RESEARCH ARTICLE

Editorial Process: Submission:11/18/2024 Acceptance:03/22/2025

Investigating the Potential Synergistic Effects of Turmeric Extract and Black Rice Bran as Cytotoxic Agents Against HeLa Cells

Rantika Silfarohana[1,2], Kuncara Nata Waskita[3], Nurul Hidayatul Mar'ah[3], Muhammad Saifur Rohman[4], Rizal Maarif Rukmana[5]*

Abstract

Objective: This study aimed to evaluate the synergistic effect of extract combinations from turmeric and black rice bran extract as anticancer agents on HeLa cells. **Methods:** Turmeric and black rice bran extracts were obtained by maceration with 96% ethanol. To determine the IC_{50} and combination index values, the cytotoxic assay of the extracts on HeLa cells was evaluated using the MTT assay, individually or in combination. The LC-HRMS analysis was employed to profile each extract. **Result:** Turmeric and black rice bran extracts yielded 23.29% and 7.57%, respectively, and LC-HRMS revealed that turmeric extract has the most ar-turmerone at 10.4%. In contrast, black rice bran extract contains many fatty acid derivative compounds. The MTT assay showed that turmeric extract had an IC_{50} value of 54 µg/mL against HeLa cells, while black rice bran extract had an IC_{50} value of 446 µg/mL. Combining ½ IC_{50} of turmeric extract and ½ IC_{50} of black rice bran extract has a synergistic effect, resulting in a lower viable cell population of 43.04% compared to the individual treatment of turmeric and black rice bran extract, which resulted in 96.92% and 79.30%, respectively. **Conclusion:** This study showed that a combination of turmeric and black rice bran exhibits greater cytotoxic activity than the individual extracts, promising an alternative anticancer treatment, particularly for cervical cancer.

Keywords: combination extract- cervical cancer- cytotoxicity- turmeric- black rice bran

Asian Pac J Cancer Prev, **26 (3)**, 1059-1067

Introduction

Cervical cancer is a disease-threatening that attacks women's reproductive system, the cervix uteri [1]. In 2022, the International Agency for Research on Cancer reported that the number of new cases of cervical cancer worldwide reached 662,401, representing 6.8% of all malignancies in females, referring to the GLOBOCAN 2022 estimates of cancer incidence and mortality. The mortality number attained 348,874 cases and evolved to the 4th most common cancer that causes death among women universally. The number of fatalities in cervical cancer increased by 2% from the previous report in 2020, which was 341,831 cases [2]. Recently, one of the standard treatments that have been recommended is chemotherapy using synthetic antineoplastic drugs. However, the program has side effects, high costs, and shows resistance, so it can relapse and even spread to other cells/tissues [3]. Other side effects of chemotherapy include directly changing the quality of cellular proteins, making them non-functional and affecting key cellular physiological pathways. Chemotherapy must also consider the dose; high doses damage healthy cells and cause disease recurrence [4].Therefore, a new therapeutic agent must be developed.

Utilization of plant extracts in cancer chemotherapy drug formulations is considered safer and has the lowest side effects [5]. Furthermore, the combination of plant extracts showed greater efficacy [6]. Various chemotherapeutic drugs are developed from natural components, including active chemicals found in plants, which show potential as anticancer medicines [7]. Potentially active phytochemicals are characterized by their perceived safety, low toxicity, diverse biological activity mechanisms, various chemical structures, and abundant natural availability [8].

*[1]Master Program of Biotechnology, Graduate School, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia. [2]Research Center for Vaccine and Drug, National Research and Innovation Agency (BRIN), Cibinong, Bogor, Indonesia. [3]The College of Health Sciences Bhakti Husada Mulia, Taman Praja street No. 25, Taman district, Madiun, East Java, Indonesia. [4]Department of Agricultural Microbiology, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia. [5]Research Center for Pharmaceutical Ingredients and Traditional Medicine, National Research and Innovation Agency (BRIN), Cibinong, Bogor, Indonesia. *For Correspondence: riza007@brin.go.id*

Turmeric (Curcuma longa L.) is prominent potential anticancer agent because it has many active compounds, such as α-curcumene, curcumenol, curcumin, curcumin II, and curcumin III [9]. One of the five phytochemicals is curcumin, which has the most effective anticancer activity [10]; however, the effectiveness is limited due to several limitations. The primary limitations of utilizing curcumin as a pharmaceutical agent include its limited bioavailability, poor water solubility, inadequate intestinal absorption, unstable molecular structure, and rapid elimination from the body [11,12]. Therefore, one way to overcome the obstacles is to combine curcumin with other extracts such as resveratrol [13], emodin [14], quercetin [15], or other plant extract.

Black rice bran (Oryza sativa L.) is one of the rice cultivars reported to have large necessary fatty acids and dietary fibers such as proteins, dietary minerals, and vitamins [16], in addition to the content of secondary metabolites such as phenolic, alkaloids, terpenoids, flavonoids, and steroids [17]. This cultivar also contained higher lipid content than other rice types [18]. Considering its tendency to be discarded and not consumed by humans, it could be explored as a concomitant treatment for cancer in conjunction with turmeric.

The potential of combining turmeric extract and black rice bran extract as an anticancer agent should be further developed. It was supported by the information from another study that black rice bran can induce apoptosis via caspase 8 activation [19]; meanwhile, turmeric inhibits cancer cell proliferation [20]. Given the multifaceted role of both extracts in anti-cancer properties, they are expected to be more effective when combined rather than relying solely on a single extract, as they may impact different mechanism targets [21,22]. Additionally, there has been no investigation into the potential of combining turmeric and black rice bran extract as chemotherapy agents for cancer. Thus, this study aims to evaluate the synergistic effect of this combination on HeLa cells.

Materials and Methods

The research was performed at the Laboratory of Traditional Medicine Raw Materials, National Research and Innovation Agency (BRIN) region Tawangmangu, Indonesia. This research has received ethical feasibility approval from the Ethics Committee of the Faculty of Medicine, Public Health, and Nursing at Gadjah Mada University, Indonesia designated by the number KE/FK/1459/EC/2023.

