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Phytochemical diversity and biological activities of *Curcuma* species from the East Coast of Peninsular Malaysia

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Abstract. *Yurasbe NQ, Din NA, Palaniveloo K, Manikam S, Nagappan T. 2023. Phytochemical diversity and biological activities of* Curcuma *species from the East Coast of Peninsular Malaysia. Biodiversitas 24: 4243-4252.* Zingiberaceae has been associated with traditional medicine for centuries. The genus *Curcuma* is traditionally famous and economically important for its therapeutic and nutritional values. Over 50 genera of 1600 species are recorded worldwide, with the highest concentration in the Malesian region, including Indonesia, Borneo, Thailand, Malaysia, Vietnam, Cambodia, Myanmar, and the Philippines. We investigated the methanolic crude extracts of *Curcuma aeruginosa*, *Curcuma caesia*, *Curcuma longa*, *Curcuma xanthorrhiza*, and *Curcuma zedoaria* from Terengganu for their phytochemicals, total phenolic content (TPC) using Folin-Ciocalteu colorimetric method, total flavonoid content (TFC) using aluminum chloride colorimetric method, antioxidant activity using the 2,2-diphenyl-1-picryl-hydrezyl (DPPH) radical scavenging activity and toxicity using brine shrimp lethality test (BSLT). Phytochemical screening revealed alkaloids, flavonoids, phenolics, saponins, tannins, triterpenoids, glycosides, lignins, and oils in all species investigated. *Curcuma longa* extract had the highest phenolic and flavonoid content with the value of 155.31±1.78 mg GAE/g and 151±8.35 mg quercetin/g, respectively as well as best antioxidative potential with IC50 value of 88.65±0.6 µg/mL, followed by *C. zedoaria* (98.61±5.23 µg/mL), *C. aeruginosa* (142.51±3.29 µg/mL), *C. xanthorrhiza* (150.01±0.63 µg/mL) and *C. caesia* (156.4±0.67 µg/mL). *Curcuma caesia* displays the lowest degree of toxicity compared to the other species, with the LC50 value of 11585 µg/mL) considered non-cytotoxic. Hence, the selected *Curcuma* species has potential therapeutic value to be developed into pharmaceutical due to its significant bioactive potentials and potent antioxidant capacity.

Keywords: BSLT, *Curcuma*, DPPH, flavonoid content, phenolic content

INTRODUCTION

Since prehistoric times, humans have relied on plants for survival, food sources, and treatment for various ailments. Using herbs and spices as treatments for various ailments dates back 4,000 years. Many medicinal scripts/ notes can be from Ayurvedic, Chinese, and Unanic medicine civilizations (Subositi and Wahyono 2019). In modern times, plant-based medications are widely used in clinical settings worldwide. With many consumers having shifted to herbal remedies, this has created a surge in demand for medicinal plants on the global market (Mohamad and Kalu 2019).

The genus *Curcuma* is traditionally famous and economically important for its therapeutic and nutritional values. It is vital to Asian cuisine due to its aromatic scent and natural food dye (Mohamad and Kalu 2019). With 93 species acknowledged taxonomically, *Curcuma* originates from the family Zingiberaceae and is widespread throughout tropical and subtropical regions, mainly distributed in India, Thailand, China, Malaysia, Indonesia, and Northern Australia (Sirirugsa et al. 2007).

The genus *Curcuma* has been an essential component of folklore medicine in numerous cultures. It has been reported to be used for the treatment of various disorders such as liver and skin problems and diseases, rheumatism, stomach ache, diarrhea, nausea, gingivitis, motion sickness, hypercholesterolemia, high levels of fatty acids, cancer, hemorrhoids, asthma, inflammation and leprosy (Naksuriya et al. 2014; Subositi and Wahyono 2019; Mahadevhi and Kavitha 2020; Walker and Mittal 2020). Among other ethnobotanical applications of *Curcuma* are condiments, food preservatives, and coloring materials. All parts of the *Curcuma* species (leaves, rhizomes, and inflorescences) have their respective benefits.

Curcuma is rich in bioactive phytochemicals, with the major constituents consisting of phenolics (diarylheptanoids, diyrenphenate, and isoflavones), essential oils (monoterpenes, sesquiterpenes, diterpenes, and triterpenoids), steroids and alkaloids among others whereas, *Curcuma* essential oil is a blend of volatile terpenes comprising of monoterpenes, sesquiterpenes, diterpenes, and triterpenes. Phenolic compounds originating from natural resources have been interesting since they have demonstrated an interesting spectrum of biological activities such as antioxidant, anti-

inflammatory, and anti-carcinogenic actions (Heleno et al. 2015; Rahayu et al. 2020).

