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The Investigation of Phytochemicals and Antioxidant Properties of *Champereia Manillana* (Blume) Merr Stem Bark

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Abstract

Introduction: *Champereia manillana* (Blume) Merr. is one of the plant species that lacks research despite many beneficial claims from consumers. Studies conducted on this species have merely discussed bioactive phytochemicals in the leaves and roots. Therefore, the objectives of this study are to screen for terpenoids, triterpenoids, diterpenoids, carotenoids, flavonoids, phenolics, and steroids, and to evaluate the antioxidant activities of *C. manillana* stem bark methanolic extract. **Methods:** The plant material was collected from the Forest of 'Ilm, IIUM. The stem bark powder was macerated in methanol. Phytochemical screening tests were utilized to determine the presence of the phytochemicals. Thin-layer chromatography (TLC) analysis was carried out on the samples using iodine vapor, ferric chloride solution, vanillin solution, and UV light. Next, the total phenolic and flavonoid content tests were conducted to obtain quantitative results, and the antioxidant activity was assessed using the DPPH assay. **Results:** *C. manillana* stem bark extract tested positive for steroid and terpenoid contents and negative for carotenoids and flavonoids. For the TLC, the extract was found positive for iodine vapor, UV light, and vanillin/H2SO4 and negative for the ferric chloride test. The flavonoid content was 0.995 mg CE/g, while the phenolic content was 12.326 mg GAE/g. For the DPPH assay, the IC_{50} value was high (26 mg/mL) compared to the positive control, ascorbic acid, which had an IC_{50} value of 6.730 μg/mL. The percentage inhibition at 10 mg/mL was 23.4%. **Conclusion:** Phytochemical screening indicates the presence of steroids and terpenoids in *C. manillana* stem bark methanolic extract. Compared to the standard (ascorbic acid), the antioxidant activity of the extract is very weak.

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Introduction

The usage of herbs and plants in the world community these days is very common, whether it is for traditional medicine, modern medicine, or cooking recipes. Based on certain beliefs, raw herbs can heal illnesses better than modern medicines. However, to prove this statement, many factors must be considered, such as hygiene and the active ingredient content within the raw herbs. Our older generation, especially from Asian countries, loves to consume herbs as part of their meals and holds a strong belief that herbs keep them healthy during old age (Musa et al., 2022). The ease with which herb plants can be grown and harvested might be the main reason why they are so commonly consumed by past generations (Welz, Emberger-Klein & Menrad, 2018). Since the use of herbs usually relies on traditional knowledge passed down from previous generations, the exact composition of the phytochemical content within the herbs remains unknown until modern medicinal technologies make discoveries (Musa et al., 2022). In recent years, the study of herbs and plants has increased significantly, aiming to achieve a better understanding of their pharmacological importance and potential health effects. Hence, to achieve a complete understanding of a plant's safety, toxicology, and compounds, proper and rigorous investigations need to be conducted.

In Malaysia, one of the famous herbs is *C. manillana (Blume) Merr.*, locally known as *'pucuk cemperai'*. It is a monotypic genus from the Opiliaceae family of plants and can be widely found from the Andaman Islands to Papua New Guinea, including other countries such as Thailand, Vietnam, Taiwan, China, Singapore, Java Island, and many more (K. Jeyaprakash & N. Balachandran, 2018). *C. manillana* is a small tree that can grow up to 20 meters tall in lowland tropical rainforests at altitudes up to 1,600 meters. It has a variety of stalk leaves, including oblong, oval, and lance shapes, while both sides of the leaf blades are hairless (Yang et al., 2017). This species can be easily found in Pahang, and the locals commonly use it to make a dish known as *masak lemak pucuk cemperai*. This species is claimed to contain a high concentration of antioxidants that help protect the body against heart disease and cancer and boost the immune system. Additionally, some people use the pounded root of *C. manillana* to make a poultice for ulcers, while the boiled roots are used for healing rheumatism. Possible extinction is not an issue for *C. manillana*

Fig. 1: *Champereia manillana* from the Forest of 'Ilm IIUM

because this species is still widely distributed in our country and can easily be found in any rainforest (Denny, Marfuah Wardani, & Adi Susilo, 2021).

Even though this plant has not received proper attention in research and studies, the leaves of *C. manillana* have been claimed to contain many beneficial phytochemical compounds that provide antioxidant activity, such as squalene, β-carotene, dietary lutein, and phytol (K. Jeyaprakash & N. Balachandran, 2018). Squalene is a natural triterpene with molecular properties directly linked to its activity against free radicals, acting as a free radical scavenger or quencher in certain cases. With its antioxidant properties, some studies have reported that squalene can suppress advanced colon cancer formation by preventing crypt multiplicity inside the intestine (Micera et al., 2020). The second phytochemical component found in *C. manillana* is dietary lutein. Lutein is a fat-soluble carotenoid phytochemical with several pharmacological properties (Rahman et al., 2023). Lutein has antioxidant, anti-inflammatory, anticancer, cardioprotective, and eye-protective activities. It exerts its ability to prevent the rapid growth of tumors and induce cancer cell apoptosis by inhibiting angiogenesis in mammary gland tumors. Another component is β-carotene, a carotenoid group compound (terpenoid) that serves as pigmentation in the plant kingdom. Its structure consists of a conjugated double bond with a retinyl group at both ends. β-carotene is the major precursor of vitamin A and contains many therapeutic properties for humans (Bogacz-