Chemical Reagent

Turmeric was collected from farmers of the "sentra tanaman obat" Karanganyar, Central Java, Indonesia and "Sembada Hitam" black rice cultivated from Sleman, Yogyakarta, Indonesia. The Laboratory of Traditional Medicine Raw Materials, BRIN, Tawangmangu, provided HeLa ATCC CCL-2 and Vero ATCC CCL-81 cell lines. Fetal Bovine Serum (F2442, Sigma Aldrich), dimethyl sulfoxide (D2650, Sigma Aldrich), Phosphate-buffered saline (P3813, Sigma Aldrich), RPMI 1640 powder (R6504, Sigma Aldrich), DMEM powder (D5648, Sigma Aldrich), MTT Reagent (M5655, Sigma Aldrich), Penicillin-streptomycin (P0781, Sigma Aldrich), fungizone (A2411, Sigma Aldrich), NaHCO3 (S5761, Sigma Aldrich) HEPES (643823, Sigma Aldrich) and sodium dodecyl sulfate (L3771, Sigma Aldrich) is purchased from Sigma Aldrich, St. Louis, Missouri. Filter paper (WHA1001090, Merck), and ethanol (64-17-5, Merck) were obtained from Darmstadt-based Merck Life Science.

Sample preparation

The turmeric rhizome was washed and sliced into small pieces. Turmeric and black rice bran samples were dried in an oven at 35-40°C to constant weight; then, the samples were ground and sieved through a 40-mesh sieve. Sixty grams of turmeric powder and 500 g of black rice bran were macerated in 96% ethanol solvent (1:10 w/v) for 2x24 hours with periodic stirring. The filtrate was collected and filtered using Whatman paper no. 1. The formula for calculating the yield (%) of evaporated dried extracts was DWextracts/DWsamples x 100%, where DWsamp is the sample's dry weight and DWext is the extract's weight after drying [23].

Cell Line Culture

HeLa cells were cultured in RPMI Media 1640, while Vero cells were in DMEM. Both media were supplemented with 10% (v/v) FBS, 1% (v/v) P/S, and 0.5% (v/v) fungizone. HeLa and Vero cells were cultured in a 25 cm^2 T-flask and incubated at 37°C and 5% CO_2 [24,25].

Test Solution Preparation

Turmeric extract was prepared as much as 2.5 mg and made a stock solution of 500 µg/mL concentration. The stock of turmeric extract was made into concentrations of 250, 125, 62.50, 31.25, 15.63, and 7.81 µg/mL. BRB extract was taken as much as 5 mg and made a stock concentration of 2500 µg/mL. The stock solution was then made into concentrations of 2500, 1250, 625, 312.5, 156.25, 78.12, and 39.06 µg/mL for analysis [26].

Cytotoxicity Assay in Single Extract

The confluent HeLa cells were harvested by trypsin-EDTA, re-suspended into 4 x 104 cells/well, and then inoculated into a 96-well plate with 100 µL per well. A 24-hour incubation period was followed by treatment of the cells with various amounts of individual extracts. Each concentration in the extract treatment was repeated 3 times and incubated for 24 hours. Then, 100 µL of MTT solution (5 mg/mL) was applied to each well and incubated for 4 hours at 37°C in a 5% CO2 incubator. To dissolve the formazan crystals, 100 µL of SDS 10% was applied to each well as a stoppan reagent. Under dark conditions, the plate was incubated overnight, and the absorbance was measured at 595 nm. The control group utilized untreated cells and achieved a 100% viability rate. One may use the following formula to calculate the percentage of cell viability by considering the absorbance value [27].

$$\text{Cell Viability (\%)} = \frac{Absorbance\ of\ treated\ cells - Absorbance\ of\ control\ media}{Absorbance\ of\ control\ cells - Absorbance\ of\ contro\ media}\ x\ 100\ \%$$

Cytotoxicity Assay in Combination Extract

The combination extract was created by combining turmeric extract and black rice bran extract, using the IC_{50} value obtained from a single cytotoxicity test. A total of 16 combinations of extracts were tested in order to identify the optimal concentration. The combination was derived by combining four different concentrations of turmeric extract (½ IC_{50}, ¼ IC_{50}, 1/8 IC_{50}, and 1/16 IC_{50}) with four different concentrations of black rice bran extract (½ IC_{50}, ¼ IC_{50}, 1/8 IC_{50}, and 1/16 IC_{50}). The combination formula assays were conducted concurrently as advised by another study to optimize the computational analysis of data [28]. The experimental design of this study is shown in Table 1. This analysis aimed to evaluate the combination index (CI) value.

LC-HRMS Profiling Analysis

The samples were acquired by combining 1 mL of MeOH with 50 mg of extract, subjecting it to vortexing for 30 seconds, sonication for 30 minutes at ambient temperature, and subsequent centrifugation for 5 minutes at a force of 1400xg. After collecting the supernatant, it was filtered using a 0.22 µm PTFE filter and injected into LC-HRMS. A liquid chromatography experiment was conducted using the Thermo Scientific™ Vanquish™ UHPLC Binary Pump and Orbitrap high-resolution mass spectrometry. The mobile phases used were MS-grade water containing 0.1% formic acid and MS-grade methanol containing 0.1% formic acid. The column utilized was Thermo Scientific™ Accucore™ Phenyl-Hexyl 100 mm × 2.1 mm ID × 2.6 µm, injected at a volume of 3 µL and a temperature of 70°C. The gradient elution reaction was conducted at a 0.3 mL/min flow rate for 45 minutes. The untargeted screening procedure used complete MS/dd-MS2 mode with positive and negative polarization ionization. The experimental setup included the following parameters: N2 gas pressure of 32, 8, and 4 AU; spray voltage of 3.3 kV; capillary temperature of 320°C; m/z range of 66.7-1000; and a resolution of 70,000 for full mass spectrometry (MS) and 17,5000 for double-diameter MS2 (dd-MS2) [29,30].

Statistical Analysis

The mean and standard deviation of the test result data were determined to conduct this investigation. The chemicals analyzed by LC-HRMS were determined using the Predicted Compositions facility of XCalibur 4.4 software and the mzCloud Search MS/MS library. The cytotoxicity test included combinations, single extracts, and confirmation using Vero cells, and the average percentage of cell viability was calculated for each extract. Next, the concentration of the extract was graphed against the percentage of cell viability to establish a linear regression equation, enabling the determination of the inhibitory concentration 50 (IC_{50}) value [31].