Recent studies in pharmacology focused on the anticancer, antioxidant, and anti-inflammation actions of several *Curcuma*'s rhizomes. The rhizomes have been transformed into numerous formulations and used in therapeutic therapy. The most famous analogs from *Curcuma* that have been exploited and commercialized for their potency are curcumin, curcuminoids, and β-sitosterol from rhizomes of *C. aromatic*, *C. manga*, *C. longa* and *C. zedoaria* (Nurcholis et al. 2016a; Aslam and Ahmad 2017). Curcumin is one of the major components that contribute to its various pharmacological activities, such as anti-parasitic, antispasmodic, anti-inflammatory, anti-carcinogenic, and gastrointestinal effects in several in-vitro studies (Khan et al. 2008; Perrone et al. 2015; Mahadeyhi and Kavitha 2020).

The phytochemicals, phenolic and flavonoid content, antioxidant activity, and cytotoxicity of several Zingiberaceae species have been a global focal point of extensive research. In Peninsular Malaysia, almost 18 genera with over 160 species of the Zingiberaceae family were recorded in 2007, with at least five species of *Curcuma* used by the local Orang Asli communities. To our best search, this report will be the first document to shed light on phytochemical content, antioxidant, and toxicity activity for the east coast of Peninsular Malaysia, especially from Kuala Nerus, Terengganu. Hence, this research aims to investigate the selected *Curcuma* species (*Curcuma aeruginosa*, *Curcuma caesia*, *Curcuma longa*, *Curcuma xanthorrhiza*, and *Curcuma zedoaria*) extracts on their phytochemicals, phenolic and flavonoid content, antioxidant activity and toxicity towards brine shrimps.

MATERIALS AND METHODS

Plant collection and extraction

About 3 kg of fresh *Curcuma* rhizomes from each species (*C. aeruginosa*, *C. caesia*, *C. longa*, *C. xanthorrhiza*, and *C. zedoaria*) were procured (April-May 2022) from a local trader in Kuala Nerus, Terengganu, Malaysia. An expert botanist, Tuan Haji Razali Salam from Universiti Malaysia Terengganu, identified the procured samples. Voucher specimens were tagged as UMTH 10010-10015 for herbarium references. The samples were cleaned from dirt, washed, and chopped (3-5 cm length) before being air-dried for 48 hours at room temperature. The air-dried rhizome was macerated in 3 L of absolute methanol (Merck, Germany) for 4 days. The solvent-containing sample was filtered using filter paper (Whatman), and the filtrate was concentrated *in vacuo* using a rotary evaporator (EYELA, Japan) to produce the crude extract. Each crude extract was desiccated with a stream of nitrogen before being weighed and stored at -20 °C before analysis (Nagappan et al. 2011).

Qualitative phytochemical screening of selected *Curcuma* extracts

The five investigated *Curcuma* species were subjected to chemical tests to determine their phytochemical content. Phytochemical tests include alkaloids, flavonoids, phenolics, saponins, tannins, triterpenoids, glycosides, steroids, proteins, lignins, coumarins, and oils. The qualitative results are indicated by (+) for the presence of phytochemicals and (-) for their absence.

Chemical tests

Alkaloids

Accurately, 1 mL of 1% hydrochloric acid (Sigma-Aldrich) and 5 mg of *Curcuma* extract were mixed in a test tube. The mixture was then gently heated for five minutes, cooled, and filtered. Two drops of Dragendorff's reagent (Merck, Germany) were added to the filtrate. Alkaloids are present when an orange-red precipitate is developed (Lawand and Gandhi 2013).

Flavonoids

Alkaline reagent test. About 10 % sodium hydroxide (NaOH) (Sigma-Aldrich, USA) solution was added to 1 mg/mL *Curcuma* methanolic extract. Flavonoids are present when a deep yellow color develops (Sawant and Godghate 2015).

Hydrochloric acid test. One mL of extracts (1 mg/mL of *Curcuma* methanolic crude extracts) was mixed with a few drops of 1% hydrochloric acid (HCl) (Sigma-Aldrich, USA). The appearance of a crimson hue on the mixture indicates the presence of flavonoids (Rao et al. 2016).

Phenolics

Five to six drops of 10% ferric chloride ($FeCl_3$) (Merck, Germany) were added to 2 mL of aqueous extract (2.0 mg extract in 5 mL distilled water). Phenolic was detected by the appearance of a bluish-black color in the mixture (Labyad et al. 2020).

Tannins ($FeCl_3$ Test)

About 10% ferric chloride ($FeCl_3$) (Merck, Germany) solution was added to aqueous extracts. The development of blackish-blue color indicates the presence of gallic tannin, whereas catechol tannin is shown by the development of a greenish-blackish hue (Lawand and Gandhi 2013).

Saponins

One mg of each *Curcuma* extract was shaken in a test tube filled with 10 mL of distilled water. After heating in a water bath for five minutes, forming foams indicates the presence of saponins (Iqbal et al. 2015).

Coumarin

Five mg of *Curcuma* extract was diluted with 2.5 mL of distilled water in a test tube. Then, 3 mL of 10% aqueous sodium hydroxide (NaOH) (Sigma-Aldrich, USA) was added to the solution. A bright yellow formation indicates the presence of coumarin (Sawant and Godghate 2015).

