cells was conducted using the *Methylthiazol Tetrazolium* (MTT) assay. The results showed the presence of a *capsaicinoid* compound group, with Retardation Factor (Rf) values of 0.76 in RCF and 0.72 in FCF, as well as orange spots due to its reaction to Dragendorff's reagent. ATR-FTIR analysis was able to identify some of typical functional groups typical of *capsaicinoid*, including -OH, CH_3, CH_2 (*stretching*), C=O, C=C, -CH_2- (*bending*), C-N, C-O, O-CH_3, and -NH with the intensity value of RCF greater than FCF. The cytotoxicity test results showed that the Inhibition Concentration (IC_{50}) value for RCF isolates was 512.37 µg/mL, while for FCF isolates was1386.82 µg/mL. These results indicate that *capsaicinoid* isolates from RCF have a higher cytotoxicity compared to isolates from FCF. A higher IC_{50} value corresponds to lower cytotoxic activity.

INTRODUCTION

Cancer occurs when the body's cells begin to split uncontrollably, typically over an extended period. Breast cancer is the most prevalent form of cancer among women, representing approximately 25% of all cancer cases worldwide [1]. Genetics, environmental conditions, and lifestyle choices are contributing factors in the development of cancer [2].

According to the World Health Organization (WHO) [3], The International Agency for Research on Cancer (IARC) reports that ten types of cancer account for two-thirds of new global cases and are the leading cause of death, with 20 million new cases and 9.7 million deaths worldwide. Lung cancer ranks highest (12.4%), followed by breast (11.6%), colorectal (9.6%), prostate (7.3%), and stomach cancers (4.9%) [4]. T47D cells are human breast cancer cells that are positive for estrogen and progesterone receptors and derived from pleural fluid. These cells express mutant p53 protein and show high sensitivity to the stimulatory effects of estradiol [5].

Cancer-related deaths and new infection-associated cases underscore the close relationship between infection and cancer. Bacteria and viruses account for approximately 2 million new cancer cases annually [6]. Chronic infections compromise the immune system, heightening the risk of cancer. Moreover, cancer treatments such as chemotherapy, radiation, and surgery can further increase patients' susceptibility to infections [7]. Inflammation caused by chronic infections also plays a significant role in cancer development [8]. The growing issue of antibiotic resistance has emerged as a critical global health concern [9], emphasizing the need for research into non-traditional therapies. The cell membrane is one of the primary therapeutic targets [10], as anticancer activity often parallels antibiotic activity, indicating a similar mechanism of action [11]. Consequently, compounds with anticancer properties hold promise as effective therapeutic agents [12]. Therefore, further research is needed on plant-based therapies that can be used in the treatment of breast cancer with minimal side effects.

Cayenne pepper (*Capsicum frutescens* L.) is one of the plants that grows a lot in Indonesia because chilies are horticultural plants that easily grow on soils which is contain plenty of water [13].

The content in cayenne pepper (*Capsicum frutescens* L.) contains capsaicinoids, carotenoids [14], ascorbic acid, essential oils, resins, and flavonoids [15].

Secondary metabolites determine the properties of the plant, with the pungency of chili peppers attributed to capsaicinoids, especially capsaicin, and dihydrocapsaicin, which account for 80-90% of the pungency [16]. Capsaicinoids are extracted using solvents such as methanol or acetone via methods such as reflux, soxhlet, and ultrasound. The benefits of capsaicin include antioxidant activity, application in pain relief, and cancer therapy [17]. Capsaicinoids effectively inhibit the growth of T47D breast cancer cells by inducing apoptosis, increasing caspase-3/7 activity, promoting cytochrome C release, and facilitating PARP cleavage. Capsaicin also suppresses cyclin D1 release, induces cell cycle arrest at the S phase, and inhibits DNA replication. At ED50 concentrations (1×10^{-5} M to 5×10^{-5} M), capsaicin reduces cell viability and slows T47D cell proliferation, highlighting its potential as an anticancer therapeutic agent [18].

Pico Paloma (PIP) genotype contains higher levels of capsaicinoids in fully ripe fruits, suggesting that maturity significantly affects capsaicinoid accumulation. Therefore, fully ripe chili peppers have a higher level of pungency than less ripe chili peppers [19]. This research aims to compare the capsaicinoid content in fresh and rotten chilies and assess their impact on cytotoxicity against T47D cells.

EXPERIMENT

Material

Dried rotten and fresh cayenne pepper samples, methanol (p.a), chloroform (p.a), Dragendorff reagent. Materials and all treatment of cytotoxicity procedures under the guidance of The Medical and Health Research Ethics Committee (MHREC) faculty of medicine, Public Health and Nursing, Gadjah Mada University, Indonesia.