Results

Yield of Extraction Samples

The study employed test materials consisting of turmeric and black rice bran extracts. The extraction process was conducted by maceration with 96% ethanol, yielding 23.29% pure turmeric extract and 7.57% pure black rice bran extract.

Cytotoxic Activity Evaluation of Single Extract

The cytotoxicity assay was performed on the HeLa cell line to ascertain the cytotoxic effects of the single black rice bran and turmeric extracts. The anticancer activity of turmeric and black rice bran extracts was assessed using the MTT Assay, as indicated by the IC_{50} value. Table 2 shows the line equation of the cytotoxic assay and the IC_{50} value. The equation of the line yields an IC_{50} value of 54 µg/mL for turmeric extract categorized as potential cytotoxic and 446 µg/mL for black rice bran extract is moderate cytotoxic. Turmeric extract has an IC_{50} value that is 8 times lower than that of black rice bran extract.

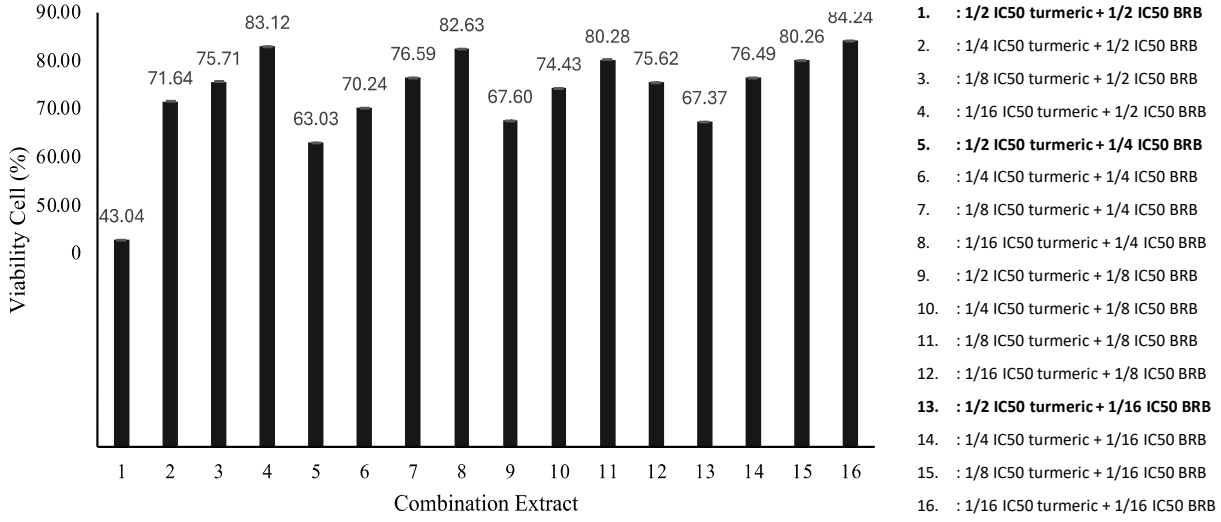
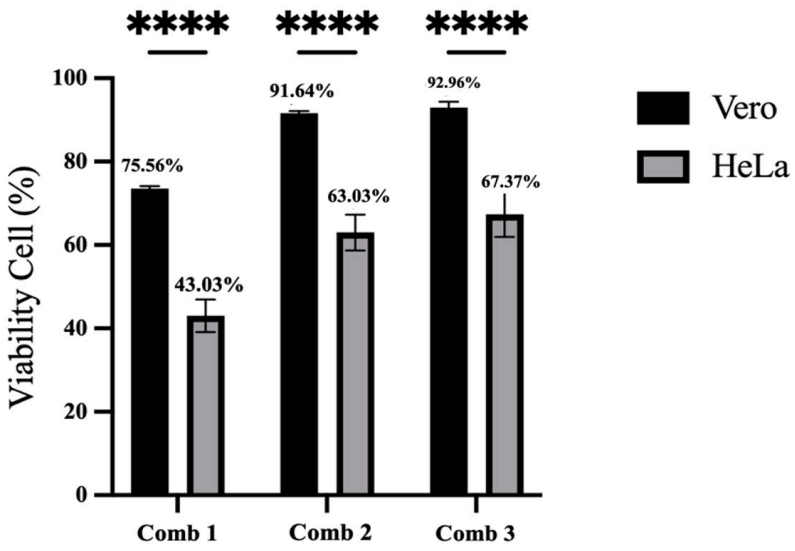


Figure 1. The Effectiveness of HeLa Cells Exposed to a 16-extract Combination



Comb 1: extract combination 1 (1/2 IC50 turmeric + 1/2 IC50 BRB)
Comb 2: extract combination 5 (1/2 IC50 turmeric + 1/4 IC50 BRB)
Comb 3: extract combination 13 (1/2 IC50 turmeric + 1/16 IC50 BRB)

Figure 2. Confirmation of the Effect of Three Selected Combination Extracts on Vero Cells Compare to HeLa Cells. The data represent the means ± SD. **** $p < 0.0001$

Table 1. The Formula of the Combination Extract from Turmeric and Black Rice Bran based on the IC_{50} Value Used in the Treatment

		Black Rice Bran (IC50)			
		1/2	1/4	1/8	1/16
Turmeric (IC_{50})	1/2	1/2 : 1/2	1/2 : 1/4	1/2 : 1/8	1/2 : 1/16
	1/4	1/4 : 1/2	1/4 : 1/4	1/4 : 1/8	1/4 : 1/16
	1/8	1/8 : 1/2	1/8 : 1/4	1/8 : 1/8	1/8 : 1/6
	1/16	1/16 : 1/2	1/16 : 1/4	1/16 : 1/8	1/16 : 1/16

Table 2. IC_{50} Value of Turmeric and Black Rice Bran against HeLa Cells

Extract	IC_{50} (mg/mL)	Line Equation	R^2
Turmeric	54	y = -54.831x+145.03	0.9616
Black Rice Bran	446	y = -55.022x+195.76	0.9357

Cytotoxic Activity Evaluation of Combination Extract

The cytotoxicity test of the combination extract was carried out using a mixture of turmeric extract and black rice bran extract at concentrations of ½ IC_{50}, ¼ IC_{50}, 1/8 IC_{50}, and 1/16 IC_{50}. From these concentrations, 16 possible variations of the combination extract were created and examined using the MTT Assay with the combination of the four concentrations listed above. Figure 1 displays the cell viability percentage at different extract concentrations from data processing generated by the MTT Assay. To evaluate the synergist between the individual extract and the combination extract, the combination index (CI) value was computed based on the percentage of cell viability, as shown in Table 3.

