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Research Article

Analysis of antioxidant and antimicrobial potentials in *Hemerocallis fulva* flower extract via thin-layer chromatography-direct bioautography (TLC-DB)

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Abstract
Hemerocallis fulva (daylily) has been traditionally used to treat a variety of ailments. This study aimed to identify the potential secondary metabolites present in Malaysian *H. fulva* (Asphodelaceae) and assess their potential antioxidant and antimicrobial activities by using thin layer chromatography-direct bioautography (TLC-DB). The dried flowers of *H. fulva* were extracted by using hexane, ethyl acetate, and methanol. Both qualitative and quantitative phytochemical analyses were performed on the crude extracts. The results indicated that alkaloids, flavonoids, quinones, proteins, and steroids were predominant in all samples extracted with the three solvents. In the quantitative analysis, methanol extract of the flowers had the highest flavonoid content (290.25 ± 0.02 mg QE/g), while ethyl acetate extract had the highest phenolic content (66.82 ± 0.06 mg GAE/g) as compared to other extracts. Additionally, the TLC-DB assay demonstrated that each extract of *H. fulva* flowers possesses significant antioxidant and antimicrobial activities. These findings provide valuable preliminary data that may contribute to the further development of this plant as a potential source of herbal medicine.

Keywords: *Hemerocallis fulva*, antioxidant, antimicrobial, thin-layer chromatography direct bioautography, phenolic

Introduction

Natural products (NPs) derived from plants, animals, and microorganisms have long been a valuable source of new drugs, playing a critical role in the development of disease-fighting agents, including anti-infectives, cancer treatments, and drugs for Type 2 diabetes and hypertension [1, 2]. These natural compounds offer structural diversity and biological activity that have been harnessed in pharmaceutical research and development. Despite this, there has been a shift in pharmaceutical companies towards synthetic alternatives, yet NPs continue to be an important source of bioactive compounds. They can be found in essential oils, herbal extracts, and dietary supplements, each possessing unique chemical profiles that contribute to their therapeutic potential. However, NPs may have negative side effects and may not be safe for all individuals. Therefore, it is crucial to consult with a healthcare provider before using them for medicinal purposes to ensure safety and efficacy [3, 4].

H. fulva, or daylily, is cultivated in Malaysia for its versatility, serving as an ornamental plant and a tool for preventing soil erosion on steep lands. It thrives in sunny, well-drained environments and is resilient to drought and humidity. The flowers and buds are edible, rich in vitamin A and iron, while the plant also has traditional medicinal uses, including diuretic and blood-purifying properties. It is valued for its adaptability and ecological benefits. *H. fulva* demonstrates diverse bioactive properties across its parts, supported by numerous studies. The flowers exhibit potent antioxidant activity due to compounds, such as chlorogenic acid, quercetin-3-O-rutinoside, linalool, and α-terpineol [5] and essential oils have shown chelating activity via DPPH and β-carotene bleaching assays. Antidepressant potential is linked to compounds like (-)-(1S,3S)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, 5-O-coumaroylquinic acid butyl ester, and prunasin, which modulate neurotransmitter levels and the HPA axis [6]. The anti-inflammatory effects are attributed to kaempferol, quercetin, myricetin, gallic acid, and ellagic acid, which regulate inflammatory cytokines and inhibit COX-2 and NF-κB [7]. Essential oils also inhibit Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), with high 1,8-cineole content being significant [5]. The leaves, extracted using ultrasound-assisted methods, contain flavonoids like rutin, hyperoside, isoquercitrin, catechin, and L-epicatechin, demonstrating antioxidant and protective effects against oxidative damage in HaCaT cells [8]. Methanol extracts reveal sedative compounds, such as kwansinone A and B and longitubanine A and B [9]. The roots, rich in diterpenes, have traditional uses as antifebrile and diuretic agents and display anti-parasitic potential against *Schistosoma mansoni* due to kwanzoquinones A-G, rhein, and dianellin [10, 11].