Triterpenoids

Salkowski's assay was carried out to verify the presence of triterpenoids. About five mg of *Curcuma* extract was added to 2 mL of chloroform (Merck, Germany) before adding 3 mL of concentrated sulphuric acid (Sigma-Aldrich,

USA) to the mixture. The presence of reddish-brown interphase indicates the presence of terpenoids (Shaikh and Patil 2020).

Glycosides

Accurately, 1 mL of distilled water was used to dissolve 5 mg of *Curcuma* extract, and then several drops of 10% sodium hydroxide (NaOH) (Sigma-Aldrich, USA) were added. The changes in color to yellow indicate the presence of glycosides (Shaikh and Patil 2020).

Steroids

About 2 mL of *Curcuma* extracts and 2 mL of chloroform (Merck, Germany) were mixed. Later, 2 mL of concentrated sulphuric acid (Signa-Aldrich, USA) was added to the mixture. The greenish-yellow of the acid layer and reddish-brown of the chloroform layer indicate the presence of steroids (Shaikh and Patil 2020).

Proteins

The heat test was used to determine the presence of proteins in the sample. About 5 mg of each *Curcuma* extract in a test tube was added with 2 mL of distilled water and shaken until the extracts were dissolved, followed by incubating the test tube in boiling water (95°C). Coagulation suggested that the sample contains proteins (Mahadevi and Kavitha 2020).

Lignins

Labat's test was used to detect the presence of the lignin compounds. About 2 mL of *Curcuma* extract was mixed with gallic acid (Thermo Scientific, USA). Changes in the color of the solution to olive green indicate the presence of the lignin compound (Shaikh and Patil 2020).

Oils

Oil in the *Curcuma* crude extracts was determined using a spot test. A small amount of *Curcuma* extracts were spotted on the filter paper. The filter paper stained with extracts was pressed onto a separate filter paper. Oil stain on the paper signifies a positive outcome (Yadav et al. 2010).

Quantitative phytochemical screening of selected *Curcuma* species.

The five selected *Curcuma* species were analyzed for their phenolic and flavonoid content. The DPPH assay was performed to determine the antioxidant activity of the extracts.

Total phenolic content (TPC)

The total phenolic content (TPC) was determined using the Folin-Ciocalteu technique with a minor modification (Johari and Khong 2019). A test tube containing 100 µL) of *Curcuma* extracts with 20-200 (g/mL concentrations and 0.75 mL of 10% Folin-Ciocalteu reagent (Sigma-Aldrich, USA) was used. After 5 minutes, the mixture was added with 0.75 mL of sodium carbonate (Na_2CO_3) (Sigma-Aldrich, USA). This mixture was kept at room temperature for an hour. The absorbance of this mixture was measured using a UV-VIS spectrophotometer (Shimadzu Corp., Japan) at 725 nm. A calibration curve of gallic acid at seven different concentrations between 10 and 200 µg/mL was used to calculate the TPC expressed as Gallic acid equivalent (GAE). Equal amounts of methanol and reagents were set as blank for this experiment.

Total flavonoid content (TFC)

The total flavonoid content (TFC) was determined using the aluminum chloride ($AlCl_3$) colorimetric technique following Rejab and Ksibi (2019) with a few minor changes. About 300 µL of 10% w/v sodium carbonate ($NaCO_3$) (Sigma-Aldrich, USA) was added to an aliquot of 100 µL of extract with concentrations of 20-200 µg/mL in 1 mL of distilled water. Then, 500 µL of $AlCl_3$ (Sigma-Aldrich, USA) (10%) was added to the mixture and left to rest for 5 minutes. About 500 µL of 1 M sodium hydroxide (NaOH) (Sigma-Aldrich, USA) was added to neutralize. The absorbance was measured using a UV-VIS spectrophotometer at 510 nm against a blank MeOH. A calibration curve was generated using a quercetin solution. The results are expressed as milligrams of quercetin (Sigma-Aldrich, USA) equivalents per gram extract (mg quercetin/g extract).

DPPH (2-Diphenyl-1-picrylhydrazyl) radical scavenging capacity

The free radical DPPH was used to evaluate the antioxidant activity of *Curcuma* extracts. The technique was modified slightly from Gul et al. (2017). DPPH stock solution was prepared by dissolving 3.94 mL of DPPH (Sigma-Aldrich, USA) in 100 mL of HPLC-grade methanol (Merck, Germany). The 96-well plate (ThermoFisher, USA) was filled with 0.1 mL of extract at a concentration of 20-200 µg/mL. Accurately, 2.9 µL of methanolic DPPH was added to each well, thoroughly mixed and shaken, and allowed to stand at room temperature for 35 minutes in the dark. Ascorbic acid served as the standard, and readings were taken in triplicate at the absorbance of 517 nm. Inhibition percentage (I %) is calculated as follows:

I = ((Abs. of control-Abs. of tested extract) x 100)/Abs. of control

Where: Absorbance of control: contains DPPH and methanol. Absorbance of test extract: contains DPPH and sample extract

Brine Shrimp Lethality Test (BSLT)