Figure 1 depicted that the more excellent combination extracts against HeLa cells were combination extract no.1, no.5, and no 13. Combination extract no.1 had a cell viability percentage of 43.04% and was considered moderately toxic. Extract no.5 had a viability of 63.03%

Table 3. Value of the Combination Index (CI) for HeLa Cells Treated with 16 Combination Extracts

		Black Rice Bran (IC50)			
		1/2	1/4	1/8	1/16
Turmeric (IC_{50})	1/2	0.25	0.27	0.22	0.16
	1/4	0.58	0.3	0.23	0.17
	1/8	0.72	0.41	0.32	0.18
	1/16	1.71	0.79	0.19	0.30

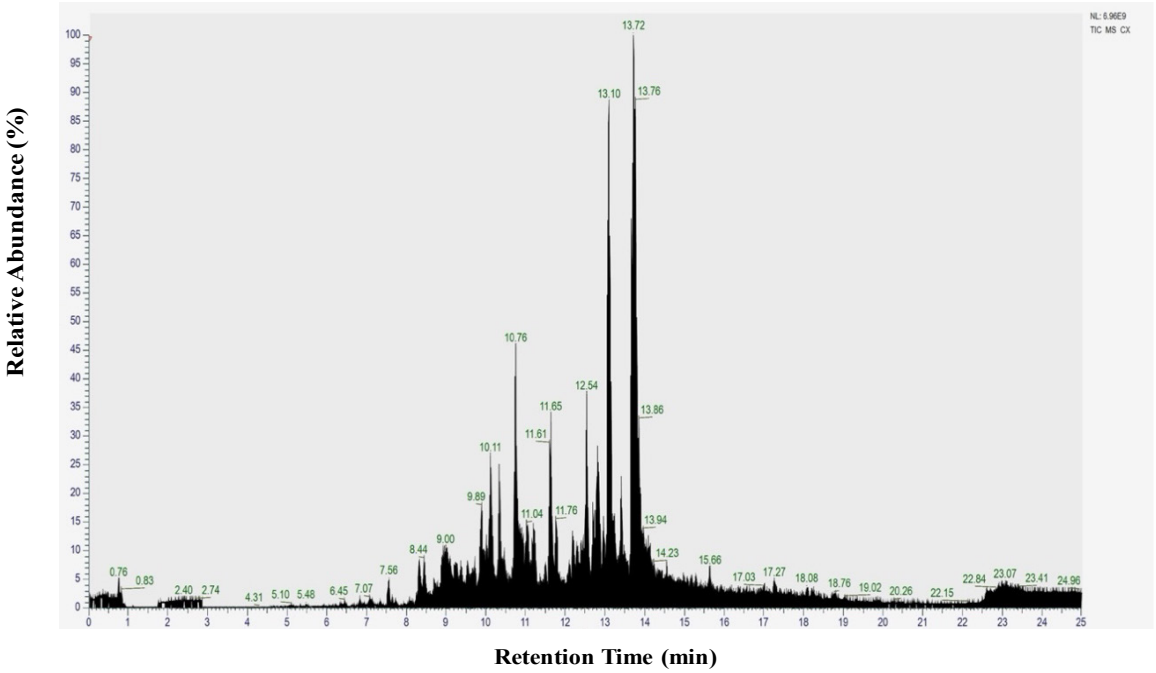


Figure 3. Total Ion Chromatogram (TIC) of the Ethanolic Extract of Turmeric

Table 4. Quantitative Analysis of Turmeric Extract and Black Rice Bran Extract with an Abundance above 0.3% Using LC-HRMS

Extract	Compound	Compound Group	Abundance (%)	Formula	RT (Min)
Turmeric	(+)-ar-Turmerone	Sesquiterpene	10.4	$C_{15}H_{20}O$	13.092
	Bisdemethoxycurcumin	Curcuminoid (phenol)	1.78	$C_{19}H_{16}O_4$	10.875
	Curcumin	Curcuminoid (phenol)	1.63	$C_{21}H_{20}O_6$	11.203
	Indole	Aromatic	0.48	C_8H_7N	13.737
Black Rice Bran	Phytosphingosine	Sphingolipid	13.41	$C_{18}H_{39}NO_3$	11.08
	Monoolein	Monoacylglycerol	5.04	$C_{21}H_{40}O_4$	14.948
	Sinensal	Sesquiterpene	4.92	$C_{15}H_{22}O$	13.674
	(+)-ar-Turmerone	Sesquiterpene	2.76	$C_{15}H_{20}O$	13.084
	(8E)-2-Amino-8-octadecene-1,3,4-triol	Sphingolipid	2.55	$C_{18}H_{37}NO_3$	10.654
	1-Piperideine	Alkaloid	1.54	C_5H_9N	0.283
	3-[(19Z)-15,16-Dihydroxy-19-do-triaconten-1-yl]-5-methyl-2(5H)-furanone	Heterocyclic	1.35	$C_{37}H_{68}O_4$	21.578
	Linoleoyl ethanolamide	Lipid	1.27	$C_{20}H_{37}NO_2$	13.666
	Choline		0.98	$C_5H_{13}NO$	0.774
	Oleoyl ethanolamide	Lipid	0.85	$C_{20}H_{39}NO_2$	14.314
	Betaine	Amino acid	0.67	$C_5H_{11}NO_2$	0.815
	2-Aminooctadec-4-yne-1,3-diol	Amino acid	0.66	$C_{18}H_{35}NO_2$	11.029
	Sphinganine	Lipid	0.55	$C_{18}H_{39}NO_2$	11.848
	(13cis)-2-Oxoretinoic acid	Vitamin A	0.48	$C_{20}H_{26}NO_3$	12.204
	15-cis-4,4'-diapophytoene	Carotenoid	0.47	$C_{30}H_{48}$	20.419
	2-Amino-1,3,4-octadecanetriol	Amino acid	0.41	$C_{18}H_{39}NO_3$	10.092
	2-[(5Z)-5-tetradecenyl]cyclobutanone	Ketone Cyclic	0.4	$C_{18}H_{32}O$	17.548
	3a,12a-Dimethyl-1-(5-methylene-2-heptanyl)tetradecahydro-7H-cyclopenta[a]cyclopropa[e]phenanthren-7-one	Vitamin D	0.37	$C_{28}H_{44}NO$	18.26
	Baicalein	Flavonoid	0.32	$C_{16}H1_4O_6$	8.769
	5-OxoETE	Arachidonate Acid (lipid)	0.31	$C_{20}H_{30}O_3$	10.408