Radomska & Harasym, 2018). Lastly, the phytol component found in *C. manillana* is identified as Acyl-CoA cholesterol acyltransferase (ACAT). Phytol is a cyclic diterpene in the plant that forms part of the structure of chlorophyll. Nowadays, it is common to include phytol in pharmaceutical products due to its antinociceptive effect, which reduces pain by blocking the pain sensory neuron (Carvalho et al., 2020). Based on previous research related to the beneficial properties of *C. manillana* leaves, we aim to explore the phytochemicals and antioxidant activity of the stem bark.

Materials and methods

Plant collection and preparation

250 g of fresh *C.manillana* stem bark (Voucher Specimen : PIIUM 0357) (Species verification by Dr Shamsul Khamis from UKMB herbarium). A voucher specimen deposited at Kulliyyah of Pharmacy IIUM was collected from the Forest of 'Ilm IIUM Kuantan on 15 October 2023. The stem bark was rinsed and dried in the oven for two (2) days with a temperature range from 40-60 ℃ (Abubakar & Haque, 2020). Next, the dried stem bark was processed into powder form using an electric grinder.

Plant extraction

150 g of the fine powder was taken to be macerated with 950 mL of methanol in the conical flask for 5 days at room temperature (Abubakar & Haque, 2020). The extract solution was filtered using filter paper and the methanol was removed using a rotary evaporator. The crude methanol extract was kept for the next usage. The percentage yield is calculated following formula (1) as below:

$$\text{Percentage yield } (\%) = \frac{\text{mass of crude methanol}}{\text{mass of sample}} \times 100 \quad (1)$$

Methanol was chosen for the extraction process due to its superior ability to extract a wide range of phytochemicals compared to ethanol. Research has shown that methanol often provides higher extraction yields of total phenolic content (TPC) and is effective at extracting various other bioactive compounds. Specifically, methanol has been reported to achieve the highest extraction yields of TPC, the second highest extraction of total flavonoid content (TFC), and the highest yield of extractable solids (Goltz et al., 2012).

Phytochemical screening

A phytochemical test is a qualitative test that indicates the absence or presence of the targeted compound. The procedures below were referred to in various articles with minor modifications.

Terpenoid Test

For the Terpenoid Test, 50 mg of the extract was weighed and dissolved in 2 mL of dichloromethane. The solution was filtered, and then 2 mL of concentrated sulphuric acid (H_2SO_4) was added to the test tube. The formation of a reddish-brown coloration at the interface indicated the presence of terpenoids (Dubale et al.,2023).

Steroid Test

For the Steroid Test, 50 mg of the extract was weighed and dissolved in 2 mL of dichloromethane. After filtration, a few drops of acetic acid were added to the sample, and the mixture was boiled in a water bath for 10 minutes. The sample was rapidly cooled using an ice bath and then 2 mL of concentrated sulphuric acid (H_2SO_4) was added. Any formation of brown ring at the junction indicated that the presence of steroid (Dubale et al., 2023).

Carotenoid Test

In the Carotenoid Test, 100 mg of the extract was dissolved in 10 mL of chloroform and filtered. A few drops of concentrated sulphuric acid (H_2SO_4) were added to the test tube containing the filtered solution. A blue color at the interface showed the presence of carotenoids (Ajayi & Ajibade O, 2011)

The Carotenoid Test utilized 100 mg of extract, double the amount used in other phytochemical tests, to ensure sufficient sensitivity and accuracy in detecting carotenoids. Carotenoids are often present in lower concentrations compared to other phytochemicals like terpenoids, steroids, and flavonoids. By using a larger quantity of the extract, the test increases the likelihood of detecting carotenoids even if they are present in small amounts. This approach enhances the test's reliability and reduces the risk of false negatives, ensuring that the presence of carotenoids can be accurately confirmed (Goltz et al., 2012).

Thin layer chromatography (TLC)

Thin-layer chromatography (TLC) facilitates the separation and identification of various components within a mixture, relying on the principles of adsorption and capillary action. The sample is applied to one end of the TLC plate and then the

plate is positioned vertically inside a sealed chamber containing an organic solvent (mobile phase). Capillary action causes the mobile phase to ascend the plate, and components of the sample migrate different distances based on their interactions with the stationary phase (the TLC plate) and the mobile phase. Once the solvent reaches the top of the plate, it is taken out of the chamber and allowed to dry (Qin et al., 2021). For this method, the TLC silica gel plate 60F254 (Merck, Germany) was cut into 10 cm x 10 cm in dimension. The 10 mg of sample was dissolved in 1 mL of methanol. The dissolved sample was applied to the TLC plate using a capillary tube as 1 cm band. Next, the plate was placed inside the TLC-developing container that consisted of ethyl acetate and methanol with a ratio of 8:2 for 10 minutes, following the method by Ameerah Shaeroun et al., (2019).

