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Original Article

SYNTHESIS OF BLACK TURMERIC EXTRACT NANOPARTICLES (*CURCUMA CAESIA*) AND ITS CYTOTOXIC ACTIVITY ON T47D CELLS

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ABSTRACT

Objective: This study aimed to formulate black turmeric into nanoparticle preparations with various concentrations of chitosan and determine its cytotoxic effect on T47D breast cancer cells.

Methods: Extraction was carried out by the maceration method. Black turmeric condensed extract was formulated into nanoparticles using the ionic gelation method, which was a method that relies on the cross-linking agent sodium tripolyphosphate (Na-TPP). The cytotoxic activity of black turmeric extract was tested using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay method.

Results: The results showed that black turmeric extract nanoparticles have a size range of 266-558 nm and were positively charged with zeta potential values ranging from 3.3 to 9.7 mV. The encapsulation efficiency of black turmeric extract nanoparticles was 63.42%. The results of the cytotoxic test showed that both black turmeric extract and black turmeric extract nanoparticles showed moderate cytotoxic activity, with the IC_{50} values of the two preparations were 78.60 µg/ml and 162.95 µg/ml, respectively.

Conclusion: The results obtained in this research indicate a promising potential of nanoparticles of black turmeric extract as a cytotoxic agent for the treatment of breast cancer.

Keywords: *Curcuma caesia*, Nanoparticles, Cancer, Cytotoxic, T47D

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INTRODUCTION

The global mortality rate for all types of cancer in women is 21.5% per year, whereas 30.5% of newly diagnosed cancer cases are detected. The incidence rate of breast cancer is 40 per 100,000 women, making it the most prevalent form of cancer affecting women globally. T47D cells in breast cancer exhibit sensitivity to chemotherapeutic drugs, including doxorubicin, despite the presence of a mutant P53 protein. T47D cells pose a significant threat to human life due to their persistent occurrence of cell damage. The treatment employed for breast cancer currently exhibits numerous limitations, including a multitude of unfavorable side effects, non-specific functionality, inadequate effectiveness, and exorbitant costs [1]. Chemotherapy exhibits limited efficacy throughout some time frames and has failed to yield satisfying outcomes. Therefore, it is crucial to select a cancer treatment that is both safe and specifically targets the disease [2].

Numerous chemicals found in nature exhibit a diverse array of pharmacological characteristics. Traditional medicine has demonstrated the practical efficacy of utilizing natural sources for disease treatment with fewer adverse effects compared to modern drugs [3]. Black turmeric is a plant that belongs to the Zingiberaceae family. *Curcuma caesia* contains many compounds, such as alkaloids, terpenoids, amino acids, carbohydrates, tannins, flavonoids, and curcumin, which are the main active components. Black turmeric possesses significant pharmacological activity, which contributes to its considerable commercial worth [4, 5].

Prior studies have indicated that black turmeric contains curcuminoids, which are composed of several components. The composition of curcuminoids is primarily made up of curcumin (77%), demethoxycurcumin (17%), and bisdemethoxycurcumin (3%) [6]. Curcumin exhibits high solubility in organic solvents such as acetone, methanol, dimethyl sulfoxide, and ethanol, while its solubility in water is comparatively lower. Curcumin is characterized by the presence of a methylene molecule (-CH2-) connecting the two ketone groups in its structure, rendering it very unstable and prone to oxidation [7].

Curcuminoids were also found to possess beneficial biological properties, including their role as antioxidants. The highest dose of black turmeric extract exhibited a remarkable free radical-scavenging capacity of over 90%. Free radicals can be generated within the body (endogenously) or introduced from external sources (exogenously). While a moderate level of free radicals can have positive effects on the body, an excessive number of free radicals can be detrimental. Antioxidants function as immunomodulators in the body, enhancing the resilience of healthy cells to counteract the harmful effects of free radicals, which possess the capacity to develop into cancer [8].

Previous research has shown that the methanol extract of *Curcuma caesia*, also known as black turmeric, has antioxidant and antimetastatic properties. It has been found to have an IC_{50} value of 90.70 µg/ml, indicating its potential as an alternative treatment for cancer and tumors. Moreover, the flavonoid content functions as a cancer inhibitor by obstructing the signal transduction pathway from the membrane to the cell nucleus [9].

