

ANTIOXIDANT POTENTIAL, TOTAL PHENOLIC CONTENT AND PROPOSED STRUCTURES OF ANTIOXIDATIVE CHEMICAL COMPOUNDS FROM *Curcuma longa* LEAVES

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Abstract

Nowadays, the importance of natural sources in modern medicine cannot be underestimated as they exhibit remarkable antioxidant properties and total phenolic content (TPC). Therefore, the aim of this research is to investigate antioxidant potential through thin layer chromatographic (TLC) dot blot assay and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay of *Curcuma longa* leaves extracts, TPC using Folin-Ciocalteu method as well as proposed structures of antioxidative compounds through Gas Chromatography Mass Spectroscopy (GC-MS). The crude extracts of *C. longa* leaves were prepared by conducting consecutive maceration using organic solvent such as hexane, ethyl acetate and acetone. According to TLC dot blot assay, the acetone extract of *C. longa* scavenges the DPPH radical at the concentration of 0.024 mg/mL. The radical scavenging activity is in concentration dependent manner for all types of extracts. Acetone extract of *C. longa* shows the lowest concentration to scavenge 50% DPPH radical (IC₅₀) at 460.40 µg/mL. Therefore, the polar extracts are known to be the most promising source of antioxidant agent compared to the less polar extract. The highest TPC value is calculated as 128.129 mg GAE/g sample. The antioxidative compounds are proposed as (Z)-9-methyl octadecenoate, Methyl hexadecanoate, 6,10,14-trimethyl-2-pentadecanone, Methyl octadecanoate, 4-pyridinol acetate, Octadecyl methacrylate and (E)-10-methyl octadecenoate. The results may be applicable to the medical and pharmaceutical industries as they provide basic scientific data for the development of new natural medicines from plant base.

Keywords: antioxidant potential, chemical compounds, *Curcuma longa*, total phenolic content

Introduction

Numerous studies have been made to examine the properties, functionality, and applications of *C. longa* in various forms. Several studies have found that *C. longa* has anti-inflammatory, antioxidant, anticancer, antibacterial and anticoagulant qualities, as well as antidiabetic benefits due to its free-radical-scavenging action (Kim et al., 2019).

Bioactive chemicals are natural plant constituents that promote health, assist in the

proper functioning of the organism, and help in the prevention and/or treatment of diseases. The primary bioactive ingredients are phenolic compounds such as flavonoids, anthocyanins and tannins, organic acids, and vitamins (Braga et al., 2018; Najafabadi et al., 2017). Consumption of these substances benefits the organism through antioxidant and cytotoxic activities, as well as effects on the immune system, diabetes, cancer, and cardiovascular disease (Braga et al., 2018). Natural flavonoid and phenolic substances are secondary metabolites found in plants that have at least one hydroxyl group attached to an aromatic ring. Because of their ability to directly influence antioxidant action through their hydroxyl groups, phenolic substances are effective electron donors (Aryal et al., 2019).

Natural antioxidants such as phenolic compounds and flavonoid are phytochemicals which possess the ability to eliminate the harmful effects of oxidative physiological processes taking place in tissues (Lukitaningsih et al., 2020). Their responsibilities are massive in the treatment of diseases linked to oxidative stress, including cancer, heart disease, diabetes, stroke, rheumatoid arthritis, Alzheimer's disease, ageing, and cataracts. As far as concerned, most phenolics and flavonoids are isolated from polar extract. Since there are not many investigations of the identification of antioxidative compounds from nonpolar extract are reported, this study also deals with the determination of proposed structural formula of antioxidative compounds from nonpolar extract along with DPPH radical scavenging assay and TPC determination.

Materials and Methods

Plant Collection and Extraction

The leaves of *C. longa* were collected in Bandar Jengka Pahang, Malaysia. The samples were air-dried at room temperature for five days and then ground into powdered form. The grinded leaves were macerated consecutively using organic solvents such as hexane, ethyl acetate (EA) and acetone followed by filtration. All the filtrates were subjected for evaporation using rotary evaporator to yield crude extracts.