Phytochemical analysis of the TLC for methanol extract

UV Lamp Analysis

The TLC plate was viewed under UV light which the wavelengths 254 and 366 nm in a dark room and the presence of aromatic compounds was seen through black and fluorescence colour on the TLC plate (chromatogram) (Ameerah Shaeroun et al., 2019).

Iodine Vapour

The TLC plate was sprayed with iodine vapour to detect the presence of a compound with double bond linkages. (Ameerah Shaeroun et al., 2019)

Ferric Chloride

The TLC plate was sprayed with ferric chloride solution to detect the presence of phenolic compounds. (Ameerah Shaeroun et al., 2019)

Vanillin/ H_2SO_4

The TLC plate was sprayed with vanillin/ H_2SO_4 solution to detect the presence of terpenoid compounds. (Ameerah Shaeroun et al., 2019).

Total phenolic content test (TPC)

TPC for the standard gallic acid and the methanol crude were performed following the method by Herald et al. (2012) with minor modification.

TPC for standard gallic acid

The stock solution of gallic acid was prepared by two-fold serial dilution in distilled water, resulting in eight different concentrations: 100, 50, 25, 12.5, 6.25, 3.125, 1.563, and 0.781 µg/mL. Each of these concentrations was then used to prepare the standards for the assay. Specifically, 20 µL of each stock solution was transferred into a 96-well plate in triplicate. To each standard well, 100 µL of Folin-Ciocalteu reagent and 80 µL of sodium carbonate were added, making a total volume of 200 µL per well. In another set of wells, the blank consisted of 20 µL of the standard solution, 100 µL of distilled water, and 80 µL of sodium carbonate. The blank was also prepared in triplicate. Finally, the well plate was incubated for 2 hours in a dark room before measuring the absorbance using a microplate reader at 750 nm (Herald et al., 2012).

Estimation of TPC for sample extract

The 15 mg sample solution was prepared into two concentrations which were 5000 ug/mL and 10000 ug/ml. These concentrations were chosen to ensure that the phenolic content could be detected even at low concentrations, which is crucial for accurately assessing the phenolic profile of the extract. Using higher volumes helps to amplify the detection signal, thereby enhancing the sensitivity of the assay (Simčič, Stibilj, & Holcman, 2011). Using larger sample volumes in phenolic content assays has been advocated in research because it enhances the reliability of detecting phenolic compounds, particularly when they are present in trace amounts. This method ensures a comprehensive analysis, capturing even minimal phenolic content which might otherwise go undetected (Simčič, Stibilj, & Holcman, 2011). Next, the 20 uL of the sample was transferred into the 96 well plate in triplicate and 100 uL of Folin-Ciocalteu reagent with 80 uL of sodium carbonate were added into the sample well. In another well, the blank extract consisted of 20 uL of standard, 100 uL of distilled water and 80 uL of sodium carbonate. The blank extract also made in a triplicate manner. The blank diluent consisted of 20 uL DMSO and 100 uL of Folin-Ciocalteu reagent with 80 uL of sodium carbonate, Na_2CO_3. Lastly, the well plate was incubated for two (2) hours in the dark room before measuring the absorbance using a microplate reader at 750 nm. The TPC value was determined from the linear regression curve of absorbance against concentration using the equation $Y = mx + c$. Results obtained were expressed as microgram gallic acid equivalence per mg.

Total flavonoid content (TFC)

TFC for the standard gallic acid and the methanol crude were performed following the method by Herald et al. (2012) with minor modification.

TFC for standard catechin

The stock solution of catechin was prepared by two-

fold serial dilution in distilled water, resulting in eight different concentrations: 100, 50, 25, 12.5, 6.25, 3.125, 1.563, and 0.781 μg/mL. Each of these concentrations was then used to prepare the standards for the assay. Specifically, 25 μL of each concentration of the stock solution of catechin was transferred into a 96-well plate in triplicate. To each standard well, 100 μL of distilled water and 10 μL of sodium nitrate were added. The well plate was incubated for 5 minutes, and then 15 μL of aluminium chloride was added to the mixture. After an additional 6 minutes of incubation, 50 μL of sodium hydroxide and 50 μL of distilled water were added. In another set of wells, the blank consisted of 25 μL of the standard solution and 225 μL of distilled water. The blank was also prepared in triplicate. Lastly, the absorbance was measured using a microplate reader at 510 nm (Herald et al., 2012).