A cytotoxic substance or drug candidate is one that effectively kills and hinders the proliferation of growing cells. Its potency is measured by its IC_{50} value. The IC_{50} value of black turmeric concerning its potential as an alternative cancer treatment remains relatively low, as indicated by multiple research. The primary focus while preparing black turmeric extract is to preserve its antioxidant activity and ensure that the antioxidant chemicals remain effective in combating cancer by preventing oxidation. This work utilized a nanoparticle-based synthesis strategy using black turmeric extract, similar to previous research on compounds with possible therapeutic effects for cancer [10].

Nanotechnology advancements have showcased the immense capabilities of nanoparticles in the medical field. Nanoparticles are ideal for targeted and regulated administration of micro-and macromolecules in disease therapy due to their high carrier capacity, ability to form stable connections with ligands, and ability to bind both hydrophilic and hydrophobic compounds [11]. Polymers can effectively preserve the antioxidant chemicals included in a medicinal extract. Furthermore, advancements in nanoparticle production for treating degenerative diseases like cancer have proven to be beneficial in enhancing the stability and accessibility of curcumin, a potent active substance. Additionally, the IC_{50} value of black turmeric extract can be increased to enhance its therapeutic efficacy [12].

This research aims to determine the feasibility of synthesizing nanoparticle formulations from black turmeric extract and to assess its potential cytotoxic activity, as indicated by the IC_{50} value, for use as an anti-cancer agent. This research aims to assess the potential of plants in Indonesia to function as cytotoxic agents, which could serve as an alternative for early-stage cancer prevention and treatment. The resulting nanoparticles, with a size range of 1-1000 nm, offer the advantage of reduced side effects. Furthermore, the black turmeric extract's remarkable metabolic stability and capacity to preserve antioxidants enhance its desirability as a target for intracellular uptake in cancer cell tissue, particularly due to its high membrane permeability. Nanoparticle technology can enhance the IC_{50} value, hence increasing the efficacy of therapy.

MATERIALS AND METHODS

The conducted research was an experimental study. The nanoparticle formulation was conducted by the ionic gelation process, which involves the creation of nanoparticles by utilizing the electrostatic interaction between the positively charged amine groups on chitosan and the negatively charged Na-TPP polyanions, resulting in the construction of a three-dimensional structure [13]. The formation of the cross-linking process using the ionic gelation method would strengthen the mechanics of the particles formed [14], while the cytotoxic test used the MTT assay method.

Materials

The materials used in the research were black turmeric rhizome (*Curcuma caesia*), was purchased from Riau (Indonesia). Ethanol pa, methanol pa, acetic acid, chitosan, Sodium Tripolyphosphate (Na-TPP), tween 80, $AlCl_3$ reagent, RPMI Media, Phosphate Buffer Saline, SDS stopper, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent were purchased from Sigma-Aldrich.

Preparation of black turmeric extract nanoparticles

The formulation used was adopted from research conducted by Samudra *et al.* [15] as in table 1. Nanoparticles were synthesized via the ionic gelation technique by dissolving 0.1 g of concentrated black turmeric extract in 150 ml of 95% ethanol. Chitosan of different weights (in milligrams) was dissolved in 1% volume/volume acetic acid, reaching a total volume of 100 ml. The solution was then agitated using a magnetic stirrer until it became uniform. Chitosan solution was added to a beaker, followed by the addition of extract. The mixture was then agitated using a magnetic stirrer for 10 min. Subsequently, 9 ml of Na-TPP was incrementally introduced in a slow and controlled manner. After achieving homogeneity, 3 ml of Tween 80 was added drop by drop. Once all the components were thoroughly combined and nanoparticles were generated, the solution underwent sonication for a duration of 20 min in order to decrease the particle size.