Qualitative Phytochemical Screening of *C. longa* Leaves Extract

The experiment was purposely conducted to screen three common phytochemicals in all types of extract of *C. longa* leaves such as alkaloid, terpenoid and phenolic compounds from developed thin layer chromatography (TLC) of extracts. The separated developed TLC of each extract were sprayed with Dragendorff's reagent, Vanillin/H₂SO₄ reagent, ferric chloride solution and DPPH solution to screen alkaloid, terpenoid, phenolic and antioxidant respectively.

Antioxidant Potential of *C. longa* Leaves Extracts

Semi-Quantitative Dot Blot Assay

This method was conducted according to Ilias Fazna et al. (2023) with slight modification. This method is the easiest way to determine antioxidant potential of extracts using square blocks marked on the TLC sheets. The evaluation of antioxidant activity was determined by observing the first appearance of yellow colour against purple background after 0.05% of DPPH solution was sprayed on square block of TLC in which each square block correspond to two-fold serial dilution of extract ranging from 100 mg/ml to 0.006 mg/ml. The first appearance of yellow color on the square block of TLC indicates the lowest concentration of extract enable to

scavenge DPPH radical.

2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity Assay

The experiment was conducted in accordance with Daud et al. (2022). A series of *C. longa* solution extract was prepared with concentrations ranging from 400 µg/mL to 12.5 µg/mL. 1 mL of each concentration was mixed with 3 mL of 0.004% DPPH solution and the mixture was kept in the dark for 30 minutes. Then the absorbance, A was measured for all mixtures using UV-VIS spectrophotometer at 517 nm. The percentage of radical scavenging activity was calculated based on the following equation (1):

$$\text{Percentage radical scavenging(\%)} = (A_0 - A_1/A_0) \times 100 \quad (1)$$

A_0 represents absorbance of DPPH solution while A_1 represents absorbance of extract/standard solution. The bar chart of (%) radical scavenging vs concentration was plotted to evaluate the trend of antioxidant potential. The IC_{50} value, which is the concentration of extract enabled to scavenge 50% DPPH radical was determined from the standard curve of (%) radical scavenging vs concentration. The equation $y = mx + c$ was implemented to calculate IC_{50} value.

Total Phenolic Content Determination (TPC)

The TPC of *C. longa* leave crude extracts was evaluated using Folin-Ciocalteu method. Gallic acid was used as a reference and Folin-Ciocalteu (FC) reagent as an oxidising agent. About 0.5 mL of 1,000 ppm of each extract was mixed with 2.5 mL of FC reagent (5 mL of FC mixed with 45 mL of distilled water) and 2 mL of 7.5% Na_2CO_3 solution.

All mixtures were kept in a dark room for 30 minutes for reaction completion. After incubation time was achieved, the absorbance, A of extracts samples was determined using a UV/VIS spectrophotometer at 765 nm. The method was repeated for standard gallic acid solution with different concentrations of 1000, 500, 250, 125, 62.5, and 31.25 ppm for standard curve development. The total phenolic content was expressed in milligrams of gallic acid equivalent per gram of sample (mg GAE/g sample) (Daud et al., 2022). The calculation of TPC of each extract was performed using Gallic acid standard curve.

Isolation of Antioxidative Compounds and GCMS Analysis

The TLC of antioxidative extract using a 10 cm × 10 cm TLC plate was developed in its suitable developing solvent system until it reached solvent front. After drying, the required band of antioxidant compound was totally scraped off and was dissolved in its dissolving solvent prior to filtration. A simple filtration processed was carried out with the use of cotton wool packed inside a tiny glass dropper. The filtrate was left at room temperature for several days until totally dried (Osman & Harun, 2019).

GCMS data was acquired on a Perkin Elmer Turbo Mass spectrometer instrument utilising a PE-WAX column (60 m X 0.32 mm, film thickness 0.25 µm). The isolated active compound was subjected to analytical process using GCMS. The temperature was set to rise by 2°C per minute to 120°C and 3°C per minute to 240°C after five minutes at 70°C. Helium was the carrier gas (Dixit & Awasthi, 2009). The mass spectrum was analyzed to determine its structural proposal.