In this study, *H. fulva* was chosen as the sample due to its recognised potential as a source of bioactive compounds. The findings collectively position *H. fulva* as a valuable plant for both medicinal and industrial applications, offering a diverse array of bioactive molecules which have been shown to exhibit antioxidant, antimicrobial, and anti-inflammatory activities [12, 13, 14]. These compounds are critical in the development of alternative therapies and natural products, supporting the plant's potential in pharmaceutical research and natural product industries [15, 16].

Materials and Methods

Preparation of plant extract

A total of 120 g of dried *H. fulva* flowers were exposed to the sun to eliminate any residual moisture. Once fully dried, the flowers were cleaned thoroughly under running water to remove any dirt, then ground into a fine powder. The plant powder was soaked in three different solvents for three days at room temperature. Following this, each extract was concentrated by using a rotary evaporator (Buchi Rotavapor R-200, Switzerland) at 35°C–40°C for 15 min, and then filtered. The resulting crude extracts were weighed for further analysis [14, 17].

Qualitative phytochemical analysis

All crude extracts were subjected to eleven phytochemical tests to identify the presence of various bioactive compounds. These tests included the alkaloid test, saponin test, tannin test (Braymer's test) [13], triterpenoid and steroid test [18], flavonoid test (H_2SO_4 test), phlobatannin test [19], quinone test [16], reducing sugar test (Fehling's test) [20], carbohydrate test (iodine test) [20] and phenolic content test (ferric chloride test) [15]. These tests provided a comprehensive assessment of the chemical constituents in the *H. fulva* extracts.

Quantitative phytochemical analysis

Total flavonoid content (TFC)

The total flavonoid content of the plant samples was measured using the aluminium chloride ($AlCl_3$) technique [21] utilizing quercetin as the standard 1 mL of 5 mg/mL plant extract and mixed with 5 mL of distilled water, then 0.300 mL of 5% sodium nitrite ($NaNO_2$) was added. After 5 min left at room temperature, 0.6 mL of 10% $AlCl_2$ was added. Then, 0.2 mL of 1 mM sodium hydroxide (NaOH) was combined to the reaction mixture in this process. Then, the reaction mixture was diluted with 1.1 mL of distilled water and incubated at room temperature for another 20 min. The absorbance was measured in a spectrophotometer at 510 nm [22]. The total flavonoid content was determined from a quercetin standard calibration curve and tested in triplicate [14, 17].

Total phenolic content (TPC)

The total phenolic content (TPC) of *H. fulva* extracts was calculated by using the Folin-Ciocalteu reagent (FCR) technique [21],[23]. About 0.5 mL (1.0 mg/L) of methanol extract and 0.1 mL of FCR (10% v/v) were poured to each of the test tubes. The mixture was set aside for 15 min. 2.5 mL of sodium carbonate solution was added and thoroughly mixed. The combination was then incubated at room temperature for another 30 min. The ultraviolet-visible (UV-Vis) spectrophotometer was used to determine the TPC at 760 nm. A calibration curve was created by creating 1 mL aliquots of gallic acid solutions containing 100 g/mL, 50 g/mL, 25 g/mL, 12.5 g/mL, 6.25 g/mL and 3.125 g/mL. The results were represented as gallic acid equivalents per gram of sample [mg GAE/g]. Gallic acid served as a standard reference chemical and the calculation must be done in triplicate [14, 22, 24].

Thin layer chromatography direct bioautography (TLC-DB)

Antioxidant assay

Thin-layer chromatography (TLC)-Bioautography was used to screen for antioxidant activity (Dot-Blot and Develop). Dot-Blot: Samples (10 μL at a concentration of 10 mg/mL) were transferred onto a developed TLC-silica plate and cold-dried. The TLC plate was sprayed with diphenyl picrylhydrazyl (DPPH) solution in methanol. A clear area will be visible around the samples after 30 min. The presence of antioxidant activity in the samples was indicated by the yellowish-white region. The antioxidant ability of one's sample shows through its color intensity [25, 26].