Table 1: Nanoparticle formulas containing black turmeric extracts

Ingredients	F1	F2	F3
Black turmeric extract	100 mg	100 mg	100 mg
Chitosan 0.75 %	18 ml	-	-
Chitosan 0.1%	-	18 ml	-
Chitosan 1.25 %	-	-	18 ml
Na-TPP 0.1%	9 ml	9 ml	9 ml
Tween 80	3 ml	3 ml	3 ml

Characterization of black turmeric extract nanoparticles

The characterization of black turmeric extract nanoparticles included the determination of particle size, percentage of transmittance, zeta potential, and identification of functional groups. The analysis of percent transmittance was conducted using UV-Vis spectroscopy (Genesys 10 UV). The particle size and zeta potential derived from observations of the sample using the Particle Size Analyzer (Horiba SZ-100) and the Fourier Transform Infrared Spectroscopy (PerkinElmer UATR Two) are utilized to determine the functional groups present in the nanoparticles.

Calculation of encapsulation efficiency of black turmeric rhizome-chitosan nanoparticles

Prior to determining the encapsulation efficiency, the nanoparticles underwent centrifugation at a velocity of 5000 revolutions per minute in order to separate the unbound curcumin in the liquid portion. The supernatant obtained was analyzed using UV-Vis spectroscopy at a maximum wavelength of 558 nm. The absorbance value obtained was then incorporated into the standard curve equation Y = 0.0995X-0.0205 and calculated using the given equation:

$$\% \text{ EE} = \frac{\text{total drug concentration} - \text{free drug concentration}}{\text{total drug concentration}} \text{ x } 100 \%$$

Cytotoxic test

Prior to conducting the cytotoxic test, all instruments were sterilized. A volume of 100 µl** of cell suspension in RPMI medium was added to each well of a 96-well plate and incubated for 24 h. The status of cell distribution was assessed by observing the cells under an inverted microscope. The cells were cultured in the incubator for a period of 24 h until they reestablished their usual condition, specifically by attaching to the bottom of the media well. The wells containing cells were retrieved from the incubator and the cell medium was extracted and rinsed using 100 µl of PBS once. A solution of MTT reagent at a concentration of 0.5 mg/ml was produced by mixing 1 ml of MTT stock with 10 ml of culture medium diluted in PBS.

A concentration series of 100.0 µl of nanoparticles derived from black turmeric rhizomes was prepared. The nanoparticles were then added to the wells in triplicate and incubated until the cytotoxic effect became observable, which took 24 h. After a duration of 24 h, the cell medium was extracted and 100.0 µl of MTT reagent was introduced to each well, including the well that served as a control for the medium (without cells). The cells were cultured for an additional 2 h in the incubator until the presence of formazen became apparent. When formazen was easily seen, 100.0 µl of 10% SDS stopper in 0.1 N HCl was introduced. The last stage entails enveloping the plate with aluminum foil and subjecting it to incubation in a light-free environment or at ambient temperature. The measurement of absorbance for each well was performed using an ELISA reader set at a wavelength of 595 nm.

$$\text{Living cells (\%)} = \frac{\text{Intervened absorbance} - \text{Control media absorbance}}{\text{Cells control absorbance} - \text{Control media absorbance}} \text{ x } 100\%$$

RESULTS AND DISCUSSION

Extraction and synthesis of black turmeric extract nanoparticles

The maceration process produced a thick extract of 19.47 g, representing a 3.894% yield. This demonstrated the appropriateness of using 96% ethanol as the solvent in the extraction procedure. The production of black turmeric is determined by the solubility of its medicinal components, with curcumin being the most abundant compound.

The production of black turmeric extract nanoparticles was conducted through the ionic gelation technique, which relies on the electrostatic interaction between the positively charged amine group on chitosan and the negatively charged Na-TPP polyanion to create a three-dimensional structure [13]. When producing nanoparticles, several concentrations of chitosan were used, specifically 0.75%, 1%, and 1.25%. This was done because changes in chitosan concentration can impact the size of the particles and the zeta potential value of the nanoparticle suspension. The nanoparticle analysis indicated that the optimal chitosan concentration is F2, which corresponds to a chitosan concentration of 1%. This was demonstrated by the clear look of the nanoparticle suspension preparation, which does not exhibit any aggregation [16]. The

production of black turmeric extract nanoparticles involved the application of the electrostatic cross-linking principle, which utilized the positive amine groups in chitosan and the non-toxic Na-TPP polyanion. Additionally, Tween 80 was incorporated to reduce interfacial tension and enhance the solubility of chitosan. Tween 80 is a surfactant with the intended effect of decreasing particle size and enhancing the stability of the nanoparticle emulsion.