Instrument

Rotary evaporator RV 8 Pro V Complete, Attenuate Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR) Shimadzu IRSpirit-T with QATR-S, Inverted microscopy Olympus CKX41, and iMark Microplate Reader Benchmark.

Procedure

Preparation of Capsicum frutescens L. Extract

Rotten and fresh *Capsicum frutescens* L. were obtained from Jombang, East Java, Indonesia. Both types of chilies were cleaned and dried in an oven at 50°C for 24 hours [20]. Subsequently, they were ground into powder and filtered through a 90-mesh sieve. For extraction, 30 g of each chili powder (rotten and fresh) was refluxed with 480 mL of methanol for 7 hours at 60°C. The extract was concentrated using a rotary evaporator to obtain the concentrated extracts of rotten and fresh cayenne peppers.

Extraction using the Reflux Method

The 30 g of cayenne pepper simplicias powders were placed in a dark bottle, followed by the addition of 480 mL of methanol. The mixture was stirred and refluxed for 7 hours at 60°C. After the refluxing, the mixture was filtered using a Buchner funnel to separate the filtrate from the residue. The residue was washed by methanol and filtered again. The combined filtrate was then evaporated using a rotary evaporator to obtain a concentrated extract of cayenne pepper.

Isolation using Preparative Thin Layer Chromatography (TLC)

Capsaicinoid compounds were separated from the recrystallized extract by using analytical thin-layer chromatography (TLC) with silica gel 60 F254 plates (10x10 cm). The crystalline powder was dissolved in methanol, applied to the silica plate, and then eluted with a mixture of chloroform and methanol (95:5 v/v) up to 20 mL. The separation spots were observed under UV light at 366 nm and then derivatized using Dragendorff's reagent, producing an orange color. The stained areas were scraped, dissolved in chloroform, and centrifuged for 15 minutes at 3000 rpm. The supernatant was evaporated in a fume hood until the solvent was completely removed, resulting in a white dry isolate based on the RF value of the capsaicinoid compound.

FTIR Identification

The identification of capsaicin compounds using ATR-FTIR begins with preparing the required samples. Once the instrument is ready and connected to the operating application, a sample is applied to cover the sample port on the instrument.

This process is followed by the analysis of IR spectra in the range of 4000-0 cm^{-1}, with measurements taken 10 times.

Cytotoxic Analysis and Data Analysis

T47D cells were obtained from the Laboratory of Parasitology, Faculty of Medicine, Public Health, and Nursing, Gadjah Mada University, Indonesia. The cells were cultured with 10% (v/v) fetal bovine serum, 3% streptomycin-penicillin, and 1 % fungizone, then incubated in a 5% CO_2 incubator at 37°C. Cells were collected after reaching 80 % confluency using 0.25% trypsin-EDTA.

T47D cells were plated into 96-well plates and incubated in a 5% CO_2 incubator at 37°C overnight. Cells were then treated with five different concentrations of Capsaicinoid isolate (7.8125; 15.625; 31.25; 62.5; 125; 250; 500; 1000 µg/mL) for 24 hours. MTT reagent of 100 µL was added to each well. The cells were re-incubated for 4 hours until formazan crystals formed. A 0.1 N SDS-HCl stop solution was also added to evaluate the color of the medium. The plates were wrapped in aluminum foil and incubated in a dark room overnight. The color absorbance was read using an ELISA reader at λ 595 nm. IC_{50} values were determined by utilizing the percentage of viable cells was determined using the results. Regression analysis was used to determine the IC_{50}. The proportion of cell viability in the treated cells relative to the untreated control is how experimental data are displayed. A graph that plotted the percentage of viable cells against the concentration of the test sample extract was used to get the IC_{50} value. Every experiment was carried out three times. Doxorubicin served as the positive control in these tests, whereas cells that were solely exposed to the solvent served as the negative control.

RESULT AND DISCUSSION

Sample preparation

The fresh and rotten cayenne pepper (*Capsicum frutescens* L.) used in this study went through a process of sorting, washing, drying, and pollination as part of the sample preparation, resulting in cayenne pepper powder with a size of 90 mesh [20]. The drying process was conducted at a controlled temperature to prevent damage to the target compounds, while the grinding process aimed to increase the sample's surface area to optimize extraction efficiency [20].

Extraction sample of RCF and FCF

The extraction process of each sample of cayenne pepper (*Capsicum frutescens* L.) uses the reflux method with the help of water bath heat at 60ºC for 7 hours using methanol solvent. This process aims to obtain the concentrated extract compound that can later be used in the isolation process of the capsaicinoid compounds group. This process aimed to produce a concentrated extract for isolating capsaicinoid compounds. Zahra et al. (2023). evaluated variations in solvent ratios and extraction times, achieving optimal results under those parameters. However, this study utilized a temperature of 60°C to minimize the risk of capsaicinoid degradation in the cayenne pepper samples.

