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Urokinase plasminogen activator inhibitory and anti-breast cancer activity of *Cananga odorata* and *Lantana camara* leaf extracts from optimization of ultrasound-assisted extraction

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Abstract: **Background**: Cancer invasion and metastasis are complex processes that depend on the degradation of the extracellular matrix, largely facilitated by proteolytic enzymes such as urokinase-type plasminogen activator (uPA). Elevated levels of uPA have been consistently correlated with increased tumor aggressiveness and poorer clinical outcomes in breast cancer patients, making this enzyme a key therapeutic target. Flavonoids found in the hydroalcoholic leaf extracts of Cananga odorata (CO) and Lantana camara (LC) have previously been associated with inhibitory effects on cancer invasion and metastasis. **Objectives**: This study aimed to investigate the influence of different Ultrasound Assisted Extraction (UAE) method parameters, including ethanol concentration (50–90%), extraction time (10–50 minute), and temperature extraction (30–60 0C). The optimal UAE condition from CO and LC on total flavonoid content (TFC) and antioxidant activity (AA) were determined using Response Surface Methodology (RSM). The biological activities of the optimized extracts were further evaluated as uPA inhibition and cytotoxicity test. **Methods**: The TFC and AA were quantified using AlCl3 and DPPH assays, respectively. Additionally, the Urokinase Inhibitor Screening Kit was utilized to assess uPA inhibition, while the PrestoBlue assay was employed to measure cytotoxicity test against MCF-7 human breast cancer cells. **Results**: The optimal UAE conditions were 74% ethanol concentration, 31 minutes of extraction time, and an extraction temperature of 41°C resulted in extracts with high bioactive content, while LC showing superior TFC and stronger AA compared to CO. The results demonstrated that LC exhibited more potent bioactivities than CO leaf extract, with significantly lower IC_{50} values for uPA inhibition and cytotoxicity test were 24.13 ± 2.68 µg/mL and 42.87 ± 2.24 µg/mL). **Conclusion**: These findings suggest that LC extract effectively suppresses uPA activity while exerting cytotoxic effects on breast cancer cells, indicating its potential as a anticancer and chemoprevention agent.

Keywords: *Cananga odorata*; *Lantana camara*; MCF-7 cells; Optimization; Urokinase plasminogen activator

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INTRODUCTION

Medicinal plants from secondary metabolites are frequently studied due to chemical constituents and the possibility of therapeutic uses with the secondary metabolites such as alkaloids, phenolics, terpenoids, flavonoids, saponins, xanthones and polysaccharides (Rajkumar *et al*., 2022). Flavonoids are polyphenolic compounds that have antioxidant activity (AA) due to the ability to scavenge free radicals, donate hydrogen atoms and chelate metal cations. The scavenged free radicals from antioxidant compounds protect the cells, delay aging and prevent related diseases such as anti-carcinogenic, regenerate cells and anti-mutagenic (Nakai and Tsuruta, 2021). Urokinase-type plasminogen activator (uPA) is a serine proteinase that catalyzes the conversion of plasminogen into plasmin and activates signaling pathways while binding to the receptor (uPAR). This activity has been proven to increase cells migration, proliferation and survival. The three parts of the uPA system are inhibitor (PAI-1), uPAR or suPAR and uPA serine protease. These elements play a significant part in different forms of cancer. For colon and breast malignancies, high uPA levels are linked to a dismal prognosis (Masucci *et al*., 2022).

A few studies revealed that *Cananga odorata* (*CO*) and *Lantana camara* (*LC*) leaf extracts contain flavonoids with high antioxidant potential (Al Kazman *et al*., 2022; Jiea *et al*., 2022). The crude methanol leaf extracts of *CO* and *LC* show positive uPA inhibition (Zha *et al*., 2013). The extraction of biological components from plants and subsequent testing of their biological activity have been used in previous investigations to identify novel medications in medicinal plants. Some drawbacks of the conventional extraction techniques for biological compounds include high solvent consumption, high energy

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requirements, thermal destruction of heat-labile components and extended extraction timeframes (Bitwell *et al*., 2023). Compared to conventional procedures, Ultrasound Assisted Extraction (UAE) is a reliable, effective extraction process and is known as green technology as this process reduces or completely eliminate the use of organic solvents, up the extraction procedure, uses less energy, thereby reducing negative impacts on the environment (Shen *et al*. 2023). UAE is an effective method of extraction, because it is able to result in a high yield of flavonoids (more than 97%) using shorter extraction time, polar solvent such as ethanol and extraction temperature (Bitwell *et al*., 2023). The choice of solvent depends on the solubility and polarity of the extract. Some common solvents used to extract polar substances, such as total flavonoids, are ethanol and water (Bitwell *et al*. 2023). To identify the ideal circumstances, this work used Box Behnken Design (BBD) and Response Surface Method (RSM) to *CO* and *LC* leaves extract by UAE. RSM is a set of statistical and mathematical techniques that minimize the expense, duration and number of experimental runs to optimize the range of variables in different experimental processes (Kumar *et al*. 2021). The mathematical models of the experimental data were fitted using analysis of variance and response surface analysis to escalate the region for both response variables. These techniques also helped to ascertain the terms regression coefficients and statistical significance (Nurcholis *et al*., 2023). Furthermore, predictive models of quadratic polynomials were selected as the best-fitted model to show the influence of the variable and interactions on the response variable. The objective of the present review is to provide the optimal TFC and AA of *CO* and *LC* leave extracts from UAE. The extracts were further assessed for anticancer activity use *In-vitro* assays as uPA inhibitor and anticancer potential on the MCF-7 breast cancer cell line.