Results and Discussion

The Screening of Phytochemicals and Antioxidant of *C. longa* Leaves Extracts

Table 1 displays the result of phytochemical screening test using certain spraying reagents as well as antioxidant screening. According to **Table 1**, all extracts are screened to contain terpenoid and phenolic except for petroleum ether extract which doesn't show any sign of phenolic's presence on its developed TLC after being sprayed with FeCl₃ solution. All extracts do not indicate any presence of alkaloid when their developed TLC were sprayed with Dragendorff's reagent. However, all extracts are antioxidative after their developed TLC sprayed DPPH. Thus, this screening result reveals that either terpenoid or phenolic is responsible for the extract's antioxidant properties. Previous study reported that phenolic compounds and flavonoids which are richer in the varieties of *Curcuma sp* are the cause of its antioxidant properties (Akter et al., 2019).

Generally, phytochemicals in plants especially herbs play important roles in their growth and development. They protect plants from harmful agents such as insects, microbes and ultraviolet (UV) irradiation as well as extreme temperatures. Each phytochemicals in plant herbs possess their own benefits and sometimes display unique properties. Moreover, they are also raising health benefits when consumed since some plant herbs are richer in phenolics compounds which are antioxidant (Abdul Majid & Harun, 2019). Hence, the presence of terpenoid and phenolic may be responsible for antioxidant properties in *C. longa* leaves.

Table 1 Phytochemicals and Antioxidant Properties of *C. Longa* Leaves Extracts

Extract	Alkaloid	Terpenoid	Phenolic	Antioxidant
Petroleum ether	x	√	x	√
Ethyl acetate	x	√	√	√
Acetone	x	√	√	√

x = absent; √ = presence

Antioxidant Potential of *C. longa* Leaves Extracts

Semi-Quantitative Dot Blot Assay

According to **Figure 1** and **Table 2**, all extracts exhibit their antioxidant properties since the square block of TLC shows the reduction of purple color to yellow color at different concentration which indicates the scavenging action of extracts towards DPPH radicals. Result from **Table 2** also reveals acetone extract as the most antioxidative with the lowest concentration to scavenge DPPH radical is at 0.024 mg/mL.

Table 2 Dot Blot Assay Analysis of The *C. Longa* Extracts of Antioxidant Activities

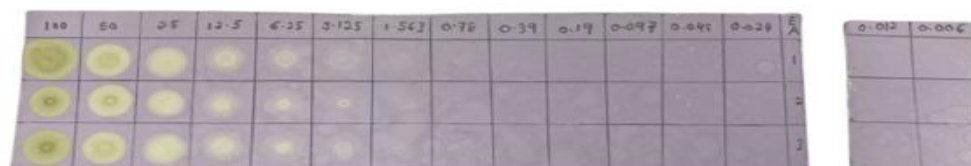
Extract	Lowest concentration of DPPH Inhibition, mg/ mL
Petroleum ether	0.097
Ethyl acetate	1.563
Acetone	0.024
Ascorbic acid (standard)	0.024

Our result is consistent with previous study who reported that more polar extract of *Curcuma sp* exhibited potent antioxidant activity compared with less polar extract (Akter et al., 2019). The presence of phytochemicals such as phenolic and terpenoid in polar extracts may be responsible for antioxidant properties of *C. longa* leaves extracts.

A. Petroleum ether extract



B. Ethyl acetate extract



C. Acetone extract



D. Ascorbic acid



Figure 1 Examples of TLC of dot blot assay results after sprayed with DPPH solution.