Physicochemical characteristics of black turmeric extract nanoparticles

Nanoparticle characterization was carried out to meet the quality of appropriate nanoparticle standards. Some of the parameters measured are transmittance (%), particle size, and polydisperse index (table 2).

Table 2: Characterization of black turmeric extract nanoparticles

Chitosan (%)	Particle size (nm)	PI (µm)	Zeta potential (mV)
0.75%	558.8±17.6	0.340±0.036	+9.7±0.7
1%	266.6±19.2	0.528±0.168	+7.0±1.5
1.2 %	298.1±375.7	0.586±0.045	+3.3±3.8

Notes: Average value±SD (triple replication)

The results of the research are in accordance with research by Samudra *et al.*, [15] that varying the concentration of chitosan in nanoparticles produced nanoparticle-sized preparations. The statistical test findings indicate that the F count (1.63229) is smaller than the F crit (5.14325), which means that there was no significant difference in the size variable. Nevertheless, in the absence of concentration change, increasing the concentration of chitosan while maintaining a steady concentration of Na-TPP is more likely to result in agglomeration or clumping, consequently leading to an increase in particle size. This is demonstrated by the particle size of an effective nanoparticle formulation, specifically Formula 2 containing 1% chitosan. In addition, the utilization of solvent ratios significantly impacts the production of nanoparticles. The three formulations demonstrate the ability to produce nanoparticles within a size range of 1-1000 nm. However, for enhanced stability, it is preferable for the nanoparticle preparation to possess a zeta potential value in close proximity to+/-30 mV [17, 18]. The polydispersity index value of the three preparations remains within the range of 0.1–0.7. This result is lower compared to the research conducted by Rabima and Sari [19].

Determination of functional groups of black turmeric extract nanoparticles

The results of the analysis of the functional groups of black turmeric extract nanoparticles were known from specific peaks and areas from the FTIR data (fig. 1).

Comparison of the three spectra from black turmeric extract, empty chitosan nanoparticles, and black turmeric extract chitosan nanoparticles all showed wide absorption at wave numbers 3000-3600 cm^{-1} with identical peaks at 3227, 3301, and 3264 cm^{-1}. These results interpreted the stretching vibration of the –OH group, which overlaps with NH. The three samples also showed the existence of an amide bond (-NHCO) as indicated by the absorption of the carbonyl group at wave numbers 1671, 1684 and 1689 cm^{-1}, while the stretching vibration of the ketone group with the C=O bond is shown at wave numbers 1602, 1630, and 1633 cm^{-1}.

The stretching vibration of the C-N bond was shown at wave numbers 1241 and 1240 cm^{-1}, originating from the amine group of the aliphatic chain. C-O-C stretching vibrations were shown at wave numbers 1130 and 1123 cm^{-1} which originated from β-1,4-glycosidic bonds. The same thing was also found in the previous research, wherein in the chitosan sample, there were glycosidic bonds from C-O-C vibrations at a wave number of 1152 cm^{-1} [20]. As explained in research by Pakkirisamy *et al.* black turmeric contains secondary metabolite compounds such as tannins, terpenoids, flavonoids, phenols, phytosterols, and saponins, which have C-O bonds and were detected at a wave number of 1077 cm^{-1} [21]. Apart from that, the sharpness of the peak is also caused by the formation of bonds between curcumin and chitosan, the formation of bonds between curcumin-chitosan and nanoparticles showed a peak indicating the P=O group at wave number of 1032-972 cm^{-1}. Peaks at wave numbers 917 and 903 cm^{-1} interpreted the formation of bonds between nanoparticles that form bonds between P=O and Na-TPP.