MATERIALS AND METHODS

Materials

The leaf samples of *C. odorata* (*CO*) and *L. camara* (*LC*) were collected from Bogor, West Java, Indonesia, and were identified at The National Research and Innovation Agency (BRIN) with identification numbers B-102/ll.6.2/Dl.05.07 /1/2023 and B-144/ll.6.2/Dl.05.07/1/2023, respectively for *CO* and *LC*. The organic solvents were of analytical grade and purchased from Merck, Germany. Chemicals such as ascorbic acid, quercetin, 2,2-Diphenyl-1-Picrylhdrazyl (DPPH), doxorubicin, Urokinase Inhibitor Screening Kit, phosphate-buffered saline (PBS), PrestoBlue™ Cell Viability Reagent, Roswell Park Memorial Institute Medium (RPMI), fetal bovine serum (FBS), Trypsin-EDTA, and Trypan Blue were obtained from Sigma-Aldrich (St. Louis, MO, USA). The phytochemical reagents were purchased from the Laboratory Supplies Office (Brataco, Indonesia).

Instruments

Ultrasound Bath (GT Sonic®), Rotary Evaporator (R-300, Buchi®, Swiss), UV-Vis spectrophotometer (Jasco V-730, Yorkshire, UK), Fluorescence Microplate Reader (200 Pro Tecah, Infinite®, Switzerland), CO_2 Incubator (1000, Thermo Fischer Scientific®, America.

Preparation of leaves

The fresh leaves were washed under running tap water, air-dried in the shade and meshed using a 40-mesh sieve to obtain sample powder (Zhang *et al*., 2022).

Phytochemical test

Phytochemical screening test of *CO* and *LC* leaf was identified using Shinoda, Dragendorff, ferric chloride, and Liebermann-Burchard reagents to determine the presence of flavonoids, alkaloids, tannins, saponins, steroids, and terpenoids, respectively (Jiea *et al*., 2022; Rajkumar *et al*., 2022). For flavonoid used Shimoda's test where 1 mL aqueous solution of the extract was treated with a piece of magnesium ribbon and a few drops of concentrated hydrochloric acid to form a reddish pink color. Alkaloid used Dragendorff's reagent to add 1 mL aqueous solution of extract and produced an orange or orange-red precipitate. To 1 mL of aqueous solution of extract, 10% alcoholic ferric chloride solution was added, and dark blue or greenish grey coloration form of the solution indicates the presence of tannins. The foam test was conducted to identify the saponins compounds. Liebermann-Burchardt test for steroids and terpenoids: To 1 mL of aqueous solution of extract were added 1 mL of chloroform, 2-3 ml of acetic anhydride, and 1 to 2 drops of concentrated sulfuric acid. Dark green coloration of the solution indicates the presence of steroids, and dark pink or red coloration of the solution indicates the presence of terpenoids.

Experimental design

TFC and AA values were obtained from *CO* and *LC* leaf extracts by optimizing UAE conditions using BBD with three levels and three variables: ethanol concentration (EC), extraction time (ET), and temperature of extraction (TE). The extraction process focused on ethanol concentration, time, and temperature extraction. BBD included 13 experimental runs using Design Expert Software version 13 for analysis model building. From Table 1, the design will run with each coded at a level of +1 (high), 0 (middle), and -1 (low). The experimental design and corresponding three response variables are presented in Table 2.

Ultrasound-assisted extraction

About 25 g samples of *CO* and *LC* leaf were added to 250 mL of ethanol concentration (%), time extraction (minute), and extraction temperature (^{0}C) according to experimental design as described in Table 1. The ultrasonic bath was set at a fixed frequency of 40 kHz. The extraction results were

filtered through Whatman No. 1 filter paper and concentrated using a rotary vacuum evaporator at 50°C to obtain a viscous extract. About 25 g samples of *CO* and *LC* leaf were added to 250 mL of ethanol concentration (%), time extraction (minute), and extraction temperature (^{0}C) according to experimental design as described in Table 1. The ultrasonic bath was set at a fixed frequency of 40 kHz. The extraction results were filtered through Whatman No. 1 filter paper and concentrated using a rotary vacuum evaporator at 50°C to obtain a viscous extract.

Total flavonoid content (TFC) determination

The aluminum chloride colorimetric method was used to quantify TFC and Quercetin was used as the standard. Each 50 mg of *CO* and *LC* was dissolved in 10 mL of ethanol. 3 mL of methanol, 2 mL of a 10% aluminum chloride, 2 mL of a 1M sodium acetate solution, and 10 mL of distilled water were mixed into 1 mL of the extract solution. Three milliliters of methanol, two milliliters of 10% aluminum chloride, two milliliters of 1 M sodium acetate and ten milliliters of distilled water were added to one milliliter of the solution. The mixture was kept at room temperature for thirty minutes in a dark area. TFC was reported as mg equivalent of quercetin equivalent (QE) in milligrams per gram (mgQE/g) of extract samples, through a standard curve obtained (y = ax + b, R^2 is the determination coefficient). The absorbance The aluminum chloride colorimetric method was used to quantify TFC and Quercetin was used as the standard. Each 50 mg of *CO* and *LC* was dissolved in 10 mL of ethanol. 3 mL of methanol, 2 mL of a 10% aluminum chloride, 2 mL of a 1M sodium acetate solution, and 10 mL of distilled water were mixed into 1 mL of the extract solution. Three milliliters of methanol, two milliliters of 10% aluminum chloride, two milliliters of 1 M sodium acetate and ten milliliters of distilled water were added to one milliliter of the solution. The mixture was kept at room temperature for thirty minutes in a dark area. TFC was reported as mg equivalent of quercetin equivalent (QE) in milligrams per gram (mgQE/g) of extract samples, through a standard curve obtained (y = ax + b, R^2 is the determination coefficient). The absorbance was evaluated using UV-VIS spectrophotometry expressed at 433 nm (Shraim et al., 2021). The total flavonoid content is expressed as means ± standard deviation (SD) and standard error (SE).