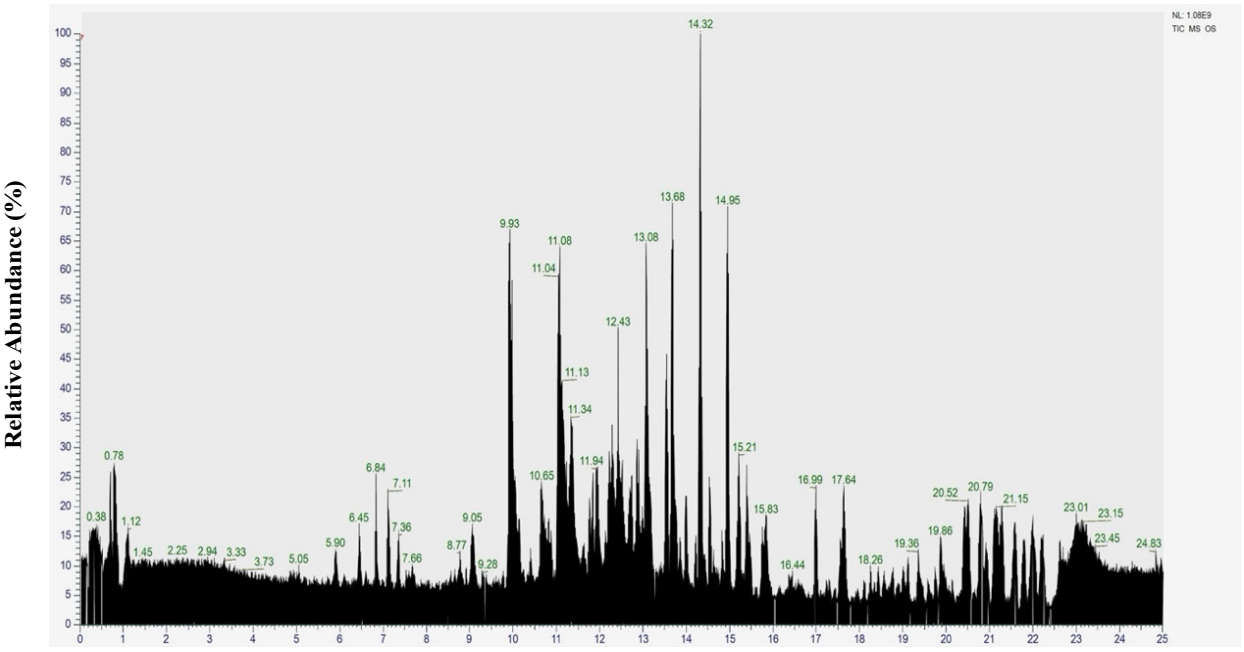


Figure 4. Total Ion Chromatogram (TIC) of the Ethanolic Extract of Black Rice Bran

and was classified as weakly toxic. Extract no.13 had a viability of 67.37% and was also classified as weakly toxic. According to CI evaluation, those combination extracts showed a strong synergistic effect (0.1 < CI < 0.3). CI value for combination extract no.1, no.5, and no. 13 were 0.25, 0,27, and 0.16, respectively.

The three combination extracts were chosen for safety analysis on normal cell lines, Vero cells (Figure 2). According to Figure 2, it is evident that the percentage of Vero cell viability in each treatment surpasses that of HeLa cells. These findings indicate that the three combination extracts do not harm normal cells, as demonstrated by the cell viability percentages of 75.65%, 91.64%, and 92.96%.

LC-HRMS Profiling of Ethanol Extracts

The chemical composition of turmeric and black rice bran extracts was analyzed using Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS). The raw data of LC-HRMS were processed using XCalibur 4.4 software, and the extract with the best total ion chromatogram (TIC) was processed for further analysis using Compound Discoverer® software. The LC-HRMS study indicated that the turmeric extract had diverse components with different concentrations. The compound ar-turmerone exhibited the most incredible abundance at 10.4%, with bisdemethoxycurcumin following at 1.78%, curcumin at 1.63%, and indole at 0.48%. Many lipid compounds have been discovered in the extract of black rice bran. Table 4 displays the results of the LC-HRMS analysis for compounds that have an abundance of over 0.3% and show potential biological activity, particularly in the context of drug development. Figure 3 displays the turmeric extract's total ion chromatogram (TIC), while Figure 4 is for black rice bran extract.

Discussion

This study aimed to evaluate the effect of the combination of turmeric and black rice bran extract as an anticancer agent on HeLa cells. The results indicated that turmeric extract is classified as potentially cytotoxic, while black rice bran extract has a moderate cytotoxic effect [13]. The extract can be categorized for its cytotoxic impact as potential cytotoxic (IC_{50} <100 μg/mL), moderate cytotoxic (IC_{50} <1000 μg/mL), or no cytotoxic (IC_{50} > 1000 μg/mL) [13]. However, as per the National Cancer Institute (NCI) in the United States, the extract can exhibit a potent cytotoxic impact when its IC_{50} value is less than 20 μg/mL [32].

In this research, the IC_{50} value of the turmeric extract against HeLa cells was 54 μg/mL. In contrast, the ethanolic turmeric extract in a previous study had an IC_{50} value of 184.5 μg/mL [33], threefold higher than the value reported in this study. The disparity in IC_{50} values is probably attributed to the varying origins of the turmeric utilized, resulting in distinct levels of active compounds and thereby demonstrating a cytotoxic effect and varying dosage requirements. Furthermore, the black rice bran extract achieved an IC_{50} value of 446 μg/mL. The results of this study are similar to those of previous studies, which stated that black rice bran extract has a cytotoxic effect on HeLa cells [34]. The IC_{50} value was determined to be 408.13 ± 51.9 μg/mL. The elevated IC_{50} value of black rice bran extract can be attributed to specific active compounds among the extract's components, which lack activity. Consequently, their capacity to decrease HeLa cell viability is diminished [34].

