Toxicity Test of Black Turmeric Extract (*Curcuma caesia* Roxb.) on *Artemia salina* Larva and Its Potential as an Anti-Cancer Agent

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ARTICLE INFO

Received: June 5, 2024
Accepted: July 11, 2024
Volume: 4
Issue: 1

KEYWORDS

Black turmeric, BSLT, GC-MS, secondary metabolite, anticancer.

ABSTRACT

Cancer is a body cell that divides non-stop and spreads to surrounding tissue. Currently, cancer treatment is carried out using surgery and therapy which can cause side effects on the sufferer's body, such as weakening the body's immune system, so people are starting to switch to using herbal plants as an alternative to traditional medicine. Black turmeric (*Curcuma caesia* Roxb.) is an herbal plant from the Zingiberaceae family which has cytotoxic activity as an anticancer. This research was conducted to determine the secondary metabolite compounds contained in black turmeric and to determine the toxicity value to *Artemia Salina* Leach larvae using the Brine Shrimp Lethality Test (BSLT) method. Black turmeric is macerated with hexane solvent. Toxicity tests were carried out on *Artemia Salina* larvae by administering 4 different concentrations of sample extract, namely 25 ppm, 50 ppm, 100 ppm and 0 ppm as a control. Toxicity test observations were carried out for 24 hours. Based on the research result, it is known that black turmeric has potential to be an anticancer agent with the LC_{50} value obtained is 58,49 ppm. In the result of GC-MS analysis of black turmeric extract, there are 15 secondary metabolite compounds have potential as anticancer agents, namely isoborneol, champhor, α-elemene, β-elemene, α-caryophyllene, β-caryophyllene, β-germacrene, γ-germacrene, curzerene, alloaromadendrene, germacrone, furanodiene, spathulenol, α-copaene, and pentadecanoic acid.

1. Introduction

Cancer is a body cell that divides non-stop and spreads to surrounding tissue (Wulandari et al., 2018). Cancer is the seventh leading cause of death in Indonesia (Rahayuwati et al., 2020). Currently, the prevalence of cancer in Indonesia is 1.4 per 1000 population (Dyanti and Suariyani, 2016). Cancer treatment is generally carried out through surgical removal and radiation therapy to cancer cell biomass followed by chemotherapy activities. Chemotherapy is the most frequently used treatment technique, but this action has weakness. It can cause side effects such as drug resistance (Rayan *et al*., 2017).

Doxorubicin is an anticancer chemotherapy agent that is widely use, but long-term use of Doxorubicin can weaken the body's immune system because this drug works non-selectively and is toxic to cancer cells and normal cells (Nuraini et al., 2015). Based on this, researchers have begun to increase the discovery and development of new anticancer drugs that come from natural ingredients and are safe for long-term consumption. Currently around 60% of anticancer drug candidates derived from plants have demonstrated significant success in clinical use (Abdalla *et al*., 2022).

Curcuma caesia or black turmeric is one of plant that needs to be studied further for its potential as an anticancer agent (Nuraeni et al., 2023). Black turmeric in Indonesia is generally used as herbal medicine. Black turmeric contains chemical compounds such as alkaloids, flavonoids phenols, curcuminoids, terpenes, and tannins. These compounds are known to act as anticancer agents (Udayani, 2022).

Preliminary test of an extract that has anticancer potential can be carried out using the Brine Shrimp Lethality Test (BSLT) method which uses *Artemia salina* larvae as test animals. The National Cancer Institute of the United States of America has long used this method because it is known that there is significance between the results of BSLT method and the inhibition of human tumor cell carried out in vitro (Anwar et al., 2021). In addition, the RNA polymerase II structure of *Artemia salina* is very similar to Hela cells, which are cancer cells derived from human cervical epithelial cells (Solis et al., 1992).

Research on the potential of black turmeric as an anticancer using the BSLT method was carried out by Nayak and Bhatnagar (2019). This research shows that the highest cytotoxic activity is found in acetone and hexane solvents with concentration of 100 µg/ml. Further research needs to be carried out to find out specifically the names of compounds contained on black turmeric which could have potential as anticancer agents.