Antioxidant activity (AA)

Antioxidant activity was performed using the protocols by Sadeer *et al*. (2020). A modification was made to the DPPH assay procedure to evaluate the free radical scavenging activity of *CO* and *LC* leaf extracts. 1mL of ethanol (1 mL; 80 mg extract/10 mL) was added to the sample to create a 0.1 mM DPPH solution. The mixture was shaken and kept in the dark for 30 min at room temperature, and then the absorbance of the reaction mixture was determined at 516.5 nm by a UV-Vis spectrophotometer. The percentage (%) of radical scavenging activity = [(A-B) / A] where A and B: absorbance of control and sample at 516.5 nm. Ascorbic acid was used as a positive control with concentrations of 2.0, 4.0, 6.0, 8.0, and 10.0 µg/mL, and DPPH solution was used as a negative control. IC_{50} values showed the sample concentration needed to scavenge 50% of the DPPH free radicals. The antioxidant activity is expressed as means ± standard deviation (SD) and standard error (SE).

Statistical analysis, predicted model, and response surface method

The experiments TFC and AA were run in duplicate, with the mean ± standard deviation (SD). Statistical analysis was performed using a one-way analysis of variance (ANOVA) and SPSS ver. 20.0 (SPSS Inc., IL, USA), followed by Duncan's test to determine the significance of differences between means with a statistical significance level of $p < 0.05$. The Design Expert Software version 13 statistical software was employed to analyze the highest TFC and AA data using RSM. The relationships between independent and dependent variables were fitted to the second-order polynomial model.

Model verification

A verification method was used to approach the optimal formula (maximum desirability) to reach the maximum yield of the TFC and AA of *CO* and *LC* leaf. This was followed by calculating the percentage residual standard error (RSE) compared with predicted values.

In-vitro test of uPA Inhibitor

uPA inhibitory activity was measured by fluorescence spectrophotometry based on the Urokinase Inhibitor Screening Kit (Cat. No. MAK220) preparation instruction. One µL each of CO and LC leaf extracts was used as a sample from the optimum condition, dilution at 3.13, 6.25, 12.5, 25.0, 50.0, 100.0, and 200.0 µg/mL, respectively, was added to the 96-well plates with Doxorubicin as the positive control. The reaction mixture consisted of Urokinase assay buffer (40 µL) and human urokinase (10 µL) were mixed in a well of 96-well microplate, then incubated at 37^{0}C for 15 min and was protected from light in the process. Subsequently, 49 mL of the enzymatic reaction mix (47 µL urokinase assay buffer and 2 µL urokinase substrate) was added to the mixture. Each experiment was performed in two replicates with appropriate blanks. The fluorescence (FLU) at λ_{ex} = 350/λ_{em} = 450 nm) in a microplate reader was examined in the 1st to 60th minutes after the reaction started (every 5 minutes). The slope of the blank sample was corrected using the % Relative Inhibition of the following equations 1 and 2.

$$\text{Slope} = \frac{(FLU2 - FLU1)}{(T2 - T1)} = \Delta \text{ FLU/ minute} \qquad \text{Eq. 1}$$

$$\%\text{ Relative Inhibition} = \frac{(Slope\ EC - Slope\ SM)}{SlopeEC} \times 100\% \qquad \text{Eq. 2}$$

Where: FLU: fluorescence for each well; Slope EC: the slope of the Enzyme Control; Slope SM: the slope of the Sample Inhibitor.

Cytotoxicity test against MCF-7 cell lines

Utilizing the PrestoBlue assay technique, anticancer activity was demonstrated against MCF-7 breast cancer cells. Human cells were taken from the ATCC (American Type Cell Cancer MCF7-HTB22) collection, which is kept at the Central Laboratory of Padjajaran University in West Java, Indonesia by the Division of Biology Activities. The cells were cultivated in RPMI liquid media supplemented with 50 µL/50 mL antibiotics and 10% Fetal Bovine Serum (FBS), with doxorubicin as the positive control. Doxorubicin was used as a positive control in clinical settings to treat a variety of cancers, including breast cancer (Mattioli *et al*. 2023). The cells used must reach a confluent at a minimum of 70% and the viability of MCF7 was determined using a trypan blue assay. A mixture was made with an equal volume of 10 µL trypan with a final density of 170,000 cells/mL in the medium (17,000 cells/well).

Cell culture preparation and cell viability assay

Cell culture in 96-well plates was incubated in a CO_2 atmosphere (5%) at 37°C for 48 hours until confluent at 70%. The dish was discharged to the media and doused twice with 1 mL Phosphate Buffered Saline (PBS), followed by adding 1 mL Trypsin-EDTA solution, and then incubated for 5 min. Furthermore, the cell layers were dispersed and appeared to float when viewed with the inverted microscope.

For 24 to 48 hours, a 96-well plate containing cell culture was incubated at 37°C with 5% CO_2 gas. By dividing the groups into medium plus MCF-7 as negative controls, doxorubicin, and dimethyl sulfoxide (DMSO) as a solvent, the viable cell count for the cytotoxicity test was performed. For each treatment sample, 10 µL of PrestoBlue® reagent was added for every 90 µL of medium. Subsequently, 100 µL of the resulting mixture is added into each well of the microplate, followed by an incubation period of 1-2 hours until noticeable color changes occur. PrestoBlue® reagent converted blue resazurin molecules with no inherent fluorescence into red, highly fluorescent resorufin when added to living cells. Conversion values may be objectively assessed to determine the absorbance spectrum used to measure resazurin and resorufin. A multimode reader was also used to detect absorbance at a wavelength of 570/600 nm.