Estimation of TFC for sample extract

The 15 mg sample solution was prepared into two concentrations which were 5000 ug/ml and 10000 ug/ml. These concentrations were chosen to ensure robust detection and quantification of flavonoids within the plant extract. The selection of higher sample volumes is supported by research indicating that it enhances the sensitivity of the assay, allowing for accurate quantification of flavonoid content, even at lower concentrations (Ghasemzadeh et al., 2010). Flavonoids are typically present in higher concentrations compared to other phenolic compounds, and using larger sample volumes ensures that the assay can detect and quantify these compounds effectively. Next, the 25 uL of the sample was transferred into the 96 well plate in triplicate and 100 uL of distilled water with 10 uL of sodium nitrate was added into the standard well. The well plate was incubated for 5 minutes and then 15 uL of aluminium chloride was added to the mixture as well. After that, the well plate was incubated again for 6 minutes and 50 uL of sodium hydroxide together with 50 uL of distilled water was added. In another well, the blank diluent consisted of the same solution with extract replaced by DMSO solution. The blank diluent consisted of 25 uL of sample and 225 uL of distilled water. Lastly, the absorbance was measured using a microplate reader at 510 nm. The TFC value was determined from the linear regression curve of absorbance against concentration using the equation Y= mx + c. Results obtained were expressed as milligram of catechin equivalence per mg.

2,2-diphenyl-1-picrylhydrazyl Assay (DPPH Assay)

DPPH assay for the standard gallic acid and the methanol crude were performed following the method by Herald et al. (2012) with minor modification**.**

DPPH assay for standard ascorbic acid

The stock solution of 1 mg/mL ascorbic acid was prepared by two-fold serial dilution in distilled water, resulting in eight concentrations: 1000, 500, 250, 125, 62.5, 31.25, 15.625, and 7.8125 μg/mL. Each concentration was carefully pipetted with 50 μL into a 96-well plate in triplicate. Subsequently, 150 μL of DPPH solution was added to each well containing the standard ascorbic acid. The blank extract was prepared similarly, with the exception that 150 μL of methanol replaced the DPPH solution. This blank was also done in triplicate to account for any background absorbance. In another set of wells, a blank diluent was created by adding 150 μL of DPPH solution with 50 μL of DMSO. Additionally, a blank control was prepared using 50 μL of DMSO and 150 μL of methanol. Both blanks were included to ensure the accuracy of absorbance readings and were also done in triplicate. Following preparation, the 96-well plate was incubated for 40 minutes in a dark room to allow the reaction between ascorbic acid and DPPH to proceed. Absorbance was then measured at 515 nm using a microplate reader.

DPPH assay for sample extract

The 10 mg/mL sample extract was prepared by two-fold serial dilution in distilled water, resulting in eight concentrations: 10000, 5000, 2500, 1250, 625, 312.5, 156.25, and 78.125 μg/mL. Each concentration was pipetted with 50 μL into a 96-well plate in triplicate. Subsequently, 150 μL of DPPH solution was added to each well containing the sample extract. The blank extract was prepared similarly, with the exception that 150 μL of methanol replaced the DPPH solution. This blank was also done in triplicate to account for any background absorbance. In another set of wells, a blank diluent was created by adding 150 μL of DPPH solution with 50 μL of DMSO. Additionally, a blank control was prepared using 50 μL of DMSO and 150 μL of methanol. Both blanks were included to ensure the accuracy of absorbance readings and were also done in triplicate. Following preparation, the 96-well plate was incubated for 40 minutes in a dark room to allow the reaction between the sample extract and DPPH to proceed. Absorbance was then measured at 515 nm using a microplate reader. The decision to use a higher starting concentration of 10000 μg/mL

for the sample, compared to the standard ascorbic acid concentration of 1000 μg/mL, was based on the observed low DPPH values during preliminary testing. In order to ensure that the antioxidant activity of the samples could be reliably detected and quantified, a higher sample concentration was necessary. Due to the complex matrix of plant extracts and the variability in flavonoid content among different species, higher concentrations are often required to achieve measurable antioxidant effects. This approach is supported by the need to overcome any dilution effects and to enhance the sensitivity of the assay, ensuring that even subtle antioxidant activities can be detected and accurately quantified (Ghasemzadeh et al., 2010). Based on the absorbance result, the calculation was made using the formula (2) to get the percentage of inhibition and IC_{50}.

$$\frac{\text{Percentage of inhibition (100\%)}}{DPPH} = \frac{\text{Control Absorbance} - \text{Extract Absorbance}}{\text{Control Absorbance}} \times 100 \quad (2)$$

Results and discussion

Percentage yield

Mass of methanol crude was 7.40 g with the percentage yield of 4.95%, indicated a very poor extraction yield from the maceration process, falling below 20%. Maceration extraction involves soaking plant material in a solvent to extract bioactive compounds over time. This method is chosen for its simplicity, cost-effectiveness, and versatility in extracting a wide range of compounds without specialized equipment (Mbah & Eme, 2017). It is particularly effective for extracting phenolic compounds and flavonoids, which are known for their antioxidant properties and health benefits (Yazdani et al., 2021). This could have been caused by various factors. Some articles agreed that maceration was not an efficient method, as it typically resulted in low yields, was time-consuming, and caused cell degradation. Instead of using maceration, Soxhlet extraction was recommended because it could produce higher yields without degrading the bioactive compounds (Aspé & Fernández, 2011). Furthermore, some articles suggested that percolation was more efficient than maceration due to its continuous process, where the saturated solvent was constantly replaced by fresh solvent (Zhang et al., 2018). Regarding the maceration process itself, the low yield production might have occurred due to improper handling during the process, such as frequent transfers to another beaker that led to a loss of yield. Other than that, it might have also been caused by a lack of stirring and insufficient time during maceration because some research suggested macerating the sample for two weeks to provide better results (Aspé & Fernández, 2011).