The lethal toxicity assay was conducted based on Sarah et al. (2017). Brine shrimp eggs were hatched in synthetic sea salt in a set-up hatching chamber. About 50 mg of the brine shrimp eggs were sprinkled inside the aquarium and left for 24 hours with constant aeration and light illumination. Using a Pasteur pipette, fifteen nauplii were transferred into petri dish (ThermoFisher, USA). Methanolic *Curcuma* extracts were tested for their lethality against brine shrimp larvae. The *Curcuma* extracts were dissolved in 1 mL of 1% DMSO (Sigma-Aldrich, USA). Toxicities of extracts were tested at 10, 50, 100, 500, and 1000 µg/mL in 20 mL

of seawater solution, while 1% DMSO was used as control. Fifteen nauplii were used in each test, and the experiment was carried out for 24 hours; the observation was carried out at intervals of 4 hours. Live nauplii were counted with white light and a 3x magnifying glass. Three replications were used for each concentration. Using dose-dependent curve analysis, GraphPad Prism was used to calculate the concentration of 50% mortality after 24 hours of exposure. The LC_{50} value greater than 1000 ppm for plant extract was considered inactive. The mortality percentage (%) was determined as follows:

% of mortality = (No. of dead nauplii after 24 hours of exposure/Initial no. of live nauplii tested) x 100

RESULTS AND DISCUSSION

Qualitative phytochemical screening

Discovering the phytochemical components of plants is essential for validating folk treatments and is the primary step in identifying the plant's therapeutic properties. Due to the presence of several phytoconstituents, the therapeutic potential to cure a wide range of diseases by *Curcuma* species has been widely acknowledged in various systems of traditional medicine (Liu and Nair 2012; Lukitaningsih et al. 2020; Subositi and Wahyono 2019; Sobrattee at al. 2021). Based on our phytochemical screening of extracts of *Curcuma aeruginosa*, *Curcuma caesia*, *Curcuma longa*, *Curcuma xanthorrhiza*, and *Curcuma zedoaria* confirms the presence of alkaloids, flavonoids, phenolics, saponins, tannins, triterpenoids, glycosides and oils as presented in Table 1. Steroids and lignins are present in all of the extracts except for *C. caesia*, and there was no detection of coumarins and proteins in all of the extracts studied. Our findings correlate with Paliwal et al. (2011) that phytochemicals in plants are used as a defensive mechanism against various pathogens and predators. Therefore, pharmacology can also be applied to create novel molecules with therapeutic potential. Hence, these initial phytochemical screening aids in identifying the major bioactive phytochemical groups present in *Curcuma*, especially *Curcuma* species from Terengganu, as the first record.

Several previous findings supported that alkaloids, carbohydrates, flavonoids, glycosides, phenolics, tannins, saponins, terpenoids, and quinones were present in *C. longa*, *C. aeruginosa*, *C. xanthorrhiza*, and *C. caesia* (Hasan et al. 2014; Pakkirisamy et al. 2014; Donipati and Hara Sreeramulu 2015; Sawant and Godghate 2015; Waras et al. 2015; Oghenejobo 2017; Irshad et al. 2018; Joshi et al. 2018) that was consistent with our findings in this study. Therefore, we can postulate that the presence of these major phytochemical groups is independent of geographical factors and can be used as chemotaxonomical markers unique to the species. Some studies reported that different solvents may affect the results of phytochemical extractability from plants. Chaturvedi et al. (2020) reported that the aqueous methanolic of *C. longa* extracts has a more diverse phytochemical profile than its aqueous acetonic extract. Donipati and Hara Sreeramulu (2015) reported that both chloroform and methanol extract of *C. caesia* contains alkaloids, phytosterols, terpenoids, carbohydrates, tannins, anthraquinones, glycosides, quinones, oils, flavonoids, amino acids, and saponins. In contrast, all phytochemicals are present in hexane extract except for tannins. Hence, it is crucial to choose the proper solvent for better extractability. Thus, this study utilized methanol as the extraction solvent due to its polarity and ability to extract most phytochemical constituents.

Table 1. Phytochemical content of the extracts of five species of *Curcuma* from Terengganu, the east coast of Peninsular Malaysia

Test	Observation	CL	CZ	KE	CX	CC
Alkaloids (Dragendorff's test)	Formation of red precipitate	+	+	+	+	+
Flavonoids						
10% NaOH	There is no formation of bright yellow	-	-	-	-	-
HCl	Immediate formation of red	+	+	+	+	+
Phenolics	Formation of bluish-black or dark brown	+	+	+	+	+
Saponins	Persistent formation of frothing	+	+	+	+	+
Tannins	Formation of bluish-black or dark brown	+	+	+	+	+
Coumarins	No formation of white precipitate	-	-	-	-	-
Triterpenoids	A yellowish color was formed at the bottom (acid layer) on all extracts except for CC	+	+	+	+	-
Steroids	Reddish brown formation at the upper layer for all extracts except for CC	+	+	+	+	-
Glycosides	Formation of yellow layer	+	+	+	+	+
Proteins	Coagulations	-	-	-	-	-
Lignins	Formation of olive-green color	+	+	+	+	-
Oil	Oil stain	+	+	+	+	+