The morphological traits were recorded with the assistance of the EVOS XL Core microscope from Invitrogen. Approximately $12x10^3$ cells were cultured in 96-well plates and incubated in RPMI media containing different doses (12.5, 25, 50, 100, and 200) /mL for a whole day. Equation 3 was used to compute the percentage of growth inhibition after the absorbance at 570 nm was measured using a multimode reader. IC_{50} was defined as the concentration of the plant extracts inhibiting 50% of the cells from equation 3. IC_{50} was determined for MCF-7 cell lines of both extracts.

$$\text{Inhibitory rate (\%)} = \frac{(Absorbantion\ control - Absorbantion\ test)}{Absorbantion\ contro} \text{x } 100\% \quad \text{Eq. 3}$$

RESULTS

Phytochemical screening

The presence of a yellow coloration, yellow precipitate, green or blackish black color, persistent frothing after warming, and a dark green sample showed results for flavonoids, alkaloids, tannins, saponins and terpenoids compounds (Parwanto *et al*. 2023).

Ultrasound-assisted extraction

It shows that the UAE method is an efficient, economical and applicable extraction method. The radiation frequency of 40 kHz is in the general frequency range of 20 kHz to 2000 kHz. Higher yields were obtained in phenolic research at 40 kHz instead of 120 kHz. The sonic cavitation from the ultrasound causes bubbles to develop, which enhances the permeability of cell walls and the surface area of interaction between solvents and plant materials (Bitwell *et al*., 2023). Previous research showed that the extract from *CO* and *LC* maceration with hydroalcohol (ethanol: water = 50:50) and petroleum ether had a lower TFC value (Jiea *et al*., 2022; Parwanto *et al*., 2023).

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Total flavonoid content

Lower extraction temperatures than 80 °C were employed in order to reduce the potential for flavonoid degradation. The major TFC of *CO* extract is 10.98 ± 0.24 mg QE/g, or the mean ± SE is 10.98 ± 0.17 mg QE/g using 70% EC, 20 minutes ET, and 45 °C TE. In LC extract results with lower temperature (40 °C), extending the extraction time (30 minutes) in the same solvent consumption, increases TFC 30.57 ± 0.40 mg QE/g, or the mean ± SE value is 30.57 ± 0.26 mg QE/g.

Antioxidant activity

AA decreased in medium-level extraction for *CO* and the *LC* leaf extracts. The 17 experimental data for all response variables differed significantly ($p<0.05$) among samples. Furthermore, the IC_{50} value of LC leaf extract was 67.92 ± 0.10 µg/mL (67.92 ± 0.07 µg/mL), stronger than *CO* leaf extract 71.22 ± 0.80 µg/mL (71.22 ± 0.57 µg/mL). The previous study of CO hydroalcohol and *LC* ethanol leaf

Table 1: Coded and experimental values of independent variables

Independent variable	Unit	Symbol	*C. odorata* leaves Coded level			*L. camara* leaves Coded level		
			-1	0	+1	-1	0	+1
Ethanol concentration (EC)	%	X_1	50	70	90	50	70	90
Extraction Time (ET)	minute	X_2	10	20	30	10	30	50
Temperature extraction (TE)	°C	X_3	30	45	60	30	40	50

Table 2: Results from the three responses of TFC and AA from *CO* and *LC*

Run	EC (%)		ET (min)		TE (ºC)		TFC (mg/g)		AA (ppm)	
	CO	*LC*	*CO*	*LC*	*CO*	*LC*	*CO*	*LC*	*CO*	*LC*
1	50	50	10	10	45	40	7.350b	10.393c	80.274i	76.320a
2	50	50	20	30	30	30	7.433a	10.262c	80.503h	77.425h
3	50	50	20	30	60	50	8.034b	9.464b	77.477i	76.906hi
4	50	50	30	50	45	40	7.845a	8.361a	80.220i	78.099h
5	70	70	10	10	30	30	9.758de	28.295i	74.154ef	71.028ij
6	70	70	10	10	60	50	9.915de	29.374j	72.419cd	69.958de
7	70	70	20	30	45	40	10.970g	30.566k	71.219b	67.921cd
8	70	70	30	50	30	30	9.154c	27.119h	75.608g	69.950b
9	70	70	30	50	60	50	9.584d	25.506g	75.132fg	69.357c
10	90	90	10	10	45	40	9.986e	20.526d	71.801bc	74.982cd
11	90	90	20	30	30	30	10.570e	21.973e	73.328e	75.251g
12	90	90	20	30	60	50	10.063e	21.120f	71.988bc	73.430g
13	90	90	30	50	45	40	9.940f	20.275d	73.881de	71.212f

Table 3: The predicted model obtained for TFC (Y1) and AA (Y2) for *CO* and *LC* were as follows