Phytochemical screening test

From the result for phytochemical screening test (Table 1), the flavonoid and carotenoid tests were negative while steroid and terpenoid tests were positive. Phytochemical screening was a laboratory technique used to identify and analyse the presence of bioactive compounds in plant extracts. These compounds, known as phytochemicals, included various secondary metabolites such as alkaloids, flavonoids, terpenoids, and phenolic compounds. From the phytochemical screening result above, the steroid and terpenoid tests were positive, while the flavonoid and carotenoid tests were negative. Based on the studies conducted for the leaves part of *C.manillana*, the compound presence included flavonoid, terpenoid, carotenoid, and steroid (Y. Ragasa et al., 2015). The presence of terpenoid and steroid aligned with the studies conducted before for the leaf part. The negative result for flavonoid and carotenoid can be explained through two possible reasons. First, flavonoid and carotenoid compounds were absent in the stem bark of *C.manillana*, which is acceptable because none of the research or studies has been done for the stem bark, so no comparison can be made, and this can be a new finding in the herbal field. Secondly, the negative result may be caused by the low amount of flavonoid and carotenoid compounds present inside the sample, causing it to fall below the detection limit. Phytochemical screening tests are qualitative tests; hence, it is predictable that certain results may not be accurate (Hashmi & Bibi, 2021). Due to that, it is suggested to conduct further investigation through quantitative methods such as total flavonoid and phenolic content tests to obtain accurate and precise results.

Thin layer chromatography

From TLC test (Table 2), the iodine vapor, vanillin/ H_2SO_4 and UV light tests were positive while ferric chloride test was negative.

Table 1: The results of phytochemical screening test

Phytochemical Test	Observation
Steroid test	Result: Positive The formation of a brown ring at the junction of two layers indicated the presence of steroids. The formation of a brown ring at the junction of two layers is indicative of the presence of steroids, as detected by the Liebermann-Burchard test in phytochemical analysis. This reaction involves the interaction of steroids with concentrated sulfuric acid and acetic anhydride, leading to the formation of a colored complex at the interface. Steroids undergo dehydration and subsequent rearrangement reactions in the acidic medium, resulting in the production of conjugated dienes which react with acetic anhydride to form the characteristic brown color (Zhang, Zheng, & Pan, 2015)
Carotenoid test	Result: Negative No blue color formed, indicating that carotenoids could not be detected inside the sample.
Flavonoid test	Result: Negative No yellowish color formed, indicating that flavonoids could not be detected in this test
Terpenoid test	Result: Positive The formation of reddish-brown color shows the presence of terpenoids. Specifically, in the case of the Liebermann-Burchard test for terpenoids, the compounds undergo dehydration and subsequent rearrangement reactions in the acidic environment. This process leads to the formation of conjugated polyenes, which absorb light at specific wavelengths, resulting in the observed reddish-brown coloration at the reaction site (Trease & Evans, 2009)

Discussion of TLC

The mobile phase consisting of ethyl acetate and methanol in a ratio of 8:2 was chosen for TLC due to its effective separation capabilities for compounds with varying polarities. Ethyl acetate, being less polar, facilitates the elution of non-polar to moderately polar compounds, while methanol, with its higher polarity, aids in separating more polar compounds. This specific ratio is well-established in chromatography literature for providing clear spot development and good resolution of natural products, including terpenoids, flavonoids, and phenolic compounds (Ezzat et al., 2014). Therefore, ethyl acetate: methanol, 8:2 is ideal for analysing complex mixtures, such as those found in the stem bark of *C. manillana*, ensuring effective compound separation and identification on TLC plates. From the TLC results, all tests were positive

Table 2: The results of TLC tests

Phytochemical Test	Observation
Iodine vapour	Result: Positive The presence of brown and yellow colors on the TLC plate after being sprayed with iodine vapor indicated the presence of compounds with double and triple bonds in the sample
Ferric chloride	Result: Negative The absence of dark blue spots after being sprayed with ferric chloride solution indicated that the phenolic compound was undetected using TLC. However, it is important to note that "undetected" does not necessarily mean absent; it could indicate that the amount present was too low to be detected by the TLC method (Wilson et al., 2014).
UV_{254} & UV_{366}	Result: Positive The presence of black and fluorescent colors on the TLC plate under UV light indicated that the sample contained aromatic compounds.
Vanillin/ H_2SO_4	Result: Positive The presence of many colors, such as pink, purple, and grey on the TLC plate after being sprayed with vanillin, shows that the sample contains terpenoid compounds.