2. Methodology

2.1 Time and place of research

This research was carried out from January to April 2024. The black turmeric was obtained from Back2nature Regenerative Farm Kuala Pilah, Malaysia. The preparation of black turmeric extract, maintenance and treatment of *Artemia salina* were carried out at the Biochemistry Laboratory of Biology, Udayana University, Bali, Indonesia. GC-MS test of black turmeric extract was carried out at Islamic University of Indonesia, Yogyakarta, Indonesia.

2.2 Methods

This research uses the Brine Shrimp Lethality Test (BSLT) method and the research design uses a Completely Random Design (CRD) consisting of 4 treatments with 5 replications, each unit filled with 10 *Artemia salina* larvae. The treatment included P0 (control, without black turmeric extract), P1 (black turmeric extract with a concentration 25 ppm), P2 (black turmeric extract with a concentration 50 ppm), P3 (black turmeric extract with a concentration 100 ppm).

Preparation of Black Turmeric Extract

Black turmeric rhizomes are washed thoroughly, then cut into smaller pieces and air-dried without being exposed to direct sunlight. The dried black turmeric rhizomes were blended into powder. 300 g of black turmeric powder were weighed and added to a beaker for extraction. Extraction was carried out using the maceration method with 3 litre of hexane solvent. Maceration is carried out for 4x24 hours and stirring occasionally. The result of maceration is filtered with filter paper and then the filtrate is concentrated using a rotary vacuum evaporator at a temperature of 35 ℃ until a concentrated extract is obtained (Senja et al., 2014).

Artemia salina Larvae Hatching

Hatching of *A. salina* Hatching is carried out in plastic container. Artificial sea water is made by dissolving 40 g of salt in 2000 mL of water. The container filled with salt water then lit by a 25 watt incandescent lamp and aerated using an aerator. 2 mg of *A. salina* eggs were add to the container and allowed to hatch for 24 hours. After 24 hours, the *A. salina* larvae transferred to another container and wait until for 2-3 days so the larvae reach instar II (Lay et al., 2022).

Preparation of Test Sample Solutions

5 mg of concentrated extract was weighed and dissolved in 5 mL of hexane to obtain a solution of 1000 ppm as a stock solution. Solutions with concentration of 25 ppm, 50 ppm, and 100 ppm were made by taking stock solutions using a micro pipette of 125 µl, 250 µl, dan 500 µl respectively and then putting them into vials. The control solution was made by adding 5 mL of salt water into vials (Nayak and Bhatnagar, 2019).

Toxicity Test using the BSLT Method

Each concentration of the test solution is put into a vial and then evaporated until the solvent evaporated. Next, each vial was filled with 1 mL of salt water and 10 *A. salina* larvae were added, then salt water added until the solution reach 5 mL. 1 mL of Saccharomyces cerevisiae was added to each vial as larva food then each concentration was replicated 5 times. The vial is placed under a lamp for 24 hours. After that, the number of died larvae were counted by observing the movement of *A. salina* larvae using loop and under lamp light. Dead larvae were identified by the absence of movement during 10 seconds of observation. Percent larval death was calculated using a formula:

$$\% \text{ larval death} = \frac{\text{number of dead larvae}}{\text{number of test larvae}} \text{ x } 100\%$$

Gas Chromatography Mass Spectroscopy **(GC-MS)**

The GCMS used was GCMS-QP2010 SE with an agilent Rtx-5MS column which had a thickness 0,25 µm; length 30 m; inside diameter 250 µm. Helium gas was used as the carrier gas at. The initial column temperature was set at 80 ℃, then temperature was increased until reached 300 ℃. The

column set in split mode with a pressure of 42,3 kPa; total flow 117.5 mL/minute; linear velocity 31,8 cm/sec; purge flow 3 mL/minute and split ratio 153. The mass spectrometer was programmed in the fragments from m/z 50 to 600. The ion source temperature was set at 250 ℃, interface temperature 300 ℃. The running time was 24 min. The library database of National Institute Standard and Technology (NIST) was used for identifying the chemical components.

2.3 Data analysis

Data on toxicity test result were analyzed statistically with One Way Anova and DMRT (Duncan Multiple Range Test) further test using SPSS program. LC_{50} value is determined by probit analysis with linier regression equations using Microsoft Excel 2010 for Windows.

3. Result and Discussion

3.1 Result

Toxicity test using the BSLT method

The result of *A. salina* larvae death during 24 hours of observations can be seen in the table below.