Extract	Measured parameter	Predicted models
C. odorata	Total Flavonoid Content	$Y1 = -22.545 + 0.302(X_1) + 0.339(X_2) + 0.603565(X_3) + 0.001 (X_1X_2) – 0.001 (X_1X_3) – 0.001 (X_2X_3) – 0.003(X_1^2) - 0.008(X_2^2) – 0.003(X_3^2)$
	Antioxidant activity	$Y2 = 154.686 – 1.496(X_1) – 1.001 (X_2) - 0.648(X_3) + 0.003 (X_1X_2) + 0.001 (X_1X_3) + 0.002 (X_2X_3) + 0.003 (X_1^2) + 0.020(X_2^2) + 0.005(X_3^2)$
L. camara	Total Flavonoid Content	$Y1 = -173.342 + 0.776(X_1) + 0.274(X_2) + 5.005(X_3) – 0.003(X_1X_3) + 0.001(X_2X_3) – 0.009(X_1^2) – 0.004(X_2^2) – 0.033895(X_3^2)$
	Antioxidant activity	$Y2 = -171.189 - 0.933(X_1) - 0.010 (X_2) – 2.296 (X_3) + 0.001 (X_1X_2) – 0.002 (X_1X_3) + 0.003 (X_2X_3) + 0.012 (X_1^2) – 0.003 (X_2^2) – 0.017 (X_3^2)$

extracted from the maceration method had weaker AA with IC_{50} values of 120.44 and 316.87 µg/mL, respectively (Jafriati *et al*., 2022). To evaluate whether TFC corrected well with AA, we adopted the Pearson correlation coefficient. The goodness-of-fit of the regression model was evaluated by the correlation coefficient (r) value (Muflihah *et al*., 2021). TFC significantly correlated with AA, the r values of *CO* and *LC* were = 0.9590 and 0.9194, respectively.

Statistical analysis, predicted model, verification model, and response surface method

Considering the importance of the extraction method and conditions, optimizing the extraction of TFC and AA could be predicted from the respective response surface plots. Models were highly significant due to a very low probability value, $p<0.0001$ as indicated by the ANOVA of the regression models. R^2 value, defined as the ratio of the explained variation to the total variation was a measure of the degree of fit. Coefficient of determination (R^2) is used to check the fitness of the models. For TFC (Y1) and AA (Y2), the anticipated models and statistical parameters computed using 3-factor BBD were obtained. Based on the quadratic model, 74% EC, 31 minutes ET, and 41 °C TE were the ideal conditions for *LC*. The expected results for TFC and AA were 30.70 mg QE/g and 67.78 µg/mL, respectively. Higher and stronger than the experiment's maximum yields for TFC and AA value at 70% EC, 30 minutes ET, and 40 °C TE. The verification results of TFC and AA as actual values were 30.64 mg QE/g and 67.77 µg/mL, very close to the result of the predicted value. The optimum TFC and AA of *LC* leaf extracts using RSM were shown in figs. 1 A and 1 B.

The predicted R^2/adjusted R^2 values for Y1 and Y2 of LC leaf extract (0.9983/0.9962 and 0.9960/0.9908 higher than *CO* leaf extract value 0.9802/0.9548, 0.97782/0.9502), respectively. It means the correlation between adjusted and

predicted values for *LC* is better than *CO* leaf extract. Predicted R^2/adjusted R^2 values greater than 0.80 or 70% are preferable. The predicted R^2/adjusted R^2 values for Y1 and Y2 of *LC* leaf extract (0.9983/0.9962 and 0.9960/0.9908 higher than the *CO* leaf extract values 0.9802/0.9548, 0.97782/0.9502), respectively. It means the correlation between adjusted and predicted values for *LC* is better than *CO* leaf extract. Predicted R^2/adjusted R^2 values greater than 0.80 or 70% are preferable. The lack-of-fit with P values for Y1 and Y2 of *CO* and *LC* leaf extracts were nonsignificant as 0.36, 0.14, 0.56, and 0.12 implied nonsignificant, relative to the pure error. The percent residual standard error (RSE) for TFC and AA were 0.20% and 0.01%, respectively. The *CO* leaf extract prediction value for TFC and AA results were lower and weaker than *LC*, namely, 11.25 mgQE/g and 70.24 µg/mL, respectively. The verification results of TFC and AA as actual values were 11.28 mgQE/g and 70.32 µg/mL. The %RSE TF and AA of *CO* were 0.27% and 0.11% higher than *LC*. Smaller values indicate better prediction and the %RSE value is used as an evaluation parameter for the correctness of the model generated by comparing the predicted value with the actual value (verification). The TFC and AA response model of *LC* has a better Adeq precision (AP) value (57.07; 36.03) than the *CO* leaf extract (17.14; 15.66) model. For the quadratic model, the AP value serves as a sufficient signal. AP values larger than 4 are favored to verify that the projected model could be utilized to navigate a design-defined space (Nurcholis *et al*., 2023).

The two regression models (Y1 and Y2) of *CO* and *LC* proposed for the responses were highly significant, as shown by $p < 0.001$ (Table 3). The results showed that the fitted model for *LC* was more reliable than *CO*. The model Y1 and Y2 for *CO* and *LC* were highly significant, with a good correlation coefficient,and adjusted R^2 was also high, which showed the significance of the model. RSM is a general-purpose technique that optimizes the expected value of a stochastic response by combining statistical methods, mathematical regression analysis, and experiment design (Carrión-Tacuri *et al*. 2013). Following the optimization process and evaluation of the TFC and AA responses, an optimal point was found. The desirability values for the *CO* and *LC* leaf extracts are 0.981 and 0.993, respectively. The primary variables influencing the extraction condition of the highest TFC and strongest AA are temperature, time, and ethanol concentration. Plots of three-dimensional model graphs are displayed in the corresponding figures. Three variables were varied within the experimental range being studied, while the remaining variables were held at the central level (0 levels) to create the model's response surface plots.

In vitro Test of uPA Inhibitor

In vitro test of uPA inhibitor carried out on spectrofluorimetry method. Doxorubicin as control positive, CO, and LC leaf extract significantly differed by ANOVA followed by Duncan test ($p<0.05$). Doxorubicin, as anthracyclines, acts on cancer cells by different mechanisms depending on drug binding to DNA. The fluorescence intensity rapidly increased during the first 25-40 min but decreased at 45 to 60 mins at each sample concentration. Percentage of relative inhibition gets increased from the concentration of 25-100 µg/mL and decreased at 200 µg/mL. The IC_{50} value of uPA inhibitor for doxorubicin, CO, and LC leaf extracts were 6.72 ± 0.10, 42.87 ± 2.24, and 24.13 ± 2.68 µg/mL), respectively. The IC_{50} values of uPA inhibition < 50 µg/mL were categorized as "very strong" (Molyneux, 2004).