Note: (+) indicates the presence, whereas (-) indicates the absence. Tests were done in triplicate. CL: *Curcuma longa*, CZ: *Curcuma zedoaria*, KE: *Curcuma aeruginosa*, CX: *Curcuma xanthorrhiza* and CC: *Curcuma caesia*

Quantitative phytochemical screening

Total Phenolic Content (TPC)

Total phenolic content quantifies the sum of phenolics in the samples as plant phenolics' redox properties allow them to serve as antioxidants (Baba and Malik 2015). The TPC was expressed as mg GAE/g extract, which was determined by the calibration curve of gallic acid ranging from 0 to 200 µg/mL. The calibration curve of gallic acid was ($y = 0.0003x + 0.0739$, $R^2 = 0.982$). The total phenolic content of the investigated *Curcuma* species ranged from 52.7 to 155.31mg GAE/g extract. Figure 2 illustrates that *C. longa* had the highest value of TPC (155.31±1.78 mg GAE/g extract, followed by *C. aeruginosa* (133.5±2.46 mg GAE/g extract), *C. xanthorrhiza* (91.59±5.80 mg GAE/g extract*), C. zedoaria* (76.37 mg ± 7.10 GAE/g extract) and *C. caesia* (52.7±1.23 mg GAE/g extract). Phenolic compounds are responsible for plants' bioactivity, leading to antioxidant effects.

Previous studies reported that *C. longa* rhizome extracts from Malaysia were 67.9 mg GAE/100 g extracts (Maizura et al. 2011) while from India ranged from 49.12 mgGAE/100g to 142.06 mg GAE/100g (Burman et al. 2020). Results in this study showed that the phenolic content of *C. longa* rhizome extract is consistent with earlier investigations. Awin et al. (2016) reported that the phenolic content of ethanolic extracts of *C. zedoaria*, *C. xanthorrhiza*, and *C. aeruginosa* were 255.7 mg GAE/g DW, 335.5 mg GAE/ g DW, and 205.5 mg GAE/g DW, respectively. Different accession of ethanolic extract of *C. aeruginosa* from Indonesia has a phenolic content value ranging from 20 mg TAE/g to 80 mg TAE/g (Jantan et al. 2012; Nurcholis et al. 2016b). A study by Sahu and Saxena (2013) showed that the phenolic content of *C. caesia* extract was 134.47 mg/g. Interestingly, the phenolic content of *C. zedoaria*, *C. aeruginosa*, *C. xanthorrhiza*, and *C. caesia* extracts in this study differs slightly from those in the literature. The radical scavenging activity of different *Curcuma* extracts could be due to polyphenols, flavonoids, and phenolic compounds, with the most antioxidant activity of plants originating from phenols (Phuyal et al. 2020). The difference in the value of the phenolic content could be affected by the temperature, geography, soil acidity, and rain volume throughout the years (Burapan et al. 2020) as well as extraction protocols, pH, and polarity of solvent used (Nour et al. 2014; Pereira et al. 2018; Nasir et al. 2021). Commonly, the protocols and solvents used in extraction to dissolve the plant's phytochemicals tend to be either polar or nonpolar. The hydroxy group in phenols is more soluble in a polar solvent and contributes to disrupting bacteria's cell membrane (Heleno et al. 2015; Vuolo et al. 2019). Other than that, free radical scavenging is facilitated by the hydroxyl groups in plant extracts (Yang et al. 2020). Phenols have an important role in enhancing the nutritional qualities of the plant, growth, reproduction, resistance to diseases, and pigmentation (Kähkönen et al. 1999; Vuolo et al. 2019).

Total flavonoid content

Aluminum chloride ($AlCl_3$) is commonly used to quantify the flavonoid content of plant extracts. Plants primarily utilize flavonoids to create yellow and other pigments crucial to developing plant hues (Rebaya et al. 2015). Total flavonoid content (TFC) is expressed as mg quercetin/g extract, which was determined by the calibration curve of quercetin ranging from 0 to 200 µg/mL ($y=0.0002x+0.0459$, $R^2 = 0.9961$).

Figure 3 shows that *Curcuma longa* has the highest total flavonoid content of all the species investigated with a value of 151±8.35 mg quercetin/g extract, followed by *C. xanthorrhiza*, *C. aeruginosa*, *C. zedoaria*, and *C. caesia* with values of 124.2±4.18, 72.47±1.76, 61±3.61, and 47.34±4.46 mg quercetin/g of extract, respectively. The flavonoid content varied significantly ($p<0.05$) among the selected *Curcuma* species.