Concentration from left to right square blocks of TLC ranging from 100 mg/mL to 0.006 mg/mL

- A: Thin layer chromatograms of petroleum ether extract
- B: Thin layer chromatograms of ethyl acetate extract
- C: Thin layer chromatograms of acetone extract
- D: Thin layer chromatograms of standard ascorbic acid

DPPH Radical Scavenging Activity

The DPPH radical scavenging activity is used to measure antioxidant power based on how effective the extract of *C. longa* leaves scavenge the DPPH radical. Generally, the lower the concentration of the extracts used for scavenging process, the more effective the extract as a scavenger. IC₅₀ value is calculated by means of evaluating which the extract acts as the best scavenger to scavenge 50% DPPH radical. According to **Table 3**, when the polarity of extract increases from petroleum ether to acetone, the value of IC₅₀ decreases. Therefore, acetone

extract is the best scavenger as it exhibits the lowest concentration at 476.40 $\mu\text{g/ml}$ to scavenge 50% DPPH radical. The lower the IC_{50} value, the more antioxidative the extract is. This result also establishes the result of dot blot assay study in **Table 2**, which also indicates acetone extract as the most antioxidative. Previous study revealed more polar extracts such as ethyl acetate, methanol and water extract exhibited lower IC_{50} value which correspond to good antioxidant activity (Illias Fazna et al., 2023; Shi et al., 2010).

Figure 2 illustrates the scavenging activity profile towards the different polarity of extracts at different concentrations. The graph depicts that as concentration of extract increases, the percentage of scavenging process also increases for all extracts. Generally, when concentration of extract increases, the concentration of antioxidative phytochemicals such as phenolic and terpenoid also increases and this phenomenon might be the cause of the findings. Among the three extracts, the acetone extract is the best scavenger as it shows the highest percentage scavenging value of 44.79% at 400 mg/mL.

Theoretically, the scavenging of DPPH radical, a stable free radical occurs when the DPPH radical is reduced by an antioxidant source. Normally the DPPH radical has an odd electron, and it gives maximum absorption at 517 nm which is purple color. When a reaction happened between DPPH radical and antioxidants, the DPPH free radical becomes paired off with the assistance of hydrogen donor from antioxidant and reduces to DPPHH form from DPPH. This transformation causes decolorization to yellow colour because of the reduction process. The more the decolorization to yellow colour, the more the reducing ability is (Dontha, 2016).

Table 3 DPPH Radical Scavenging Activity of *C. longa* Leaves Extracts (IC_{50} Values)

Extracts/standard	IC_{50} values ($\mu\text{g/ml}$)
Petroleum Ether	764.71
Ethyl Acetate	587.43
Acetone	476.40
Standard Ascorbic Acid	89.71