The cytotoxicity test of the combination extract was carried out using a mixture of turmeric extract and black rice bran extract at concentrations of ½ IC_{50}, ¼

IC_{50}, 1/8 IC_{50}, and 1/16 IC_{50}. According to the formula combination, three combination extracts were identified as potential anticancer drug candidates using HeLa cells, as determined by cell viability data from cytotoxicity experiments conducted with combination extracts. The combination extracts were chosen based on the lowest cell viability, below 70%, and the strongly synergistic CI value ($0.1 < CI < 0.3$) [28]. The cytotoxicity test is classified as non-toxic if the percentage of cell viability exceeds 80%, weakly toxic if the percentage is between 80% and 60%, moderately toxic if the rate is between 60% and 40%, and strongly toxic if the percentage is less than 40%, according to the provisions of ISO 10993-5 [35]. Three combination extracts were chosen for analysis. Combination extract number 1 (further mentioned as combination extract no. 1) had a cell viability percentage of 43.04% and was considered moderately toxic. Combination extract number 5 (further mentioned as combination extract no. 2) had a viability of 63.03% and was classified as weakly toxic. Combination extract number 13 (further mentioned as combination extract no. 3) had a viability of 67.37% and was also classified as weakly toxic, as depicted in Figure 1.

Additionally, the selection of the three extracts was determined by the combination index (CI) value, which indicated a strong synergistic effect ($0.1 < CI < 0.3$). An extract is considered to possess synergistic activity when combined with other substances, resulting in greater efficacy than when administered alone [22]. The effectiveness of combining a compound or extract can be achieved through various mechanisms. These include targeting different signaling pathways, enhancing bioavailability in the body, overcoming drug resistance, and improving efficiency [36]. Combined extract no. 1 was derived from these two parameters by combining ½ IC_{50} turmeric with ½ IC_{50} BRB. Combination extract no. 2 was formed by combining ½ IC_{50} turmeric with ¼ IC_{50} BRB, while combination no. 3 was obtained by combining ½ IC_{50} turmeric with 1/16 IC_{50} BRB.

According to Figure 2, it is evident that the percentage of Vero cell viability in each treatment surpasses that of HeLa cells. These findings indicate that the three combination extracts do not harm normal cells, as demonstrated by the cell viability percentages of 75.65%, 91.64%, and 92.96%. A preparation is deemed safe for cells if it exhibits a viability percentage above 80% [35]. From a statistical standpoint, it is evident that the administration of the combination extract has distinct effects on Vero cells and HeLa cells. This conclusion is supported by the alpha level 0.05 and a p-value of less than 0.0001 (****).

The identification of black rice bran extract components did not reveal the existence of anthocyanin compounds such as cyanidin-3-glucoside and peonidin-3-glucoside, as reported in other research [34, 37]. The variation in the identification approach with LC-HRMS is probably the cause of this discrepancy. This study employed an untargeted methodology [30]. The total ion chromatogram (TIC) analysis involved using Compound Discoverer software to identify the compound corresponding to the mzCloud library. This was done by considering the intensity of the ions automatically fragmented with DDA fragments. Automated DDA fragments may not accurately align with the fragmentation pattern for identifying anthocyanin compounds. Table 4 displays the results of the LC-HRMS analysis for compounds that have an abundance of over 0.3% and show potential biological activity, particularly in the context of drug development.

This investigation revealed that turmeric extract primarily comprised ar-turmerone and curcuminoid compounds. Turmerones are principal sesquiterpenes obtained from turmeric. Ar-Tumerone has demonstrated potential as an anticancer, immunomodulatory, and chemopreventive agent [38]. Previous research has shown that ar-Tumerone, isolated from Turmeric (Curcuma longa), possesses anti-cancer properties to inhibit the growth of various cancer cell lines, including K562, L1210, U937, and RBL-2H3 cells, by inducing apoptosis [39].

Additionally, ar-Tumerone has been demonstrated to inhibit the proliferation of cancer cells by reducing the expression of cathepsin B (CTSB) [20]. Notably, CTSB deficiency has been associated with the downregulation of cyclin B1 and the upregulation of the cyclin-CDK inhibitory protein P27kip1 in the G2 phase of the cell cycle, thereby contributing to growth inhibition in cancer cells [40]. Furthermore, curcuminoids have gained widespread recognition as potential therapeutic agents, particularly in cervical cancer treatment [41].

The black rice bran extraction results were dominated by phytosphingosine compounds, which were 13.41% abundant (Table 4). Phytosphingosine compounds have been known to inhibit the growth of lung cancer by inducing apoptosis via a mitochondria-mediated pathway, arresting the cell cycle in the G2/M phase, and increasing the expression of Bax/Bcl-2 proteins. This causes a decrease in mitochondrial membrane potential, encouraging the release of cytochrome C, caspase 9, and 3, and degrading PARP in A549 cells [42]. Both extracts play a role in preventing cancer growth at distinct targets, indicating the possibility for synergistic multi-target effects [22] that boost efficacy when combined [28], as seen by the reduced vitality of HeLa cells compared to the single treatment. However, more research is required to clarify the mechanisms of growth inhibition by this combination extract, particularly in cervical cancer.

Author Contribution Statement

All authors contributed equally in this study.

Acknowledgements

General

The authors thank the Laboratory of Traditional Medicine Raw Materials, National Research and Innovation Agency (BRIN) area Tawangmangu, Indonesia for supporting this study.

Funding Statement

This study was funded and supported by the Rumah Program scheme with contract number 23/III.9/HK/2023, administered by the Organisation Research for Health,

National Research and Innovation Agency (BRIN).

Approval

This research was conducted as part of Rantika Silfarohana's thesis during the Master Program of the Graduate School at Universitas Gadjah Mada, Indonesia.

Ethical Declaration

This research has received ethical feasibility approval from the Ethics Committee of the Faculty of Medicine, Public Health, and Nursing at Gadjah Mada University, Indonesia designated by the number KE/FK/1459/EC/2023. The Medical and Health Research Ethics Committee (MHREC) states that the documents meet The Officer for Human Research Protections (OHRP) under the U.S. Department of Health and Human Services regulations.

Availability of data

Data will be made available on request to the corresponding author.

Conflict of Interest

The authors state that there are no potential conflicts of interest.