Cytotoxicity test against MCF-7 cell lines

According to a cell viability test, an increase in concentration results decrease in cell viability (a dose-dependent phenomenon). IC_{50} values for *CO*, *LC* leaf extract and doxorubicin were 173.76 ± 0.63, 131.05 ± 0.14, and 1.66 ± 0.09 ppm, respectively, determined from the regression equations of Y= - 0.2837x + 99.04, Y = - 0.3021x + 89.591, and - 15.181x + 74.592. Samples were determined from the regression equation with a coefficient of determination (R^2) value. The trend of average cell death of *CO* leaf extract ($R^2 = 0.9489$) is more linear than *LC* leaf extract ~~with~~ ($R^2 = 0.8104$) and doxorubicin ($R^2 = 0.5265$). This result suggested a better fit for the data in *CO* cytotoxic test. The concentration of *LC* extract required for a 50 % reduction in cell viability was determined to be 50 µg/mL, while the *CO* leaf extract needed a higher concentration (100 µg/mL). Increased cytotoxic effects due to the *LC* leaf extract were the expected result. No discernible cell death was seen before 100 µg/mL, which may be related to the initial cell media's normal appearance in the absence of therapy.

The cytotoxic effect of the *LC* leaf extract was more active against MCF-7 cells compared to the *CO* leaf extract. The higher cytotoxic recorded for *LC* leaf extract was more active (almost twofold) against MCF-7 cells than *CO* leaf extract (>80% cell death). The response of MCF-7 cells after treatment with *LC* leaf extract and doxorubicin showed higher apoptosis compared to doxorubicin itself (Figs. 2A and B). Morphological observations of cells due to the treatment of *LC* leaf extract enhanced the cytotoxic effect of doxorubicin treatment. No discernible cell death was seen before ~~to~~ 100 µg/mL, which may be related to the initial cell media's normal appearance in the absence of therapy. Consequently, after adding LC leaf extract to the medium at concentrations of 100 and 200 µg/mL, apoptosis started.

DISCUSSION

According to literature~~s~~ of Carrión-Tacuri *et al*., 2013, Jiea *et al*., 2022, three main factors, including ethanol concentration, extraction time, and temperature extraction can affect the content of flavonoid and antioxidant activity. The presence of polar compounds in ethanol, which have