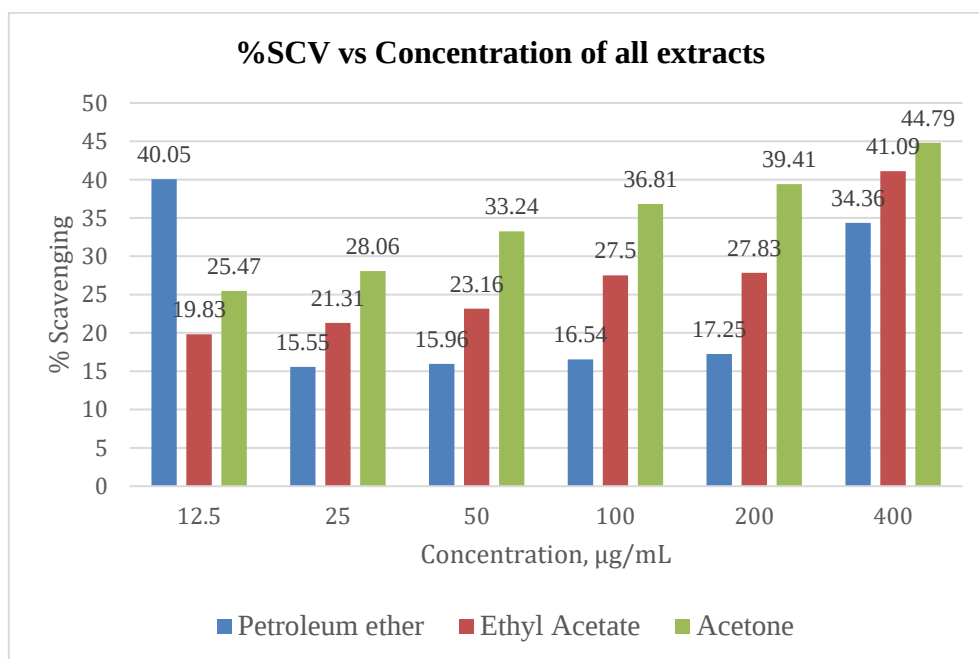


Figure 2 Percentage Scavenging Activity of *C. Longa* Leaves Extracts

Total Phenolic Content of *C. longa* Extracts

The total phenolic content is quantitatively determined from the standard curve of Gallic acid as shown in **Figure 3** using Folin-Ciocalteu method. Theoretically, when phenols are present in the extracts, a complex blue compound can be observed, as it absorbed at 765 nm (Everette et al., 2010). Oxidation of phenol group leads to the conversion of heteropoly acid in the Folin-Ciocalteu reagent into a molybdenum-tungsten complex. The presence of NaHCO₃ supports this complex in alkaline medium which causes protons in phenolic compounds to dissolve into phenolic ion (Martono et al., 2019).

According to **Table 4**, the highest TPC is 128.129 mg GAE /g sample belongs to petroleum extract followed by ethyl acetate extract and acetone extract containing the lowest value of TPC. The results of TPC for acetone and ethyl acetate are consistent with the result of phytochemical screening in **Table 1** which records the presence of phenolic compound in both extracts. Generally, primary antioxidant such as phenolic compound is well known as free radical scavenger by means to delay or interrupt the process of lipid oxidation, thus decreasing the formation of volatile decomposition products (Shahidi & Ambigaipalan, 2015).

Table 4 Total Phenolic Content (TPC) of *C. Longa* Leaves Extracts

Extract	TPC Value (mg GAE / g sample)
Petroleum ether (PET)	128.1291
Acetone	46.0589
Ethyl acetate (EA)	114.7528