except for the ferric chloride test. The iodine vapor test detects the presence of double bond compounds, indicated by the formation of a brown colour on the TLC plate. Aromatic compounds are detected using the UV test, appearing as black or fluorescent colours under UV light. The vanillin test is employed to detect terpenoid compounds, characterized by the formation of various colours such as grey, pink, purple, and blue. The ferric chloride test, typically used for detecting phenolic compounds, shows as blue spots on the TLC plate (Yahyaoui et al., 2017). TLC serves as a preliminary screening method to identify major compounds qualitatively (Wilson et al., 2014). However, due to the complex nature of the sample containing numerous active biochemicals, the spots on the TLC plate may not be clearly defined. This complexity indicates that the sample does not need to be

purified thoroughly for this study's purposes. The negative result from the ferric chloride test may not accurately reflect the presence of phenolic compounds, as their concentration might be too low to detect with this method. Hence, for a more precise determination of phenolic content, additional quantitative tests such as TPC and TFC are essential. Therefore, TLC serves as a general guidance in this study for screening major compounds, providing preliminary insights into the presence of double bond compounds, aromatic compounds, and terpenoids. However, for accurate quantitative analysis, TPC and TFC tests are necessary to determine the exact composition and concentration of phenolic and flavonoid compounds in the stem bark of *C. manillana*.

Total phenolic and flavonoid content test

Total phenolic content test

From the observation of Fig. 2: The 96 well plate of the sample for TPC, the blue colour solution formed indicated the presence of phenol compound. The intensity of the blue colour was very low which might be caused by the low amount of phenol presence inside the extract (Aryal et al., 2019).

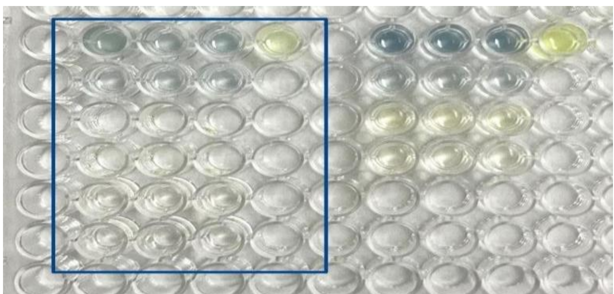
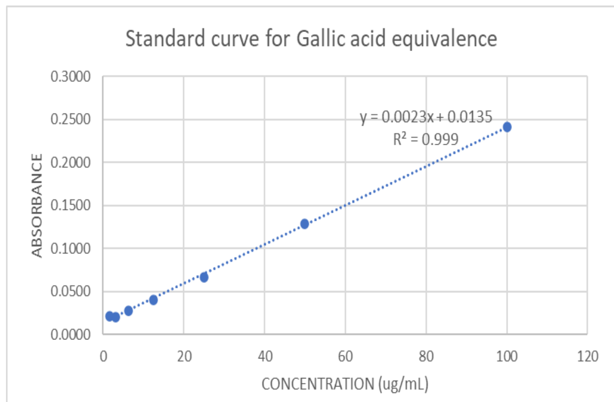


Fig. 2: The 96 well plate of the sample for TPC



Graph 1: The standard curve for Gallic acid equivalence

Based on the data interpretation by using the standard curve of gallic acid (Graph 1) the absorbance for TPC 10000 ug/mL (10 mg/mL) is 0.297 and Further calculation gave the value of TPC is 12.326 mg GAE/g. Further calculation of the mean TPC (mg GAE/g) for 10000 ug/mL is 11.630 ± 3.480 mg GAE/g and for 5000 ug/mL is 3.990 ± 0.260 mg GAE/g.

Total flavonoid content test

From the observation of **Error! Reference source not found.**, the yellow color solution formed indicated the presence of flavonoid compound. The intensity of the yellow color was very low which might be caused by the low amount of flavonoid presence inside the extract.

Based on the data interpretation by using the standard curve of catechin (Graph 2), the value of absorbance for TFC 10000 ug/mL (10 mg/mL) is 0.136. Further calculation gave the value of total flavonoid content is 0.995 mg CE/g. Further calculation of the mean TFC value for 10000 ug/mL is 0.937 ± 0.250 mg CE/g and for 5000 ug/mL is 0.428 ± 0.180 mg CE/g.