Isolated sample

Capsaicinoid compounds can be isolated using Thin Layer Chromatography (TLC) (**Figure 1**) with the elution process stopped once the sample reaches the upper limit line, after which the plate was marked at each spot (**Table 1**). A1 cm section was taken for the spraying process using Dragendorff reagent. The spraying process was performed due to the absence of a capsaicinoid control compound solution, producing an orange spot that confirms the presence of capsaicinoid compounds in the cayenne pepper sample. Spots that are not exposed to the spray reagent can be scraped off to isolate the capsaicinoid compounds bound to the silica gel RCF and FCF are observed at an Rf (Retardation Factor) value of 0.76 [21], marked by orange spots visible under a UV lamp at 366 nm.

Table 1. Spot TLC of RCF and FCF.

Spot	Rf value	Spot color	Spot color under UV_{366nm} lamp	*Rf reference
1	0.26	-	Red	-
2	0.35	-	Green	-
3	0.40	Red	Black	-
4	0.47	Orange	Black	-
5	0.60	-	Grey	-
6	0.76	Blue	Deep blue	+

Figure 1. Thin layer chromatography.

The capsaicinoid compound isolates adsorbed on silica gel were washed with ethanol solvent and then evaporated to obtain a white powder, which is presumed to be the capsaicinoid compound isolates from fresh and spoiled cayenne peppers. Before conducting cytotoxicity tests on cancer cells, the capsaicinoid isolate was characterized using FTIR to confirm the presence of capsaicinoid compounds based on their specific functional group characteristics.

FTIR Identification

Identification using ATR-FTIR shows absorption bands that match the characteristics of capsaicinoid functional groups [22]. The functional groups include -OH, CH_3, CH_2 (stretching), C=O, C=C, -CH_2- (bending), C-N, C-O, O-CH_3, and -NH on **Figure 2**. The absorption data obtained, presented in **Table 2**, confirm that the isolated compounds belong to the capsaicinoid group.

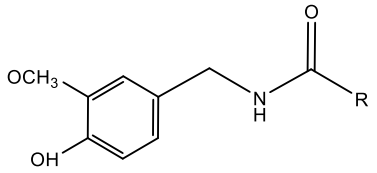


Figure 2. Capsaicinoids compound [16].

Based on **Figure 3**, it can be seen that the difference in the spectrum of the rotten cayenne pepper isolate sample and fresh cayenne pepper is in the intensity of the valley resulting from the identification process. The sharper the intensity of the valley of a spectrum, the more dominant a compound is identified.

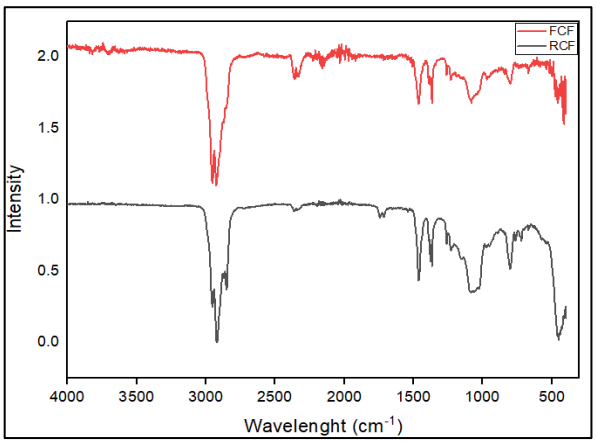


Figure 3. ATR-FTIR spectrum of RCF and FCF.

Table 2. Interpretation of identification absorption using ATR-FTIR.

Isolate of RCF		Isolate of FCF		Vibration	Range (*)
Wavelength (cm^{-1})	Δ%Transmition (%)	Wavelength (cm^{-1})	Δ%Transmition (%)		
3351.59	28.05	3330.20	27.54	-OH *stretch*	3500-3200
2919.45	85.82	2950.83	82.55	CH_3 *stretch*	2990-2850
2852.42	51.62	2923.73	83.90	CH_2 *stretch*	2926-2853
1717.15	6.59	1715.73	2.81	C=O *stretch*	1900-1500
1540.31	2.11	1635.86	1.78	C=C *stretch*	1475-1680
1463.29	43.53	1459.01	28.91	-CH_2- *bend*	1465
1366.31	30.16	1366.31	26.43	C-N *stretch*	1350-1000
1229.39	19.64	1227.97	10.35	C-O *stretch*	1260-1000
1092.48	21.00	1082.49	14.89	O-CH_3 *stretch*	1250-1050
798.67	23.45	802.96	8.27	-NH *stretch*	909-606

Cytotoxic analysis

The cytotoxic test of RCF and FCF isolates was conducted in vitro against T47D cancer cells using the MTT method (**Table 3**). This test includes cancer cell preparation, harvesting, calculation of cells, toxicity test, MTT reagent administration, and absorbance reading using an ELISA reader. T47D cancer cells were revived and cultured until they reached 80% confluent. After that, cells were harvested, counted using a hemacytometer, and placed in a plate to incubate for 24 hours.