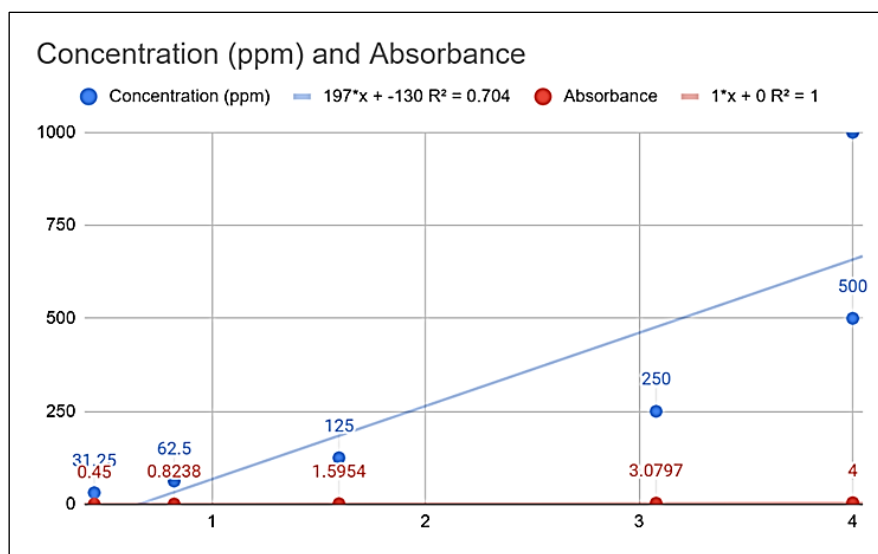


Figure 3 Standard Curve of Gallic acid

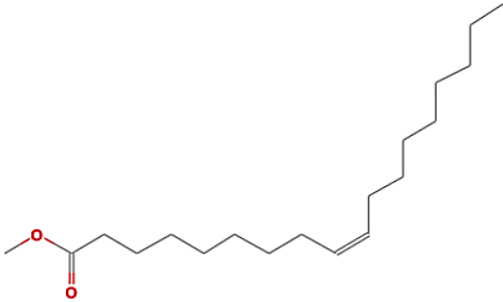
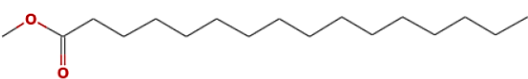
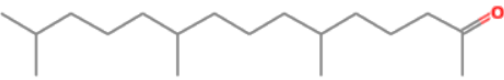
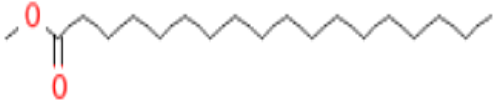
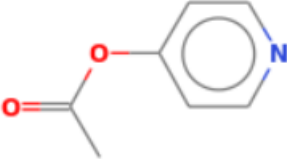
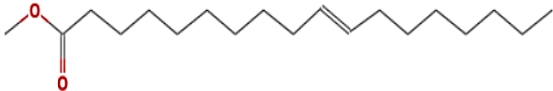
Proposed Antioxidative Chemical Compounds from Petroleum Ether Extract

Table 5 depicts seven proposed antioxidative chemical compounds from petroleum ether extract analysed by Gas Chromatography Mass Spectroscopy (GC-MS). This extract is nonpolar and is chosen because the compounds are easier to be analysed by GC-MS compared to other polar extracts.

According to **Table 5**, all listed proposed structural formulas of antioxidative compounds are considered as nonpolar compounds as most of the structures contained longer

hydrocarbon tail which is hydrophobic. The proposed antioxidative compounds are known as (*Z*)-9-methyl octadecenoate, Methyl hexadecanoate, 6,10,14-trimethyl-2-pentadecanone, Methyl octadecanoate, 4-pyridinol acetate, Octadecyl methacrylate and (*E*)-10-methyl octadecenoate. All proposed structures contain electronegative elements such as oxygen and nitrogen whereby these elements contain lone paired electrons which serve as radical scavenger by electron donating mechanism or by hydrogen donating mechanism (Rahman et al., 2015).

Table 5 Antioxidative Chemical Compounds from Petroleum Ether Extract of *C. Longa* Leaves

Retention time (RT)/%area	Name of isolated chemical compounds	Structural formula
22.3238 /(22.324%)	(<i>Z</i>)-9-methyl octadecenoate	
17.1839 /(17.522%)	Methyl hexadecanoate	
11.1701 (11.17%)	6,10,14-trimethyl-2-pentadecanone	
19.1286/ (10.485%)	Methyl octadecanoate	
21.3355/ (5.993%)	4-pyridinol acetate	
20.7746 /(5.8695%)	Octadecyl methacrylate	
20.5544 /(5.072%)	(<i>E</i>)-10-methyl octadecenoate	

Conclusion

The antioxidant activity from TLC dot blot assay of *C. longa* leaves extract was highest in acetone extract with the lowest concentration of 0.024 mg/ml. The DPPH radical scavenging activity quantitatively showed that acetone extract exhibited the highest antioxidant activity with the lowest IC₅₀ value of 476.40 µg/ml. The contribution of phytochemicals such as phenolic and terpenoid are the remarkable reason for the antioxidant activity of *C. longa* leaves. The proposed structural formula of antioxidative chemical compounds were (Z)-9-methyl octadecenoate, Methyl hexadecanoate, 6,10,14-trimethyl-2-pentadecanone, Methyl octadecanoate, 4-Pyridinol acetate, Octadecyl methacrylate and (E)-10-methyl octadecenoate. This is the first time this finding has been reported from nonpolar extract and the structures can be established in future work using NMR spectroscopy.

Ethics Statement

The research does not require research ethics approval.

Authors Contribution

Writing – Original draft preparation, Jamin, S; Literature Review, Daud, S; Methodology, Abdul Aziz, N; Writing – Review and editing, Harun, A.”

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Conflict of interests

The authors declare that there is no conflict of interest regarding publication of this paper.

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