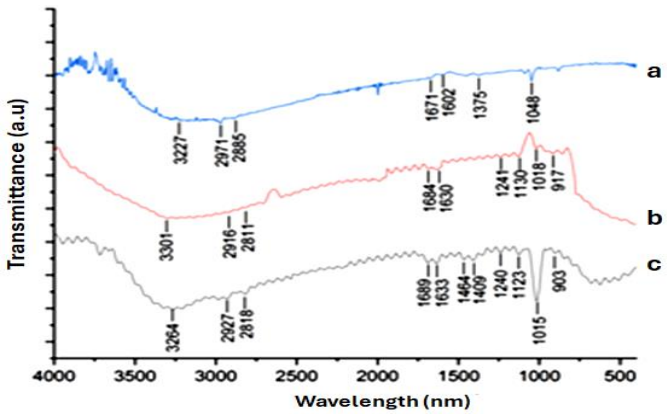

Fig. 1: FTIR spectra of black turmeric extract (a), empty chitosan nanoparticles (b), black turmeric extract chitosan nanoparticles (c)

Encapsulation efficiency (EE) of black turmeric rhizome nanoparticles

EE refers to the proportion of the active material that is absorbed in the nanoparticle preparation. The equation Y = 0.0995X – 0.0205, derived from the standard curcumin solution's linear standard curve, was employed to calculate the EE. The calculation findings indicate that the EE value obtained from three successive replications of formula 2 was 64.94%, 62.16%, and 63.16%, with an average value of 63.42±1.41%. This suggested that not all of the curcumin was utilized. The research conducted by Rabima and Sari reveals that the curcumin was discovered to be perfectly

encapsulated. According to their findings, an optimum EE value was near 100% [19]. According to research conducted by Arozal *et al.*, if the ratio of chitosan concentration as an encapsulation agent to the active substance curcumin is increased, it can result in insufficient space for the nanoparticles to absorb the curcumin. As a result, a significant amount of curcumin remains unabsorbed or free [22].

Cytotoxic activity of black turmeric extract nanoparticles

The IC50 value is a metric that indicates the concentration at which 50% of the surviving cell population can be inhibited, thereby reflecting the cytotoxic capability. Under microscopic examination, distinct morphological disparities were seen between viable and non-viable T47D cells (fig. 2). The untreated cells exhibited a spherical morphology with distinct cell nuclei, as the cytoplasm, which allows light transmission under the microscope, formed clusters and adhered to the wells. Dead T47D cells exhibited a spherical shape, with their cell nuclei appearing dark and distinct from the cell cluster. Additionally, the nuclei did not be connected to the wells, as demonstrated in the doxorubicin positive control depicted in fig. 2f. Fig. 2b depicted the visual representation of T47D cells, which indicated the occurrence of cell death. In fig. 2e, there was a noticeable increase in the quantity of deceased cells. The observed cell death in T47D cells was most likely attributed to the presence of secondary metabolites, specifically flavonoids and curcumin, found in the extract of black turmeric [23].

Based on the results of the cytotoxic test, it has been determined that black turmeric extract exhibits moderate cytotoxic potential (table 3).