Antimicrobial assay

A streak plate of stock bacteria was prepared on an appropriate solid agar media (nutrient agar or Mueller-Hinton agar) before the incubation process for 24 h at 37 °C. Next, a pure isolated colony poured into the broth and incubated for another 24 h at the same temperature. Then, the bacterial culture was poured with sterile nutrient broth, approximately 1:1, into a sterile petri dish. The developed TLC extract was dipped into the bacterial suspension for 10 s and dried at room temperature for 2 min. The bio-autogram was placed on a sterile petri dish containing wetted paper (under humid conditions) for 18-24 h at 37 °C. Then, the TLC plate was sprayed with MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromi -de) until all was covered. Another incubation process took place for 2 h–5 h at the same temperature. When a clear white zone appeared on the TLC plate, it proved the antimicrobial activity. Gram-positive: *Bacillus subtilis* and *Staphylococcus aureus* and Gram-negative: *Escherichia coli* and *Salmonella* were the most frequently used bacteria [27, 28, 29].

Results and Discussion

Metabolites screening analysis

Eleven phytochemical screening tests were conducted on each crude extract to identify the secondary metabolites present in *H. fulva* extracts, with a specific focus on differentiating compounds based on their polarity. **Table 1** reveals the result of each test towards the crudes. Secondary metabolites play an important role in the biological activities of medicinal plants, including hypoglycemia, antioxidant, and antimicrobial properties. In the present study, saponins were detected in all three crudes. Previous studies have confirmed that saponins have the unique ability to precipitate and coagulate red blood cells [30]. Similarly, all samples contained alkaloids that were capable of affecting the central nervous system, reducing appetite, and acting as diuretics [17, 31] were revealed as positive results too. Also, steroids that have been illustrated to have antibacterial properties in the previous report were positive on all three crudes [32]. Overall, the phytochemical screening results confirmed that all three crudes are potential sources of bioactive compounds and should be further investigated for use in the food, pharmaceutical, and agrochemical industries.

Unfortunately, some of the tests resulted in negative since the experimental tests were conducted using dried *H. fulva* that differed in regions and environments from the *H. fulva* used in published journals [24, 28]. Hence, incompatible humidity and growing sites contribute to the growth of the same genus of *H. fulva*, which exhibits different metabolites [33]. However, the *H. fulva* flavonoids and phenolics have shown strong positive findings, indicating its potential as an antioxidant agent [34]. This theory can be confirmed further since total flavonoid content (TFC) and total phenolic content (TPC) were investigated in quantitative analysis.

Quantitative phytochemical analysis

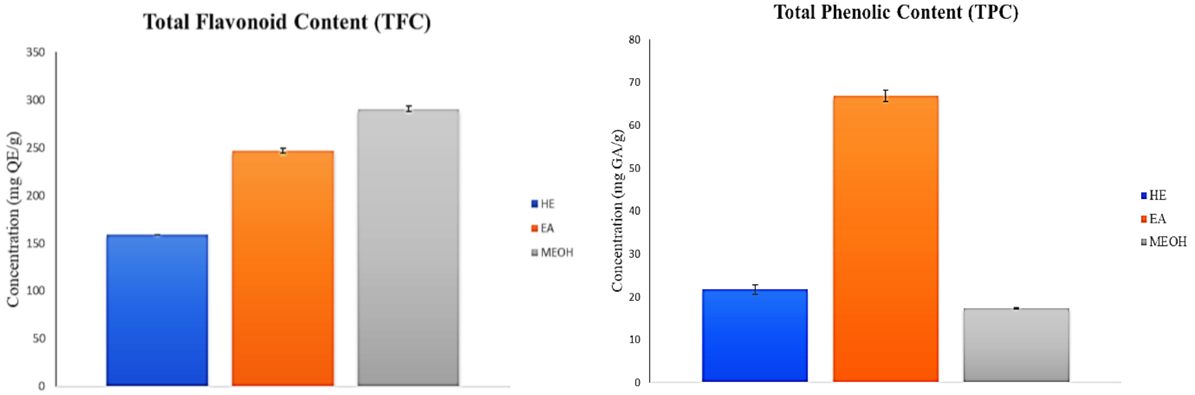
Total flavonoid content (TFC)