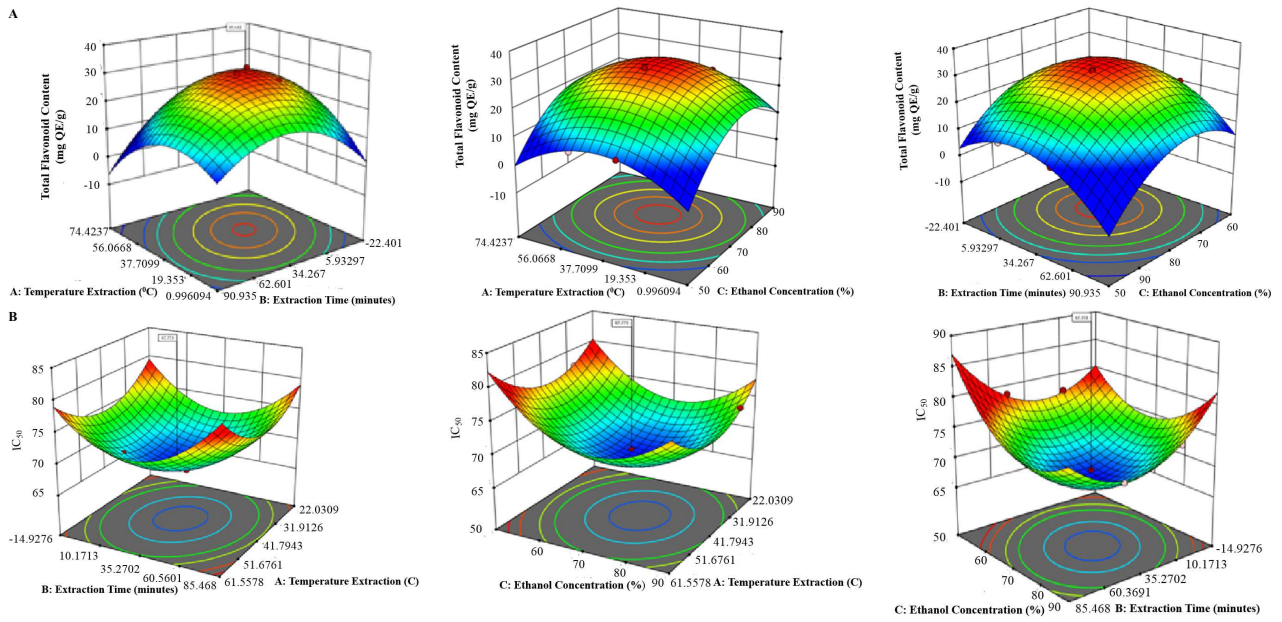


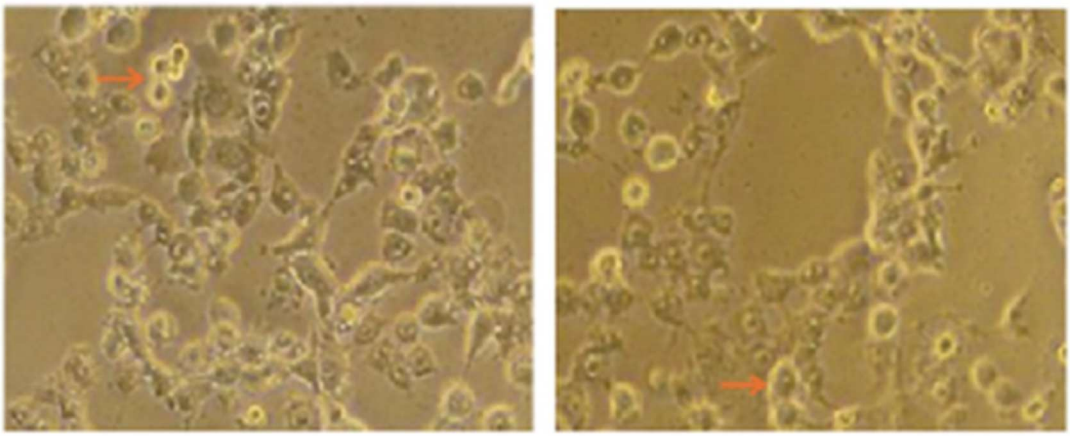
Fig. 1: Response surface plots of TFC and AA from LC leaf extract (A and B); Variable of temperature and time extraction (a); Variable of temperature extraction and ethanol concentration (b); Variable of time extraction and ethanol concentration (c)

hydroxyl groups in their backbone, can dissolve polar molecules. Plant bioactive compounds are very useful for humans due to their wide range of therapeutic activities (Roy *et al*., 2022). Flavonoids, alkaloids, tannins and saponins could be potential anticancer agents as reported in the literature (Al Kazman *et al*. 2022; Jiea *et al*. 2022).

Some main advantages of extracting bioactive compounds using UAE are in low temperature and ET, but a longer ET provides a greater opportunity for mass transfer between the solvent and sample. Our results showed that the best ET was 30 minutes for LC leaf extract with $p<0.05$. Temperature variation significantly impacts the extraction efficiency of different bioactive compound classes. Lower temperatures afforded more efficiency in extracting high yields of flavonoids (Bitwell *et al*., 2023). Differences in ET and TE were determined through preliminary experiments and a literature review that *LC* at high temperatures (50 °C) showed permanent damage to membrane rupture and the loss of cellular contents (Carrión-Tacuri *et al*., 2013, Koragi *et al*., 2023). Solvents for extraction, such as ethanol and ethanol/water mixtures (hydroalcohol) have been widely recognized and selected based on the target compound through several studies. It may be possible to extract chemicals like isoflavones, flavanones, flavones, and flavanols more effectively under the medium run experiment condition with a less polar solvent. It is preferable to extract more polar chemicals, such glycosides, and aglycones, using 50% ethanol (Muflihah *et al*. 2021). Higher ethanol concentration of the extraction condition was due to the essential oil, monoterpene, and sesquiterpene content isolated in *CO* and *LC* leaf, such as canangafruticosides A-E and β-caryophyllene as hydrophobic compounds (Vimaladevi *et al*. 2021; Sarma *et al*. 2020). Extraction using ultrasound produced ultrasonics that applied the thermal, mechanical and cavitation effects of UAE. The ultrasonic could disrupt the cell wall, facilitating the release of cellular content and enabling efficient extraction of the target (Shen *et al*. 2023).

Flavonoids as phenolic compounds, are important natural antioxidants due to their capacity to scavenge free radicals, donate electrons and hydrogen atoms, and chelate metal cations (Hassanpour, Doroudi, 2023). The antioxidant activity was related to the compounds of both extracts. Flavonoids were derived from the ability to scavenge reactive oxygen species directly. This is due to the ability to chelate free radicals immediately and donate one hydrogen atom or one electron transfer.

These studies showed that *L. camara* had anticancer activity against MCF-7 cells. Flavonoids have the potential to inhibit cancer cell proliferation by targeting various cellular mechanisms. Research indicates that flavonoids suppress cyclin-dependent kinases (CDKs), which are crucial for cell growth and division, inhibit angiogenesis, and regulate MDR1 activity. Additionally, flavonoids influence angiogenesis and the activity of reactive oxygen species (ROS) in tumor cells, thereby preventing cancer cell growth. Their functional properties enhance the activity of ROS-scavenging enzymes, contributing to the disruption of the cell cycle and promoting autophagy and apoptosis, which ultimately suppress cancer cell proliferation and invasion (Yusuf *et al*., 2023).



A B

Fig. 2: Cytotoxic effect on morphological changes of the MCF-7 cell treated by *LC* leaf extract 100 µg/mL (a) and 200 µg/mL (b) (the red arrows indicated the cell death).

Alkaloid compounds can reduce lipid peroxidation and exhibit antioxidant-like activity, thereby reducing oxidative stress. The outcome implied that the formation of reactive oxygen species (ROS) and other oxidants might occur during regular physiological activities (Chrysargyris *et al*. 2024). Alkaloids and tannins are compounds that exhibit precipitation of proteins and are associated with the physiological actions of herbal remedies. Saponins consist of amphiphilic glycoconjugates compound, which produces soap-like foams in water. Additionally, terpenoids are significant phytochemicals with a wide spectrum of antioxidants that are essential for preventing disease by lessening the oxidative damage that free radicals and reactive oxygen species inflict on cellular components. (Dhivya *et al*., 2022). A strong AA value with a decrease in IC_{50} showed that the TFC value increased equivalently. Ethanol concentrations used for UAE include the most (50%), semi (70%), and least polar (90%). Stronger AA showed a favorable correlation with ethanol content. Chemical compounds in *CO* and *LC* were distributed in semi-polar ethanol. Organic compounds with non-polar properties, such as terpenoids and steroids, were dissolved properly in a non-polar solvent, such as 90% ethanol. In contrast, organic compounds with polar properties, such as phenols, were highly distributed in the polar ethanol. Extended extraction duration had an adverse impact with a high IC_{50} value, most likely because the extended heat exposure that followed led to a decrease in the concentration of the desired antioxidant chemicals.