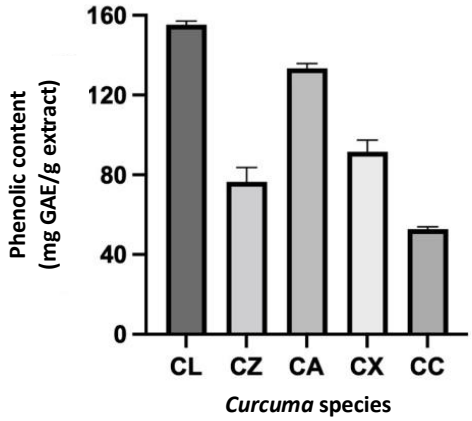

Figure 2. Total phenolic content of methanolic crude extracts *C. longa* (CL), *C. zedoaria* (CZ), *C. aeruginosa* (CA), *C. xanthorrhiza* (CX), and *C. caesia* (CC). The results are expressed as mean ± SD. All experiments were done in triplicates. All extracts are significantly different from each other, with $p<0.05$

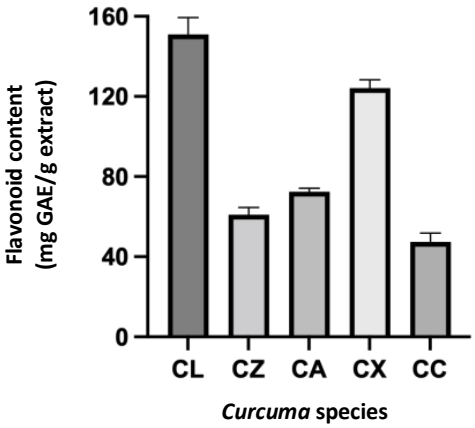

Figure 3. The total flavonoid content of methanolic crude extracts of *C. longa* (CL), *C. zedoaria* (CZ), *C. aeruginosa* (CA), *C. xanthorrhiza* (CX), and *C. caesia* (CC). The results are expressed as mean ± SD. The experiments were done in triplicates. All extracts are significantly different from each other, with $p<0.05$

According to Panche et al. (2016), the number of hydroxyls (-OH) group positions affects the potency of flavonoids with antioxidant capacity. *Curcuma xanthorrhiza* has been reported to possess higher flavonoid content than *C. zedoaria*, with a value of 797.5 mg rutin/g extract and 15.3 mg rutin/g extract, respectively (Akter et al. 2019). Meanwhile, Alafiatayo et al. (2014) stated that *C. longa* has higher flavonoid content than *C. xanthorrhiza* and *C. aeruginosa* extracts with a value of 741.36 mg/g DW, 220.53 mg/g DW, and 14.97 mg/g DW, respectively. The flavonoid content of *C. longa* and *C. caesia* extracts was reported at 79.36 mg/g and 40.6 mg/g, respectively. These previous findings are partially consistent with our current results. Nurcholis et al. (2016b) reported that different accession of *C. aeruginosa* has flavonoid content ranging from 7 mg/g to 22 mg/g, while methanolic extract of *C. caesia* contained flavonoid content of 2.752 mg/100 mg of dried extract. These results are quite contradictory to our reports. The differences in TPC, TFC content, and antioxidant properties may be due to genetic diversity and biological, environmental, seasonal, and yearly variations. (Kumar et al. 2018; Phuyal et al. 2020).

2,2-diphenyl-1-picrylhydrazyl's scavenging capacity (DPPH)

Free radical DPPH (2,2-Diphenyl-1-picrylhydrazyl) was used to assess the investigated *Curcuma* species' antioxidant or free radical scavenging activity. This method is widely accepted because it is quick and practical (Li et al. 2013). The interactions between DPPH and antioxidants stabilize the free radicals. The reactions cause the purple coloration of DPPH to change into yellow by producing a more stable 2,2-Diphenyl-1-pikrilhydrazine (DPPH-H) molecule. The intensity of the color change depends on how effective the antioxidants are (Baliyan et al. 2022).

The concentration of *Curcuma*'s extract required to scavenge 50% of the DPPH radicals' activity is reported as 50% free radical scavenging capacity or IC_{50} (Alinaghi and Kamalinejad 2008). The lower the IC_{50} value, the higher the free radical scavenging activity. Figure 4 shows the results obtained after *Curcuma* extracts and ascorbic acid were treated with DPPH. It showed that the higher the concentration of the extracts, the higher the radical scavenging activity (%). Figure 5 shows the IC_{50} value of ascorbic acid and *Curcuma* extracts. *C. longa* extract had the highest antioxidant activity, as it had the lowest IC_{50} value at 88.65±0.6 µg/mL followed by *C. zedoaria* extracts with an IC_{50} value of 94.9±5.23 µg/mL, *C. aeruginosa* extracts at 142.51±3.29 µg/mL, *C. xanthorrhiza* extracts at 150.01±0.63 µg/mL and lastly *C. caesia* extracts at 156.4±0.67 µg/mL.