The total flavonoid content (TFC) obtained was 158.80 ± 0 mg QE/g, 246.50 ± 0.02 mg QE/g and 290.25 ± 0.02 mg QE/g for hexane, ethyl acetate and methanol crude, respectively. Thus, methanolic extract has the highest TFC followed by ethyl acetate and then hexane, as shown in **Figure 1(a)**. Hence, it proved that the flavonoid compounds were abundant in the daylily flower and since flavonoid compounds are polar, that explains why methanol crude has the highest TFC. Also, these results were aligned with value of TFC in the reported study [31],[35], supporting the statement that *H. fulva* flower is an antioxidant agent.

Table 1. Results of the phytochemical screening test

Phytochemical Test	Hexane Crude	Ethyl Acetate Crude	Methanol Crude
Alkaloid	+	+	+
Flavonoid	+	+	+
Saponin	+	+	+
Phenol	-	+	+
Protein	+	+	+
Tannin	+	-	-
Steroid	+	+	+
Triterpene	+	+	+
Anthocyanin	-	-	-
Quinone	+	+	+
Carbohydrate	-	+	+

Indicators: + = positive result - = negative result



(a) (b)

Figure 1. (a) Total flavonoid content (TFC) of the crudes of *H. fulva* (n = 3), (b)Total phenolic content (TPC) for the crudes of *H. fulva* (n = 3)

Total phenolic content (TFC)

The total phenolic content (TPC) of hexane, ethyl acetate, and methanol crude was estimated to be 21.74 ± 0.13 mg GAE/g, 66.82 ± 0.06 mg GAE/g, and 17.34 ±0.02 mg GAE/g, correspondingly, using the standard curve. According to the results, ethyl acetate crude had the highest TPC, followed by hexane and methanol, as shown in **Figure 1(b)**. This was inconsistent with previous research [31, 35]. Thus, the errors were attributed to the techniques used during the experiment. While performing maceration extraction techniques, methanol solvent may not extract all of the polar compounds from the sample residue. In fact, handling well-plates has always been tedious, and poor pipetting skills led to the errors. In contrast to the former studies, explained that when compared to non-polar solvents, extraction in highly polar solvents resulted in a higher extract yield. However, this resulted in a lower phenolic content, in parallel with TPC methanol and ethyl acetate findings [36].

Correlation between total flavonoid and phenolic content amongst plant crudes

The quantitative analysis of *H. fulva* flower extracts, measuring total flavonoid (TFC) and phenolic content (TPC) in different solvents as stated in **Table 2**. The total flavonoid content (expressed in mg QE/g) was highest in methanol extracts (290.25 ± 0.02), followed by ethyl acetate (246.50 ± 0.02) and hexane (158.80 ± 0.01). Conversely, the total phenolic content (expressed in mg GAE/g) was highest in ethyl acetate extracts (66.82 ± 0.06), with lower amounts in hexane (21.74 ± 0.13) and methanol (17.34 ± 0.02). This data highlights the influence of solvent polarity on the extraction efficiency of bioactive compounds.

Phenolic and flavonoid compounds are well-recognized for their antioxidant properties, which play a vital role in neutralizing harmful free radicals in the body. Free radicals are highly reactive molecules with unpaired electrons, and when their levels exceed the body's ability to neutralize them, they can cause oxidative stress. This stress is linked to the development of numerous chronic diseases, including diabetes, cardiovascular diseases, and cancer [37]. These diseases are associated with damage to cellular components, such as lipids, proteins, and DNA, which can lead to inflammation, tissue damage, and even mutation of cellular genetic material.