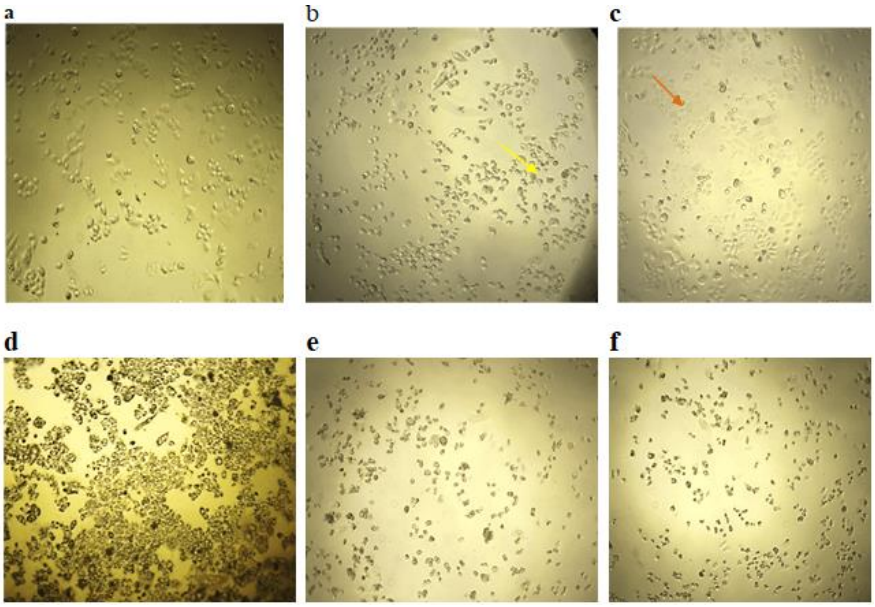

Fig. 2: Morphology of T47D cells before treatment (a), after adding black turmeric extract nanoparticles (b), after adding empty nanoparticles (c), formazan crystals (d), after adding black turmeric ethanol extract (e), after adding doxorubicin (f). Yellow and orange arrows indicate cells that are dead and alive, respectively

Table 3: Linear regression equation for each sample and IC_{50}

Sample	Linear regression	IC_{50} (µg/ml)
Black turmeric extract nanoparticles	Y =-0.3077x+100.14 (R^2 = 0.9299)	162.95
Black turmeric extract	Y =-0.3862x+80.356 (R^2= 0.7019)	78.60
Empty nanoparticles	Not detected	Not detected

This conclusion was drawn from research involving the IC_{50} values. It is observed that concentrations below 10 µg/ml are considered very strong, concentrations between 10-20 µg/ml are classified as strong, and concentrations between 50-100 µg/ml are considered weak but still exhibit activity against cancer cells. Conversely, nanoparticles that are devoid of any substance do not exhibit any harmful effects on cells. According to a study conducted by Islam *et al.*, chitosan is a natural polysaccharide that is non-toxic and has a composition that is well-suited for biomedical treatment [24]. These data suggest that there is a dose-dependent relationship, where the percentage of cell viability decreases as the concentration increases. The IC_{50} value was closely correlated with antioxidant activity, which can be affected by phenol chemicals, pentacyclic triterpene compounds, and dyes such as chlorophyll, curcumin, and nitrogen [25]. The rhizomes of black turmeric contained a high concentration of several phytochemicals, such as curcuminoids, alkaloids, terpenes, amino acids, carbohydrates, tannins, flavonoids, steroids, anthracene protein, and turmigone [4].

The research conducted by Nayak and Bhatnagar focused on the development of black turmeric extract. They used different solvents, including hexane, acetone, and methanol, and employed the DPPH method to assess its antioxidant activity. The results revealed an IC_{50} value of 23.45 µg/ml, indicating a relatively high level of activity [8]. The study conducted by Saefudin *et al.* investigated the antiproliferative and antioxidant properties of white turmeric extract. The results showed that although it is classed as having compensatory action, high dosages of white turmeric extract still effectively inhibited the proliferation of Hela cells, with an IC_{50} value of 37.6 µg/ml. A study comparing the cytotoxic effects of curcumin using a nanoparticle system and a non-nanoparticle system against MCF-7 cancer cells found that the IC_{50} value of curcumin with the nanoparticle system was 1.7 µg/ml. In contrast, the IC_{50} value of non-nanoparticle curcumin was 11.7 µg/ml. The nanoparticle approach enhances curcumin molecules' cellular absorption, improving its efficacy compared to non-nanoparticle curcumin. Multiple conducted experiments have yielded suboptimal outcomes for this research. However, black turmeric and black turmeric extract nanoparticles are identified as powerful cytotoxic agents [26].

CONCLUSION

The analysis revealed that nanoparticles derived from black turmeric extract have a size range of 266-558 nm and possess a

positive charge, with zeta potential values ranging from 3.3 to 9.7 mV. The encapsulation effectiveness of nanoparticles containing black turmeric extract was 63.42%. The cytotoxic test results indicated that both black turmeric extract and black turmeric extract nanoparticles exhibited a moderate level of cytotoxic activity, with IC_{50} values of 78.60 µg/ml and 162.95 µg/ml, respectively. The findings from this study demonstrate the favorable capability of black turmeric extract nanoparticles as a cytotoxic agent for the management of breast cancer.

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AUTHORS CONTRIBUTIONS

All authors discussed the results and contributed to the final manuscript. Conceptualization and funding acquisition: MD and EW; performing the experiment and data collection: NA and AR; data analysis: MD and SN.

CONFLICT OF INTERESTS

The authors declare no conflict of interest

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