Table 1. The result of *A. salina* larvae death during 24 hours of observations

Treatment	Extract Concentration	Mean (%) ± Standard Deviation
P0	0 ppm	0 ± 0^{a}
P1	25 ppm	$6 \pm 0,89^{a}$
P2	50 ppm	$38 \pm 1,38^{b}$
P3	100 ppm	$86 \pm 1,67^{c}$

Note: the numbers accompanied by different letter notations in the same column show significant differences average values based on the DMRT (Duncan Multiple Range Test) at the level of 5% after statistical test using ANOVA.

Table. 1 show that increasing the concentration of black turmeric extract is directly proportional to increasing the mortality of *A. salina* larvae, which means that higher the concentration of the extract, the greater the toxicity (more toxic). The highest percentage of *A. salina* larvae death was found at a consentration of 100 ppm. The treatment of black turmeric extract gave significantly different result to the death of *A. salina* larvae with a $p<0,05$. Further test showed that P1 is not significantly different from P0, but it is significantly different with P2 and P3. Treatment P2 was significantly different with P3, and each of these treatments is also significantly different from P0 and P1.

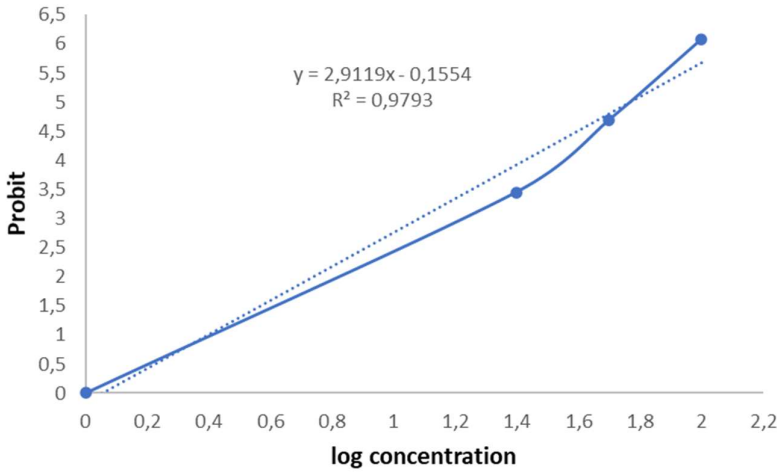

Figure 1. Linear regression graph of black turmeric extract against probit value

Based on the graph in figure 1, it is known that the determinant coefficient or R^2 value is 0,9793 (close to number 1) so it can be interpreted that influence of the probit value or death of *A. salina* larvae is getting stronger on the log concentration of extract. The linier regression equation from the graph is y= 2,9119x – 0,1154, then the x value is equal to 1,7794. The LC_{50} value is antilog of x, so the LC_{50} is 58, 49 ppm.

Gas Chromatography Mass Spectroscopy (GC-MS) Analysis

The compound present in the hexane extract of black turmeric were identified by GC-MS analysis (figure 2).

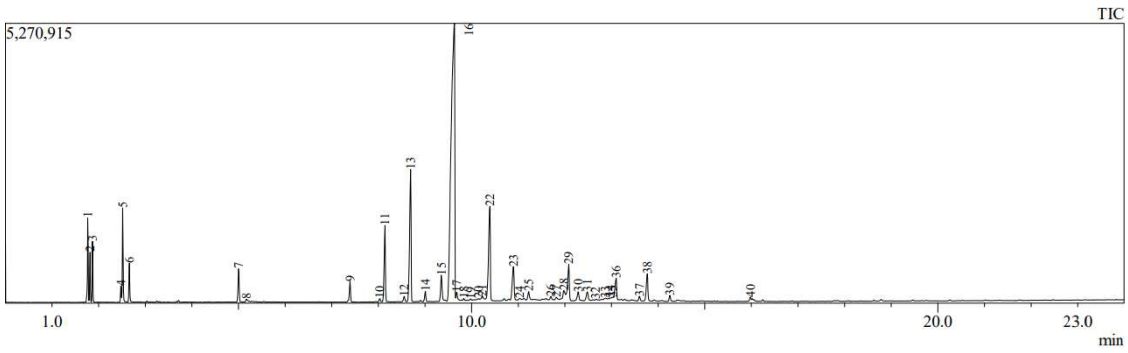

Figure 2. GCMS chromatogram of black turmeric of hexane extract

Fifteen compounds were identified by GC-MS. The major components present in black turmeric were Curzerene (44.66%), β-germacrene (8.49%), β-elemene (4.18%) and various other compounds were identified as low level. These compounds are potential for various pharmacological activities, especially as an anticancer agent (table 2).