Phenolic compounds, such as flavonoids, act as potent antioxidants by donating hydrogen atoms or electrons to free radicals, effectively neutralizing their reactivity. This process prevents free radicals from causing further damage to cells and tissues. Flavonoids, in particular, are a class of polyphenolic compounds found abundantly in fruits, vegetables, and herbs, and have been extensively studied for their ability to reduce oxidative damage. Research has shown that flavonoids not only act as antioxidants but also exhibit anti-inflammatory, anticancer, and neuroprotective properties [38].

Studies confirm that the intake of flavonoids and phenolics from plant-based sources can provide significant health benefits, helping to mitigate the risks associated with oxidative stress and its related diseases. For example, reported study emphasizes the role of dietary antioxidants in managing metabolic and cardiovascular conditions by reducing oxidative damage [37]. As such, phenolic-rich plants like *H. fulva* are of interest due to their potential in therapeutic applications aimed at preventing or treating oxidative stress-related diseases. Thus, natural antioxidants like *H. fulva* flower are vital in protecting human health.

Table 2. Summary of quantitative analysis of *H. fulva* flower

	Total Flavonoid Content (mg QE/g)	Total Phenolic Content (mg GAE/g)
Hexane	158.80 ± 0.01	21.74 ± 0.13
Ethyl acetate	246.50 ± 0.02	66.82 ± 0.06
Methanol	290.25 ± 0.02	17.34 ± 0.02

Antioxidant assay via thin layer chromatography-direct bioautography (TLC-DB)

Previous qualitative and quantitative analyses provide strong evidence for further investigation into the antioxidant activity of *H. fulva* flowers. After identifying the optimal solvent system for each of the three crude extracts and selecting the compounds most suitable for separation, the developed thin layer chromatography (TLC) method was utilised. The TLC plates were then sprayed with 1,1-diphenyl-2-picrylhydrazyl (DPPH) reagent to assess the ability of the active compounds to scavenge free radicals (**Table 3**). This reaction was indicated by a yellow spot formation on the TLC, signifying the presence of antioxidants capable of neutralising the DPPH free radical [37]. This method is widely used to evaluate the radical scavenging properties of plant compounds, supporting the exploration of *H. fulva* as a potential source of natural antioxidants.

The data revealed that methanol crude produced the highest number of spots on the thin layer chromatography (TLC) plates, followed by ethyl acetate and hexane after treatment with 1,1-diphenyl-2-picrylhydrazyl (DPPH) reagent (**Table 3**). The TLC analysis showed that specific compounds in all three crudes appeared at the same retention factor (R_f) value of 0.54, responding to the DPPH reagent by turning yellow. This suggests that these compounds are likely non-polar or semi-polar, as they were present in crudes with increasing polarity and positioned towards the centre of the TLC plate, consistent with previous findings [39, 40]. Additionally, compounds at R_f values of 0.09, 0.17, 0.25, 0.54, and 0.89 were found in both ethyl acetate and methanol extracts, further supporting their classification as polar compounds.

The appearance of yellow spots on the TLC plates indicated that *H. fulva* flower has antioxidant potential. The yellow colour resulted from the active compounds on the TLC plate scavenging the free radicals from the DPPH reagent, transforming them into DPPHH [41, 42]. This reduction of the violet colour of DPPH to yellow confirms the antioxidant activity of the flower. These observations aligned with the high levels of flavonoid and phenolic content in the crude extracts and are consistent with the phytochemical screening results, further supporting the hypothesis that *H. fulva* flowers possess significant antioxidant properties.