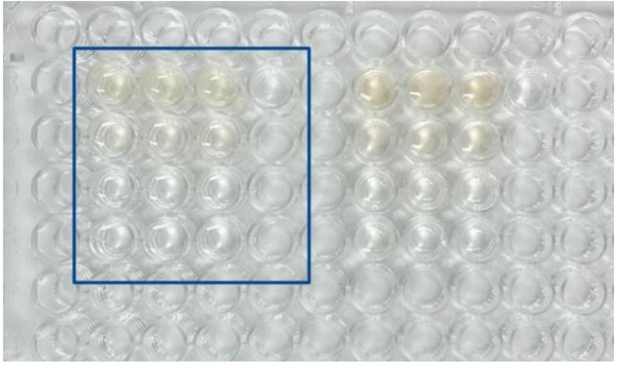
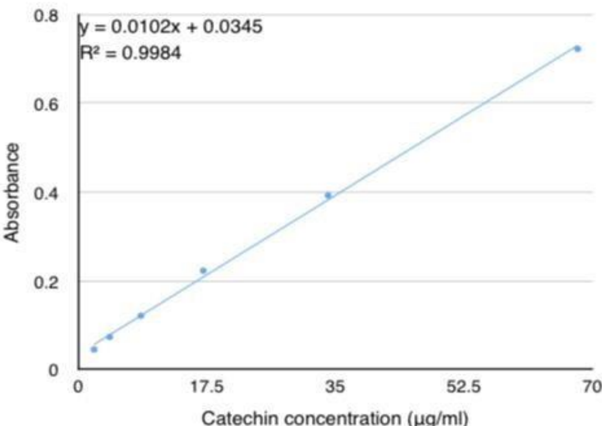


Fig. 3: The 96 well plate of the sample for TFC

Catechin Standard Curve



Graph 2: The standard curve of catechin equivalent

Discussion

The TPC and TFC test was a quantitative method that provided an accurate measure of the phenolic

and flavonoid compounds present in a plant. This assay was significant in measuring the total antioxidant capacity, as high phenolic and high flavonoid content have been linked to high antioxidant capacity (Molole et al., 2022). In the case of *C. manillana* stem bark, the tests revealed a total phenolic content of 12.33 mg GAE/gram and 0.99 mg CE/gram for flavonoids. Since no prior studies on *C. manillana*'s stem bark existed for comparison, a reference value could not be established, making this a new finding in the herbal field. However, when comparing it with *Opilia amentacea*, a plant from the same family (Opiliaceae), its stem bark exhibited a significantly higher total phenolic content of 55.08 mg GAE/gram and a flavonoid content of 23.62 mg CE/gram (Ollo Youl et al., 2023). These large differences were expected since they belong to different species. One certainty from the TPC and TFC tests was that the presence of phenolic compounds and flavonoid compounds in the stem bark could be verified. Unlike the TLC and phytochemical test, which doesn't capable of detecting phenolic and flavonoids compound, the TPC and TFC tests quantitatively validated the existence of phenolic compounds and flavonoids. The negative results from the phytochemical tests and TLC do not conclusively indicate the absence of phenolic and flavonoid compounds. Instead, these results suggest that these compounds may be present but at levels below the detection limits of these qualitative methods. This further underscore the importance of employing quantitative tests such as TPC and TFC, which are capable of accurately detecting and quantifying even the lowest amounts of these bioactive compounds. Regarding colour changes for TPC, the reaction between the Folin-Ciocalteu reagent and phenolic compounds formed a blue-coloured solution. The blue color intensity was very low since the compound was also low in amount. For the TFC color changes, the yellow color formed from the formation of a complex between the aluminium ion and the carbonyl and hydroxyl groups of flavonoids. The mean TFC value was 0.937 ± 0.25 mg CE/g for 10000 μg/mL and 0.428 ± 0.18 mg CE/g for 5000 μg/mL. For TPC values, the mean for 10000 μg/mL was 11.630 ± 3.480 mg GAE/g and for 5000 μg/mL was 3.990 ± 0.260 mg GAE/g.

DPPH assay

Ascorbic acid

Ascorbic acid was used to serve as standard in this assay.

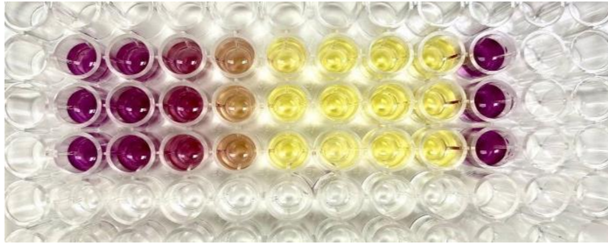


Fig. 4: The 96 well plate of the ascorbic acid for DPPH (from left; the lowest concentration to the highest concentration)

From the observation of Fig. 4, as the concentration of the sample increased, the purple color started to change to yellow, indicating the presence of antioxidant activity. The change in color occurred when the stable free radical compound in purple DPPH was scavenged, causing it to turn yellow. Based on the calculations, the IC_{50} for ascorbic acid is 6.730 μg/mL, and the mean IC_{50} is 7.100 ± 1.489 μg/mL.

Sample extract

From the observation of Fig. 5, the yellow colour indicates the presence of antioxidant activity. As the concentration of the sample reduced, the yellow colour has no longer seen, since the amount of antioxidant activity is too low scavenged the free radical of DPPH solution.