Assessment of total phenolic and flavonoid content by DPPH scavenging capacity on *Curcuma* extracts is important in determining the free radical scavenging activity or antioxidant. Phenolic and flavonoid molecules are crucial antioxidant components responsible for deactivating free radicals based on their ability to donate hydrogen atoms to free radicals (Phuyal et al. 2020). Furthermore, it is commonly known that the antioxidant capability of phenolic compounds depends on the number of hydroxyl moieties in their ring structure and arrangement. An ortho-position of hydroxyl groups confers high stability to radical formation after neutralization (Martinez et al. 2022; Saeed et al. 2023). Previous studies on *C. longa* extracts showed high antioxidant activity with IC_{50} values ranging from 8-30 µg/mL (Widowati et al. 2011; Swain and Rautray 2021), while the IC_{50} value of *C. zedoaria* ethanolic extract was 165 µg/mL. Different accession of *C. caesia* ethanolic extracts results in IC_{50} values ranging from 89.91 to 505.65 µg/mL (Nurcholis et al. 2017). Curcuminoids in *Curcuma* species exhibit high antioxidant activity (Masuda et al. 1992; Mošovská et al. 2016). Curcuminoids are polyphenol compounds comprising curcumin, demethoxycurcumin, and bisdemethoxycurcumin (Widowati et al. 2011; Abdul Zahar et al. 2020). Other compounds that contribute to radical scavenging ability are 1-(4-hydroxy-3,5-dimethoxyphenyl)- 7-(4-hydroxy-3-methoxyphenyl)-(lE,6E), 6-heptadiene-3,4-dione (Swain and Rautray 2021). Polyphenol compounds in plants can donate hydrogen atoms or electrons and capture free radicals (Widowati et al. 2016). These compounds were also responsible for other pharmacological abilities in *Curcuma* species, such as antimicrobial, anticancer, antidiabetic, and antiinflammation (Ramsewak et al. 2000; Akram et al. 2010; Basnet and Skalko-Basnet 2011; George and Britto 2015; Adamczak et al. 2020). These findings offer crucial guidelines for determining *Curcuma*'s rhizomes for cultivation and use in the production of pharmaceuticals.

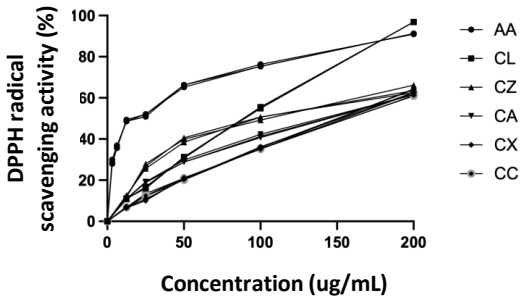

Figure 4. DPPH scavenging activity of *C. longa* (CL), *C. zedoaria* (CZ), *C. aeruginosa* (CA), *C. xanthorrhiza* (CX), *C. Caesia* (CC) and Ascorbic acid (AA)

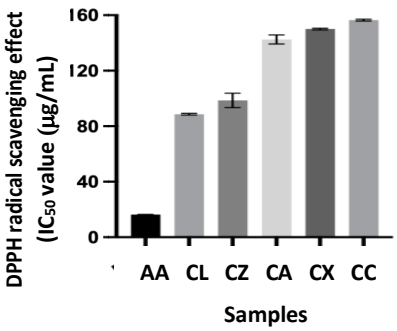

Figure 5. The IC_{50} value of *C. longa* (CL), *C. zedoaria* (CZ), *C. aeruginosa* (CA), *C. xanthorrhiza*, *C. caesia* (CC) and Ascorbic acid (AA)

Brine Shrimp Lethality Test (BSLT)

Artemia salina, or brine shrimps, is a crustacean in saltwater worldwide. These species are widely used as test organisms in many toxicological experiments. The brine shrimp lethality bioassay was frequently used to assess the toxicity of heavy metals, herbicides, drugs, and natural plant extracts (Theanphong et al. 2015; Omeke et al. 2018). Lethal concentration 50 (LC_{50}) is the chemical needed to kill 50% of brine shrimp larvae during a specific observation period (Wu 2014). The LC_{50} value indicates the toxicity level of the extract.

The methanolic crude extracts of *Curcuma aeruginosa, Curcuma caesia, Curcuma longa, Curcuma xanthorrhiza,* and *Curcuma zedoaria* were tested for their toxicity against brine shrimps at intervals of 24 hours. Table 2 shows the number of dead nauplii after 24 hours of *Curcuma* extracts' exposure at concentrations ranging from 10 to 1000 µg/mL. The number of dead nauplii correlated with *Curcuma* extract concentrations. The higher the concentration of the extract, the higher the mortalities of the brine shrimps. Figure 6 shows the variation in the mortality rate of the brine shrimps against *Curcuma* extracts.

The LC_{50} readings are frequently compared to either Meyer's or Clarkson's toxicity index to determine the toxicity of herbal extracts. Meyer et al. (1982) determined that extracts with LC_{50} values lower or equal to 1000 µg/mL are considered harmful, whereas extracts with values greater than 1000 µg/mL are considered non-toxic. Clarkson's toxicity classification for assessing the toxicity of plant extracts are as follows: LC_{50} values of 0 to 100 g/mL are highly toxic, 100 to 500 g/mL is medium toxic, 500-1000 g/mL is low toxic, higher than 1000 µg/mL is considered non-toxic (Clarkson et al. 2004).