Antimicrobial assay via thin layer chromatography-direct bioautography (TLC-DB)

Szewczyk et al. [5] speculated in 2020 that *H. fulva* flowers may possess antimicrobial properties. This hypothesis is supported by another researcher [43]. The current study's phytochemical screening revealed the presence of alkaloids and phenolics, which are often associated with antimicrobial activity. However, despite these promising indicators, the antimicrobial testing performed via thin layer chromatography-direct bioautography (TLC-DB) against four bacterial strains-two Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and two Gram-negative (*Escherichia coli* and *Salmonella sp.*), yielded poor results, as shown in **Table 4.**

In contrast to previous report [5], which suggested that *H. fulva* flowers possess antimicrobial properties, the experimental results in the current study showed positive antimicrobial activity only for hexane and ethyl acetate extracts against *B. subtilis*, with no activity observed against other bacterial strains, particularly with methanol extract. While this does not entirely invalidate the hypothesis that *H. fulva* has antimicrobial potential, it suggests that the antimicrobial activity may be selective or dependent on the specific bacterial strain tested. Additionally, the thin layer chromatography-direct bioautography (TLC-DB) method used in this study may not be the most suitable for accurately assessing antimicrobial properties, as it has limitations in detecting low levels of antimicrobial compounds or in certain extraction solvents [44, 45].

The use of TLC-DB, while a useful preliminary screening method for antimicrobial activity, may not provide the sensitivity and reproducibility needed to assess the full antimicrobial potential of *H. fulva* extracts. In future studies, methods such as the agar well diffusion assay, which measures the zone of inhibition around an extract, would be more reliable for evaluating antimicrobial activity in a broader range of bacterial species. This method has been widely recommended for evaluating antimicrobial agents because it allows for more accurate quantification of inhibition zones and can detect a wider spectrum of antimicrobial compounds [46, 47]. Such methods

Table 3. Details of thin layer chromatography-direct bioautography (TLC-DB) for antioxidant assay

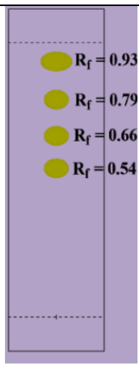
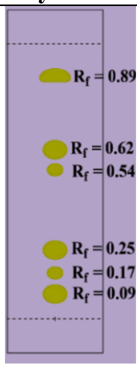
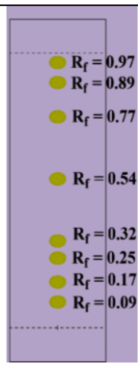
Crude	Hexane	Ethyl acetate	Methanol
Indicator: H = hexane E = ethyl acetate T = toluene			
Solvent system	H: E	H: E	T: E
Ratio	9:1	6:4	7:3
Number of spots	4	6	8

Table 4. Results of antimicrobial assay of *H. fulva* flower crudes

Thin Layer Chromatography (TLC) Plate	Gram-positive Bacteria		Gram-negative Bacteria	
	Staphylococcus aureus	***Bacillus subtilis***	***Escherichia coli***	***Salmonella sp.***
Hexane extract	-	+	-	-
Ethyl acetate extract	-	+	-	-
Methanol extract	-	-	-	-

Indicator: + = positive result - = negative result

could provide clearer insights into the effectiveness of *H. fulva* extracts as antimicrobial agents.

Conclusion

Overall, the study demonstrated that the polar extracts of *H. fulva* flowers, specifically ethyl acetate and methanol, contain various phytochemicals, including alkaloids, flavonoids, saponins, terpenoids, and proteins. These compounds are known for their potential medicinal properties, which support the use of *H. fulva* as an alternative treatment for various diseases and infections. The high levels of flavonoids and phenolics in the flower contribute to its antioxidant activity, as confirmed by the positive results in the thin layer chromatography-direct bioautography (TLC-DB), where the yellow coloration on the TLC plate indicated the presence of antioxidant bioactive compounds. Moreover, the abundance of alkaloids and terpenoids in *H. fulva* flowers also supports its antimicrobial potential, demonstrated through its activity against *Bacillus subtilis* in antimicrobial assays. These findings on the phytochemical profile, antioxidant, and antimicrobial properties of *H. fulva* are promising and are being increasingly utilized in research and pharmaceutical industries for the development of new drugs targeting a variety of diseases.

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