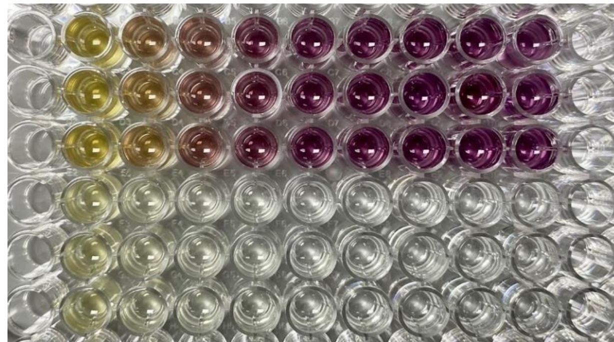
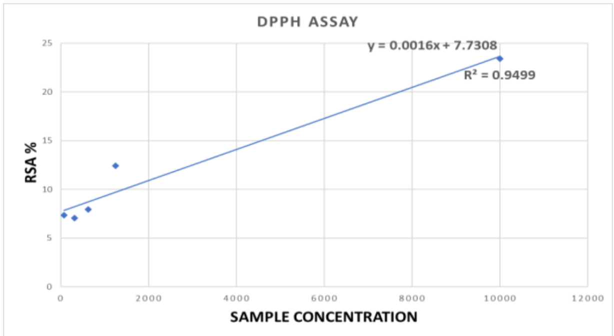


Fig. 5: The 96 well plate of the sample for DPPH (from left; the highest concentration to the lowest concentration)

The DPPH assay was conducted to measure the total antioxidant activity present in the plant extract. DPPH stands for 2,2-diphenyl-1-picrylhydrazyl, which exists in both solid and solution forms as a monomer. It is a stable free radical compound with a purple color at 517 nm that turns yellow when scavenged. The color change occurs due to the neutralization of DPPH when it receives an electron donated by an antioxidant compound, acting as an indicator of antioxidant activity (Munteanu & Apetrei, 2021). For this project, the graph was



Graph 3: The DPPH assay graph for sample

created using five concentrations instead of eight to achieve an R value above 0.95. As seen in Graph 3, the R value of 0.95 was achieved with concentrations of 10000, 5000, 2500, 1250, and 625 µg/mL.

The antioxidant activity by the DPPH neutralization method is often reported as IC_{50}, which is defined as the inhibitory concentration of the antioxidant necessary to scavenge 50% of the DPPH initial radical. The lower the IC_{50} value, the higher the potency of the antioxidant. From the data obtained from Graph 3, the IC_{50} calculated for the ascorbic acid was 6.730 ug/mL, while the IC_{50} calculated for *C. manillana* stem bark was 26 mg/mL. This showed that the antioxidant value in ascorbic acid was higher than the one present inside *C. manillana* stem bark. Besides, it can be said that the antioxidant properties of stem bark were very weak since it was above 0.1 mg/mL (Welz et al., 2018). Hence, it can be concluded that the stem bark of *C. manillana* is not a good source of antioxidants. The highest percentage inhibition was 23.4% at 10000 ug/mL. None of the studies have been made for the stem bark, so this might be a new finding available. Comparing to the studies done for the leaves part, the percentage inhibition was higher with 31% compared to the stem bark. The low antioxidant activity may be associated with the low amount of flavonoid and phenolic compound since they are considered as natural potent antioxidants (Tungmunnithum et al., 2018).

Conclusion

From the tests that have been conducted, the phytochemical screening showed a positive result for the presence of steroids and terpenoids, while yielding negative results for flavonoids and carotenoids. In the TLC test, the iodine vapor test, vanillin test, and UV light test were positive, indicating the presence of double bond compounds, terpenoids, and aromatic compounds respectively. However, the ferric chloride test was negative, suggesting the absence of phenolic compounds. Despite this, both TLC and phytochemical screening tests are qualitative methods, and their outcomes may be limited in accuracy. Next, the TFC and TPC result shows the presence of flavonoid with 0.995 mg CAE/g and phenolic with 12.326 mg GAE/g. The antioxidant activity for *C. manillana* stem bark was low, with an IC_{50} at 26 mg/mL (>0.1 mg/mL), and the highest percentage inhibition is 23.4% at 10,000 µg/mL. For future studies, multiple extractions should be performed to extract and quantify targeted compounds that provide antioxidant activity, such as flavonoids, phenolic compounds, and terpenoids. The maceration process can also be replaced with a Soxhlet or percolation process. Lastly, instead of using methanol as the solvent, ethanol can be considered for better safety reasons. In conclusion, this study can provide valuable information for future research and may serve as a basis for authorities to consider preserving this plant due to its beneficial value.

Authors contributions

Study design, A.S.A. Direction and Coordination, S.Z.M.S. Investigation, A.S.A. Resources, S.Z.M.S. Writing-Original Draft, A.S.A Writing-Review and Editing S.Z.M.S., and A.S.A. Supervision, S.Z.M.S. Project Administration, S.Z.M.S. and A.S.A.

Conflict of interest

The authors claim that there is no conflict of interest associated with this work.

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