References

1. Putri AR, Khaerunnisa S, Yuliati I. Cervical Cancer Risk Factors Association in Patients at the Gynecologic-Oncology Clinic of Dr. Soetomo Hospital Surabaya. Indones J Cancer. 2019;13(4):1-26. https://doi.org/10.33371/ijoc.v13i4.610
2. Andinata B, Bachtiar A, Oktamianti P, Partahi JR, Dini MSA. A Comparison of Cancer Incidences Between Dharmais Cancer Hospital and GLOBOCAN 2020: A Descriptive Study of Top 10 Cancer Incidences. Indones J Cancer. 2023;17(2):119-22. https://doi.org/10.33371/ijoc.v17i2.982
3. He M, Xia L, Li J. Potential mechanisms of plant-derived natural products in the treatment of cervical cancer. Biomolecules. 2021;11(10):1-26. https://doi.org/10.3390/biom11101539
4. Anand U, Dey A, Chandel AKS, Sanyal R, Mishra A, Pandey DK, et al. Cancer chemotherapy and beyond: Current status, drug candidates, associated risks and progress in targeted therapeutics. Genes Dis. 2023;10(4):1367–401. https://doi.org/10.1016/j.gendis.2022.02.007
5. Maarif Rukmana R, Puniawati Soesilo N, Pratiwi R. The Effect of Ethanolic Extract of Black and White Rice Bran (Oryza sativa L.) on Cancer Cells. Indones J Biotechnol. 2016;21(1):63-9. https://doi.org/10.22146/ijbiotech.26814
6. Rukmana RM, Sawal AA, Wibawa DA. Combination of antibacterial activity of ethanol extract of meniran leaves and kenikir leaves against Shigella dysenteriae. In1st International Conference for Health Research–BRIN (ICHR 2022) 2023 Mar 1 (pp. 7-20). Atlantis Press.
7. Seran AA, Peranginangin JM, Rukmana RM. Cytotoxic Activity and Antiangiogenesis of Extract Fraction of Coccinia grandis (L.) Voigt) Base Towards T47D Breast Cancer Cells and Chorio Allantoic Membrane (CAM) of Chicken Embryo Induced by bFGF. Jurnal Ilmiah Sains. 2020;20(1):37-48. https://doi.org/10.35799/jis.20.1.2020.27032
8. Talib WH, Awajan D, Hamed RA, Azzam AO, Mahmod AI, AL-Yasari IH. Combination Anticancer Therapies Using Selected Phytochemicals. Molecules. 2022;27(17):1–50. https://doi.org/10.3390/molecules27175452
9. Kusuma SMW, Utomo DH, Susanti R. Molecular Mechanism of Inhibition of Cell Proliferation: An In Silico Study of the Active Compounds in Curcuma longa as an Anticancer. J Trop Biodivers Biotechnol. 2022;7(3):1–16. https://doi.org/10.22146/jtbb.74905
10. Nocito MC, De Luca A, Prestia F, Avena P, La Padula D, Zavaglia L, et al. Antitumoral activities of curcumin and recent advances to improve its oral bioavailability. Biomedicines. 2021;9(10):1–38. https://doi.org/10.3390/biomedicines9101476
11. Lopresti AL. The problem of curcumin and its bioavailability: Could its gastrointestinal influence contribute to its overall health-enhancing effects?. Adv Nutr. 2018;9(1):41–50. https://doi.org/10.1093/advances/nmx011
12. Nelson KM, Dahlin JL, Bisson J, Graham J, Pauli GF, Walters MA. The Essential Medicinal Chemistry of Curcumin. J Med Chem. 2017;60(5):1620–37. https://doi.org/10.1021/acs.jmedchem.6b00975
13. Arena A, Romeo MA, Benedetti R, Masuelli L, Bei R, Gilardini Montani MS, et al. New insights into curcumin-and resveratrol-mediated anti-cancer effects. Pharmaceuticals. 2021;14(11):1-2. https://doi.org/10.3390/ph14111068
14. Guo J, Li W, Shi H, Xie X, Li L, Tang H, et al. Correction to: Synergistic effects of curcumin with emodin against the proliferation and invasion of breast cancer cells through upregulation of miR-34A. Mol Cell Biochem. 2021;477(3):979–80. https://doi.org/10.1007/s11010-021-04312-0
15. Srivastava NS, Srivastava RAK. Curcumin and quercetin synergistically inhibit cancer cell proliferation in multiple cancer cells and modulate Wnt/β-catenin signaling and apoptotic pathways in A375 cells. Pytomedicine. 2019;52:117–28. https://doi.org/10.1016/j.phymed.2018.09.224
16. Yu Y, Zhang J, Wang J, Sun B. The anti-cancer activity and potential clinical application of rice bran extracts and fermentation products. RSC Adv. 2019;9(31):18060–9. https://doi.org/10.1039/c9ra02439e
17. Wang W, Li Y, Dang P, Zhao S, Lai D, Zhou L. Rice secondary metabolites: Structures, roles, biosynthesis, and metabolic regulation. Molecules. 2018;23(12):1-50. https://doi.org/10.3390/molecules23123098
18. Fatchiyah F, Sari DRT, Safitri A, Cairns JRK. Phytochemical compound and nutritional value in black rice from Java Island, Indonesia. Syst Rev Pharm. 2020;11(7):414–21. https://doi.org/10.31838/srp.2020.7.61
19. Park MT, Kang JA, Choi JA, Kang CM, Kim TH, Bae S, et al. Phytosphingosine Induces Apoptotic Cell Death via Caspase 8 Activation and Bax Translocation in Human Cancer Cells 1. Clin Cancer Res. 2003;9:878–85.
20. Cao W, Chen X, Xiao C, Lin D, Li Y, Luo S, et al. Arturmerone inhibits the proliferation and mobility of glioma by downregulating cathepsin B. Aging. 2023;15(18):9377–90. https://doi.org/10.18632/aging.204940.
21. Imming P, Sinning C, Meyer A. Drugs, their targets and the nature and number of drug targets. Nat Rev Drug Discov. 2006;5:821–34. https://doi.org/10.1038/nrd2132
22. Pezzani R, Salehi B, Vitalini S, Iriti M, Zuñiga FA, Sharifi-Rad J, et al. Synergistic effects of plant derivatives and conventional chemotherapeutic agents: An update on the cancer perspective. Medicina. 2019;55(4):1-6. https://doi.org/10.3390/medicina55040110
23. Maharani AA, Puspawati N, Rukmana RM. The phytochemical composition of medicinal plants in Indonesia and their potential as antibacterial agents against Salmonella typhi ATCC 13311. InIOP Conference Series: Earth Environ