The chili pepper samples were dissolved in DMSO and added to the cells at various

concentrations. After 24 hours of incubation, the cells were observed using a microscope to see the interaction between the samples and the cells. MTT solution was then added to detect live cells through the formation of formazan crystals, which were observed under an inverted microscope.

Testing the activity of the capsaicinoids compound group was carried out using the MTT-assay method with 8 variations in concentration, it can be seen from the treatment that has been carried out that the capsaicinoids compound group in rotten cayenne pepper has cytotoxic activity which is directly proportional to the number of concentrations of isolates given to T47D cancer cells. The higher the concentration given, the more T47D breast cancer cells die. The same treatment is carried out on the sample of fresh cayenne pepper isolates as a comparison and it can be seen that the results of the IC_{50} value in rotten chili are smaller, which means that the higher the cytotoxicity of the activity of the *capsaicinoids* compound group in rotten cayenne pepper against T47D breast cancer cells compared to the isolate of the *capsaicinoids* compound group in fresh cayenne pepper.

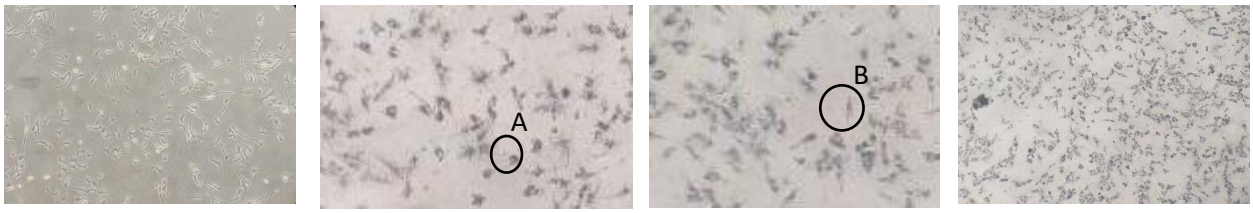
Table 3. Cytotoxic activity of IC50 value.

Sample	IC_{50} (µg/mL)	Cytotoxic activity
RCF	512.37	Moderate (IC_{50}=100-1000 µg/mL)
FCF	1386.81	Non-toxic (IC_{50}>1000 µg/mL)
Doxorubicin	0,70	Potential (IC_{50} <1000 µg/mL)

According to cytotoxicity categories value [24], a bioactive compound that has an IC_{50} value of less than 100 µg/mL means that it has potential cytotoxicity, moderate cytotoxicity if the IC_{50} value is 100-1000 µg/mL, and non-toxic if the IC_{50} value is more than 1000 µg/mL. Compounds with high cytotoxicity can be utilized as cytotoxic agents, while compounds with moderate cytotoxicity can be utilized as chemoprevention agents (only inhibit and prevent the development of cancer cells).

Capsaicinoids play a role in inducing apoptosis in breast cancer cells. The process of apoptosis is a more targeted mechanism of cell inhibition and death triggered by cytotoxic agents that can be obtained from natural isolates [25].

Cancer cells can be observed from an *inverted microscope* to show the morphological differences between dead and living cells. Dead cells are characterized by round, small, and shrunken. While the surviving cells are needle-shaped formazan formed from the disruption of the tetrazole core ring and the formation of a purple solution of the surviving cell, cells will be needle-shaped formazan needle, the difference in shape can be seen in **Figure 4.**



(a) (b) (c) (d)

Figure 4. Sel T47D (a) cell control; (b) Treatment control; (c) RCF ; (d) FCF; (A) living cells; (B) dead cells.

CONCLUSION

The results indicated that *capsaicinoid* isolates from RCF exhibit greater toxicity towards T47D breast cancer cells compared to isolates from FCF, as demonstrated by lower IC_{50} values. This conclusion was further supported by FTIR analysis, which revealed that the intensity of the functional groups in capsaicinoid compounds from RCF was higher than in FCF. This suggests that the increased content of capsaicinoid compounds correlates directly with the heightened toxicity effect against T47D breast cancer cells.

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