The optimization point based on the RSM from the Design Expert 13.0 software may be determined with a degree of certainty thanks to this desirability value parameter. The outcomes demonstrate that the *LC* procedure's confidence level is 99.3%, making it more reliable than the *CO* leaf extract method in determining validity. Because the %RSE value of the *LC* leaf extract was lower than that of the *CO* leaf extract, it was utilized as a measure of the model's accuracy, with smaller values denoting better prediction, and the model's correctness was determined by comparing the predicted value with the actual value (verification) (Nurcholis *et al*., 2023). Because it is greater than 4, the AP value of the *CO* and *LC* leaf extract suggests a signal that is sufficient signal for the model. Compared to *CO*, the *LC* leaf extracts RSM model, which was developed to predict TFC and AA, fits the data better. The desirability value of TFC and AA for *LC* is higher than *CO* leaf extract. Desirability (D=1) denotes that the answer within the ideal interval is an ideal approximation (Azahar *et al*. 2017). RSM can be used to accurately predict EC, ET, and TE parameters in optimal conditions.

The reaction of uPA inhibition in vitro test relies on a specific fluorogenic substrate reaction, 7-amino-4-trifluoromethylcoumarin (AFC), based serine substrate is cleaved, resulting AFC as high fluorescence quantum. In previous research, it was identified that extracts from ethyl acetate and chloroform fraction of *CO* and *LC*, respectively, have inhibited uPA (Zha *et al*., 2013).

A synthetic AFC substrate was cleaved to produce AFC, a fluorescent product (λex = 350/λem = 450 nm) that was proportionate to the amount of enzymatic activity present. This was used to assess the uPA activity. This reaction occurred based on the release of 7-aminocoumarin from the 7-peptidyl-coumarin through the amide bond's protease-catalyzed hydrolysis. Furthermore, the reaction of the response of AFC on the primary amine at the 7-position as an electron-donating group produced high fluorescence quantum yields.

A multipurpose serine protease called uPA is crucial to the process of tumor cell metastasis. Plasminogen is converted to plasmin when uPA attaches to its receptor, or uPAR, starting a proteolytic cascade. Migration of cancer cells is induced by plasmin formation. The IC_{50} inhibitory uPA value of the two extracts (*CO* and *LC*) is generally recognized to be below 100 µg/mL, which makes the

extracts results promising for further chemical exploration (Zha *et al*., 2013). Quercetin as a bioactive in LC leaf extract, significantly suppressed GC cell viability, migration, and invasion activities via decreasing expression of uPA and uPA receptor (uPAR) proteins, which are strongly associated with gastric cancer metastasis (Vafadar *et al*., 2020).

According to a cell viability test, a concentration rise results in a drop of cell viability (a phenomenon that is dose-dependent). The higher cytotoxic recorded for the LC leaf extract was more active (almost twofold) against MCF-7 cells than the CO leaf extract (>80% cell death). The larger the IC_{50} value, the cytotoxic rise, and the higher the quantity of LC leaf extract utilized, the lower the percentage of cells that are viable. Doxorubicin was used as a positive control in clinical settings to treat a variety of cancers, including breast cancer (Mattioli *et al*. 2023). The phase of MCF-7 cells apoptosis in response to *LC* leaf extract was reliant on the inhibitory concentration. The higher the concentration of *LC* leaf extract utilized, the stronger the increase in cytotoxicity and the corresponding decrease in cell viability percentage.

CONCLUSION

The authors should kindly indicate in this study that UAE of TFC and AA from *Cananga odorata* (*CO*) and *Lantana camara* (*LC*) leaves extract were successfully optimized using RSM. This is one of the novelties to achieve high result of TFC and AA in *CO* and *LC* leaf extracts. TFC and AA in the *LC* leaf extract have stronger inhibitory activity of uPA and cytotoxicity against MCF-7 cells than in the *CO* leaf extract.

The presence of inhibitors of uPA, inhibitory activity of *LC* with an IC_{50} value of 24.13±2.68µg/mL, was stronger than the *CO* leaves extract (42.87±2.24µg/mL). The *LC* leaves extract also showed stronger cytostatic activity over MCF-7 cells with an IC_{50} value of 131.05±0.14 µg/mL than the *CO* leaves extract (173.76±0.67µg/mL). As we know, research on *CO* and *LC* leave extracts with optimized extraction (EC, ET, TE), as an inhibitor of urokinase and cytotoxicity against MCF-7, has never been conducted.

For promising drug candidates to prevent cancer metastasis, these extracts can be isolated and structural elucidation. It is indicated that LC leaf extract has potential as a chemopreventive agent. It is important to continue the research to ensure a safe and non-toxic extract.

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Author's contributions
Bina Lohita Sari: conceived of the presented idea, Dien Puji Rahayu: Developed the theory of *in vitro* analysis, Euis Julaeha: Verified the analytical methods, Syaikhul Aziz: Developed the theory of *Cananga odorata*, Aulia Ilmiawati: Developed the theory of *Lantana camara*, Eli Yulia Sukmawati: Analyzed the optimization for *Cananga odorata*, and Laili Salsabila Suddin Putri: Analyzed the optimization for *Lantana camara.*

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Data availability statement
The datasets used in this research are available from the corresponding author on request.

Ethical approval
Not applicable.

Conflict of interest
The authors declare no conflict of interest.

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