Sci. Institute of Physics; 2024:1–8.
24. Pincigher L, Valenti F, Bergamini C, Prata C, Fato R, Amorati R, et al. Myrcene: A Natural Compound Showing Anticancer Activity in HeLa Cells. Molecules. 2023;28(18):1-6. https://doi.org/10.3390/molecules28186728
25. Fadholly A, Ansori ANM, Proboningrat A, Nugraha AP, Iskandar RPD, Rantam FA, et al. Apoptosis of hela cells via caspase-3 expression induced by chitosan-based nanoparticles of Annona squamosa leaf extract: In vitro study. Indian J Pharm Educ Res. 2020;54(2):416–21. https://doi.org/10.5530/ijper.54.2.47
26. Qurishi Y, Seshadri VD, Poyil MM, Franklin JB, Apte DA, Alsharif MHK, et al. Anticancer activity in HeLa and MCF-7 cells via apoptopic cell death by a sterol molecule Cholesta-4,6-dien-3-ol (EK-7), from the marine ascidian Eudistoma kaverium. J King Saud Univ Sci. 2021;33(4):1-0. https://doi.org/10.1016/j.jksus.2021.101418
27. Zamrus SNH, Akhtar MN, Yeap SK, Quah CK, Loh WS, Alitheen NB, et al. Design, synthesis and cytotoxic effects of curcuminoids on HeLa, K562, MCF-7 and MDA-MB-231 cancer cell lines. Chem Cent J. 2018;12(1):1-0. https://doi.org/10.1186/s13065-018-0398-1
28. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev. 2006;58(3):621–81. https://doi.org/10.1124/pr.58.3.10
29. Rochat B, Mohamed R, Sottas PE. LC-HRMS metabolomics for untargeted diagnostic screening in clinical laboratories: A feasibility study. Metabolites. 2018;8(2):1-7. https://doi.org/10.3390/metabo8020039
30. Windarsih A, Suratno, Warmiko HD, Indrianingsih AW, Rohman A, Ulumuddin YI. Untargeted metabolomics and proteomics approach using liquid chromatography-Orbitrap high resolution mass spectrometry to detect pork adulteration in Pangasius hypopthalmus meat. Food Chem. 2022;386:1-9. https://doi.org/10.1016/j.foodchem.2022.132856
31. Pratiwi R, Amalia AR, Anindito Sri Tunjung W, Rumiyati. Active fractions of black rice bran cv cempo ireng inducing apoptosis and S-phase cell cycle arrest in T47D breast cancer cells. J Math Fundam Sci. 2019;51(1):47–59. https://doi.org/10.5614/j.math.fund.sci.2019.51.1.4
32. Somaida A, Tariq I, Ambreen G, Abdelsalam AM, Ayoub AM, Wojcik M, et al. Potent cytotoxicity of four cameroonian plant extracts on different cancer cell lines. Pharmaceuticals. 2020;13(11):1–19. https://doi.org/10.3390/ph13110357
33. Kurniawan C, Siagian JW, Hutomo S. Cytotoxicity of Ethanolic Extract of Curcuma Longa on Hela Cells, In Vitro Study. Yogyakarta; 2016 Sep.
34. Pratiwi R, Anindito Sri Tunjung W, Rizqi Amalia A. Black Rice Bran Extracts and Fractions Containing Cyanidin 3-glucoside and Peonidin 3-glucoside Induce Apoptosis in Human Cervical Cancer Cells. Indones J Biotechnol. 2015;20(1):69-79. https://doi.org/10.22146/ijbiotech.15271
35. López-García J, Lehocký M, Humpolíček P, Sáha P. HaCaT Keratinocytes Response on Antimicrobial Atelocollagen Substrates: Extent of Cytotoxicity, Cell Viability and Proliferation. J Funct Biomater. 2014;5(2):43–57. https://doi.org/10.3390/jfb5020043
36. Rahmawati DR, Nurrochmad A, Jenie RI, Meiyanto E. The Synergistic Cytotoxic Effect of Pentagamavunon-1 (PGV-1) and Curcumin Correlates with the Cell Cycle Arrest to Induce Mitotic Catastrophe in 4T1 and T47D Breast Cancer Cells. Indones Biomed J. 2023;15(5):318–27. https://doi.org/10.18585/inabj.v15i5.2594
37. Sari DRT, Paemanee A, Roytrakul S, Cairns JRK, Safitri A, Fatchiyah F. Black rice cultivar from Java Island of Indonesia revealed genomic, proteomic, and anthocyanin nutritional value. Acta Biochim Pol. 2021;68(1):55–63. https://doi.org/10.18388/abp.2020_5386
38. Nair A, Amalraj A, Jacob J, Kunnumakkara AB, Gopi S. Non-curcuminoids from turmeric and their potential in cancer therapy and anticancer drug delivery formulations. Biomolecules. 2019;9(1):1-36. https://doi.org/10.3390/biom9010013
39. Ji M, Choi J, Lee J, Lee Y. Induction of apoptosis by arturmerone on various cell lines. Int J Mol Med. 2004;14:253-6.
40. Bian B, Mongrain S, Cagnol S, Langlois MJ, Boulanger J, Bernatchez G, et al. Cathepsin B promotes colorectal tumorigenesis, cell invasion, and metastasis. Mol Carcinog. 2016;55(5):671–87. https://doi.org/10.1002/mc.22312
41. Zhang X, Zhu L, Wang X, Zhang H, Wang L, Xia L. Basic research on curcumin in cervical cancer: Progress and perspectives. Biomed Pharmacother. 2023;162:1–0. https://doi.org/10.1016/j.biopha.2023.114590
42. Cai Q, Chen M, Wang B, Wang J, Xia L, Li J. Phytosphingosine inhibits the growth of lung adenocarcinoma cells by inducing g2/m-phase arrest, apoptosis, and mitochondria-dependent pathway cell death in vitro and in vivo. Chem Biol Interact. 2024;387:110795. https://doi.org/10.1016/j.cbi.2023.110795.