Table 2. Secondary metabolites identified in black turmeric by GC-MS

Name of the compound	Area %	Compound nature	Activity	Source
Isoborneol	0.22	Monoterpene	Antitumor, antibacterial, anti-inflammatory	(Pakkirisamy *et al*., 2017; Masyita *et al*., 2022)
Camphor	1.68	Monoterpene	Anticancer, relieves asthma, rheumatism	(Hamidpour *et al*., 2013)
γ-elemene	1.12	Sesquiterpene	Anticancer, antimicrobe, anti-inflammatory	(Fan *et al*., 2023)
β-elemene	4.18*)	Sesquiterpene	Anticancer, anti-inflammatory	(Viveiros *et al*., 2022)
α-caryophyllene	0.55	Sesquiterpene	Anticancer, anti-inflammatory	(Viveiros *et al*., 2022)
β-caryophyllene	1.13	Sesquiterpene	Anticancer, antibacterial, anti-inflammatory	(Fan *et al*., 2023)
β-germacrene	8.49*)	Sesquiterpene	Anticancer	(Sharma *et al*., 2022)
γ- germacrene	1.74	Sesquiterpene	Anticancer, antimicrobe	(Sharma *et al*., 2022; Sitarek *et al*., 2017)
Curzerene	44.6*)	Sesquiterpene	Anticancer, antioxidant, antifungal	(Da Costa *et al*., 2020; Wang *et al*., 2016)
Alloaromadendrene	0.28	Sesquiterpene	Antitumor, antibacterial	(Pakkirisamy *et al*., 2017)
Germacrone	2.86	Sesquiterpene	Anticancer, anti-inflammatory, hepatoprotector	(Wu *et al*., 2017)
Furanodiene	0.65	Sesquiterpene	Anticancer, anti-inflammatory	(Zhu *et al*., 2019)
Spathulenol	0.27	Sesquiterpene	Anticancer, antimicrobe	(Bomfim *et al*., 2016; Paksoy *et al*., 2015)
α-copaene	0.19	Sesquiterpene	Anticancer, antioxidant	(Turkez *et al*., 2014)
Pentadecanoic acid	0.35	Fenol	Anticancer, antimicrobe	(Venn-Watson *and* Schork., 2023)

Note: *) The major components present in black turmeric

3.2 Discussion

Toxicity test using the BSLT method

The highest average mortality of *A. salina* shown at 86% when given an extract with a concentration of 100 ppm. Previous research conducted by Nayak and Bhatnagar (2019) who testes the toxicity of turmeric extract using various solvents shown that the highest average mortality of *A. salina* shown when the extract was given a concentration of 100 ppm with value of 90,99% in acetone solvent, followed by hexane solvent with an average mortality of 80,12%. The difference in results occurred because the black turmeric sample were taken from different places. Environmental factors in area such as climate, topography, and soil nutrient content can influence the content of plant secondary metabolite compounds (Yani et al., 2023).

According to Meyer et al. (1982) an extract has bioactivity in the toxicity test if the LC_{50} value of the extract shows <1000 ppm. Toxic potential concists of three categories, if the LC_{50} value is <30 ppm then the extract is very toxic, if 31 ppm < LC_{50}> 1000 ppm the extract is toxic and if LC_{50} value >1000 ppm the extract is not toxic. The result of this research obtained that the LC_{50} value of black turmeric extract is 58,49 ppm. It is categorized as toxic which could have potential as an anticancer agent or be developed as a new cytotoxic drug (Megawati et al., 2021).

Artemia salina larvae have thin skin and are sensitive to environmental changes so they can be used in toxicity test (Sharififar et al., 2017). The *A. salina* larva that uses in the test are 48 hours old larvae called nauplii. In the nauplii phase, active mitotic division occurs which identical to cancer cells. Apart from that, larvae in the nauplii phase have a completely formed digestive tract epithelium so their lives depend on the external environment to obtain food (Manopo et al., 2023).