Notably, the lower the IC_{50} value of extracts, the higher the toxicity effect. The toxicity of *Curcuma* extracts was found in the following order: *C. xanthorrhiza* > *C. zedoaria* > *C. longa* > *C. aeruginosa* > *C. caesia.* All extracts were categorized as non-toxic (LC_{50} value higher or equal to 1000 µg/mL) (Meyer et al. 1982). The non-toxic category of all *Curcuma* extracts (more than 1000 g/mL) may imply that low toxicity is related to broader dietary supplement and meal usage.

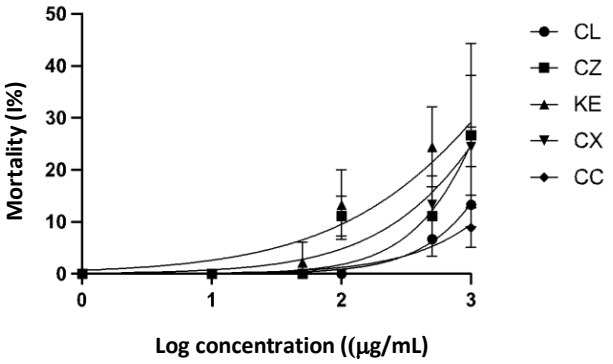

Figure 6. Mortality percentage (%) of brine shrimps against *Curuma* methanolic extracts

Table 2. The average death rate of brine shrimps at various concentrations against methanolic *Curcuma* extract after 24 hours of exposure

Plant extracts	Concentration (µg/mL)	Number of dead nauplii after 24hr			Total number of death	% Mortality	LC_{50} value (µg /mL)	Remark
		S1	S2	S3				
C. longa	10	0	0	0	0	0±0.00	4384	Inactive
	50	0	0	0	0	0±0.00		
	100	0	0	0	0	0±0.00		
	500	1	1	1	3	6.67±0.00		
	1000	2	2	2	6	13.33±0.00		
C. zedoaria	10	0	0	0	0	0±0.00	3965	Inactive
	50	0	0	0	0	0±0.00		
	100	2	2	1	5	11.11±3.85		
	500	3	1	1	5	11.11±7.70		
	1000	5	2	5	12	26.67±11.58		
C.aeruginosa	10	0	0	0	0	0±0.00	4471	Inactive
	50	0	1	0	1	2.22±3.85		
	100	3	2	1	6	13.33±6.67		
	500	3	3	5	11	24.44±7.70		
	1000	2	3	7	12	26.67±17.64		
C. xanthorrhiza	10	0	0	0	0	0±0.00	2391	Inactive
	50	0	0	0	0	0±0.00		
	100	0	0	0	0	0±0.00		
	500	2	2	2	6	13.33±0.0		
	1000	4	4	3	11	24.44±3.85		
C. caesia	10	0	0	0	0	0±0.00	11585	Inactive
	50	0	0	0	0	0±0.00		
	100	0	0	0	0	0±0.00		
	500	1	1	1	2	6.67±0.0		
	1000	2	1	1	4	8.89±3.85		

Note: No death was observed in control (salt water + DMSO). This experiment was repeated three times and expressed as mean ± SD

A previous study by Aly and Gumgumjee (2011) showed that *C. longa* extract up to a concentration of 400 µg/mL is non-toxic. However, previous studies by Sharma and Kharel (2019) and Khattak et al. (2005) showed that crude extract of *C. longa* is considered toxic because of the low LC_{50} value, ranging from 33 g/mL to 62 g/mL. Several studies conducted on *C. zedoaria*, *C. aeruginosa*, *C. xanthorrhiza*, and *C. caesia* showed that these species are considered to be non-toxic when tested on brine shrimps with the LC_{50} value lower than 400 µg/mL (Nurcholis et al. 2012, 2016b; Hossain et al. 2012; Syahbirin et al. 2017; Nayak and Bhatnagar 2018; Fitria et al. 2019). Notably, the solvent used for extraction might slightly influence the results (Hamidi et al. 2014).

In conclusion, the investigated *Curcuma* species have good phenolic and flavonoid content, responsible for their antioxidant capabilities. The phytochemical diversity in the extract plays a significant role in determining the biological activity of *Curcuma* extract. The high antioxidant activity of investigated *Curcuma* species may help to stop detrimental oxidation in nutraceuticals or drugs used to treat cardiovascular problems. The high phenolic and flavonoid content in *C. longa* relates to high antioxidant activity, suggesting that this species is the most promising compared to the other *Curcuma* species in this study. The findings of this study indicated that the methanolic extract of *C. longa* rhizomes has a high antioxidant capability, which is crucial for preventing several diseases caused by oxidative stress. The results could be used as baseline data on phytochemical diversity, antioxidant, and toxicity of *C. zedoaria*, *C. aeruginosa*, *C. xanthorrhiza*, and *C. caesia* from the east coast of Peninsular Malaysia. Further investigation is needed to isolate and identify the phytochemical and determine the mechanism of action of antioxidant components to understand better the effectiveness of antioxidants from *Curcuma* species to prevent diseases that significantly affect the quality of life.

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