The death of *A. salina* in the BSLT test occurred to the presence of secondary metabolite compounds in black turmeric extract which are toxic, consisting of saponins, alkaloids, terpenoids, and flavonoids. If this compound is swallowed, it can disrupt the digestive system by inhibiting the taste receptors in the mouth of *A. salina* larvae, which result in the larvae failing to receive a taste stimulus so they are unable to recognize the food. This causes larva to be unable to eat and then starve to death (Meyer et al., 1982). The present of secondary metabolite compounds can disrupt the active Na^+ K^+ transport system so that Na ions enter the cell uncontrollably. Na ions that continuously enter the cell will trigger the rupture of the cell membrane and result in cell death (Aris and Adriana, 2022).

***Gas Chromatography Mass Spectroscopy* (GC-MS) Analysis**

Generally, the main secondary metabolites produced by turmeric plants are curcuminoids and essential oil components. Curcuminoids were not found in this research which used hexane as solvent because curcuminoids are difficult to dissolve in non-polar solvents such as hexane. This is inversely proportional to essential oils which are easily soluble in non-polar hexane. The essential oil in turmeric consists of more than 250 terpenoid compounds (Zhang and Kitts, 2021). The compounds included in essential oils are the opposite of curcuminoids or curcumin free compounds so they are called non-curcuminoids components (Aggarwal *et al*., 2013).

Non-curcuminoids components have not been studied much by researchers, but several studies have shown that they have the same potential as curcuminoids, so these compounds are interesting to study for their potential as anti-inflammatory, antioxidant and anticancer. Non-curcuminoids components have a broad spectrum of anticancer activity for different cells, including in malignant and drug-resistant diseases (Nair et al., 2019). Currently, several non-curcuminoids compounds such as germacrone, β-elemene, curcumol, curdione, and neocurdione have been isolated and identified (Zhong et al., 2011).

Elemene (1-methyl-1-vinyl-2, 4-diisopropenyl-cyclohexane) is a new anticancer agent extracted from traditional Chinese herbs and derived from *Curcuma zedoariae*. Elemene extract consists of a combination of β-, γ-, dan δ-elemene compounds. Among the three, β-elemene is the main anticancer compound that can cause apoptosis and has broad clinical spectrum of activity in various cancer treatments (Xie at al., 2011). China has used β-elemene to treat certain types of cancer and has been shown to have fewer side effects than other anticancer agenrs (Liu et al., 2011). The use of β-elemene has been approved by the Chines State Food and Drug Administration for anticancer treatment based on several small-scale clinical trials in China. Until now, β-elemene has been used clinically to treat carcinoma in the brain, breast and liver (Guan et al., 2014).

Curzerene is known to be able to induce apoptosis and antiproliferation in lung cancer cells. Curzerene also provided significant result in inhibiting cancer growth in mice transplanted with cancer cells, curzerene even showed more promising result compared to the use of β-elemene (Wang et al., 2016). Another compound, namely pentadecanoic acid also is reported to be able to to induce apoptosis in various types of cancer and inhibit migration, invasion, and formation of breast cancer cells (To et al., 2020).

Chen at al. (2011) conducted research on the synergistic effect of germacreone and furanodiene compound on breast cancer cell. Previously, germacrone and furanodiene were separately known for their anticancer potential. The combination of germacrone and furanodiene in this research showed significant results in inhibiting cancer cell proliferation through the apoptosis induction process.

α-caryophyllene is a compound that is usually found together with its isomer, namely β-caryophyllene. The interaction between the two is known to have anticancer and anti-inflammatory potential (Yeo et al., 2021). α-caryophyllene plays role in increasing the production of Reactive Oxygen Species (ROS) which is responsible for its cytotoxic activity against cancer cells. This increase in ROS will increase significantly if α-caryophyllene is associates with β-caryophyllene (Viveiros et al., 2022).

4. Conclusion

The LC_{50} of black turmeric hexane extract is 58,49 ppm in the toxic category. This can be used as guideline that black turmeric has potential to be an anticancer agent. Apart from that, black turmeric contains 15 secondary metabolite which have potential as anticancer agents consisting isoborneol, champhor, α-elemene, β-elemene, α-caryophyllene, β-caryophyllene, β-germacrene, γ-germacrene, curzerene, alloaromadendrene, germacrone, furanodiene, spathulenol, α-copaene, and pentadecanoic acid.

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