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Determination of total flavonoid content and antibacterial activity of solvent fractions from 'Cacao' (*Theobroma cacao* L.) leaves

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Abstract

Cacao is renowned for its rich phytochemical composition, especially in its pods, which exhibit significant antibacterial properties. However, the leaves remain underexplored for such potential. This study seeks to examine the antibacterial properties of cacao leaves by fractionating its ethanolic extract, identifying bioactive compounds, and evaluating their activity against common pathogens, *Escherichia coli* and *Staphylococcus aureus*, in light of the growing challenge of antimicrobial resistance. Dried and pulverized cacao leaves were macerated in 80% ethanol to extract flavonoids. The crude extract was partitioned using water, hexane, and ethyl acetate. Each fraction was subjected to qualitative phytochemical screening and quantification of flavonoid content (TFC). The fraction with the highest TFC was further tested for antibacterial activity using the disk diffusion assay at concentrations of 50, 75, and 100 µg/mL. The positive control was cefoxitin (30 µg/disc), while the negative control was 1% DMSO. All three fractions tested positive for flavonoids, glycosides, tannins, and phenols. The ethyl acetate fraction (EAF) exhibited the highest TFC at 581.67 mg QE/g. Despite its high flavonoid content, the EAF exhibited minimal antibacterial activity, with maximum zones of inhibition of 6.83 ± 0.00 mm for *E. coli* and 6.84 ± 0.00 mm for *S. aureus* at 100 µg/mL. Cefoxitin showed significantly larger zones: 20.46 ± 0.08 mm and 31.45 ± 0.03 mm, respectively. The findings underscore the need for optimized extraction and compound characterization methods. Further research is recommended to explore synergistic effects with antibiotics and investigate additional biological activities of cacao leaf extracts.

Keywords: Antibacterial; Flavonoid; Ethyl acetate; *Theobroma cacao*

1. Introduction

One of the most pressing health concerns in the world today is the emergence of antimicrobial resistance, which progressively undermines the effectiveness of life-saving antimicrobial agents [1]. The rapid emergence of drug-resistant strains, largely due to the misuse of antibiotic misuse, necessitates urgent remedies to combat infectious diseases. However, the slow pace of discovering and developing novel antimicrobial agents has prompted a shift toward alternative options, such as natural products, to address this growing crisis [2]. Plants have traditionally been a reliable source of novel drugs because they produce secondary metabolites that are relatively safe, cost-effective, and offer a wide range of therapeutic benefits. Flavonoids, a class of polyphenols that includes over 4000 compounds found in plant-based products such as tea and nuts, serve as a common example. These compounds have demonstrated antibacterial properties, with mechanisms that include cytoplasmic membrane damage and the inhibition of metabolism and nucleic acid synthesis [3].

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Cacao, a member of the Sterculiaceae family, has long been valued for its diverse effects, owing to compounds such as tannins, saponins, and glycosides, all of which are known to exhibit antibacterial activity. Historically, cacao has been associated to various advantages, such as anti-inflammatory, neuroprotective, and antineoplastic properties [4]. Recent studies have shown that extracts from its shell and husk exhibit antibacterial effects against *Escherichia coli* and *Staphylococcus aureus*, both of which are harmful pathogens that cause nosocomial infections and have exhibited resistance towards numerous treatments [5].

In response to the growing need for alternative agents, this study seeks to determine the bioactive compounds present in solvent fractions from cacao leaves and investigate its antibacterial potential against known microbes, such as *E coli* and *S aureus*.

2. Materials and Methods

2.1. Ethical Considerations

There were no ethical concerns regarding human or animal subjects, as this study involved *in vitro* experiments using bacterial strains. Standard laboratory protocols and guidelines for *in vitro* research were strictly followed throughout the study.

2.2. Reagents

Ethanol (MW: 46.07 g/mol; GPR quality), hexane (MW: 86.18 g/mol; 95% purity), and ethyl acetate (MW: 88.11 g/mol; GPR quality) were obtained from Belman Laboratories (Quezon City, Philippines). Dimethyl sulfoxide (MW: 78.13 g/mol; ≥99.5% purity) was sourced from Sigma-Aldrich (Missouri, USA). Reagents used for phytochemical testing were procured from the laboratory of Lourdes College, Inc. (Cagayan de Oro, Philippines). All materials and reagents, unless otherwise specified, were of analytical grade.

2.3. Instruments

The dried leaves were ground using a cutting mill (Wiley™ Model 4, Thomas Scientific, USA). After percolation, the resulting extract was passed through cellulose paper (Whatman® Grade 1, Sigma-Aldrich, USA). A rotary evaporator (RV 10™, IKA®, Malaysia) was used to concentrate the crude extract and subsequent fractions. The aqueous fraction was dried using a lyophilizer (FSF-18N, Faithful Instrument Co. Ltd., China). The flavonoid content in the plant fractions was quantified using a UV-Vis spectrophotometer (LAMBDA 365+, PerkinElmer®, USA). The test organisms for the antimicrobial assay were cultivated in an electro-thermostatic incubator (DRP-9082, Senxin®, Shanghai, China).

2.4. Preparation of Plant Material

Matured leaves of *Theobroma cacao* L. were harvested in February 2021 from a designated lot located in Balingasag, Misamis Oriental. A plant specimen was submitted to the herbarium of Far Eastern University for confirmation of its botanical identity. The harvested leaves were rinsed with distilled water, air-dried seven days under shade, and subsequently ground using a cutting mill.

2.5. Preparation of Crude Ethanolic Extract

Approximately 717.18 grams of air-dried leaves were immersed in 80% ethanol and left to macerate for 36 hours in a stainless steel percolator. Afterwards, the mixture was filtered through cellulose filter paper to eliminate plant residues. The organic solvent was removed from the crude extract using a rotary evaporator set at 60 °C, yielding a thick, green syrup-like product. The crude ethanolic extract was kept in an amber-colored, tight container at a cold temperature until further use. The formula below was used to calculate the percentage yield, where CEE represents the weight of the crude ethanolic extract and DL denotes the weight of the dried leaf material.

$$percentage\ (\%)\ yield = \left(\frac{CEE}{DL}\right) x\ 100$$

2.6. Fractionation of Crude Ethanolic Extract

Approximately 100 grams of CEE was transferred into a 250 mL separatory funnel along with 50 mL of distilled water. The mixture underwent liquid-liquid partitioning with 25 mL of hexane. The funnel was shaken three times and then allowed to separate on an iron stand until clear separation between the aqueous and hexane layers was observed. The aqueous portion was carefully drained into one container, while the hexane layer was collected separately. This process

was repeated two more times to ensure thorough extraction, and the combined hexane and aqueous layers were reserved for subsequent concentration. The pooled aqueous layer was partitioned with 25 mL of ethyl acetate, following the same procedure. The pooled hexane fraction (HF) and ethyl acetate fraction (EAF) were concentrated using a rotary evaporator at 60°C, while the aqueous fraction (AF) was lyophilized using a freeze dryer.

2.7. Qualitative Phytochemical Analysis

To determine the presence of various phytochemicals, 1 mL of each plant fraction was placed in labeled test tubes and subjected to a series of standard qualitative tests, as outlined below:

2.7.1. Flavonoids

A few drops of 10% sodium hydroxide were added to the fraction, resulting in a deep yellow coloration. Subsequent addition of diluted hydrochloric acid caused the yellow hue to diminish, indicating the presence of flavonoids.

2.7.2. Glycosides

The fraction was combined with 1 mL of glacial acetic acid, several drops of concentrated sulfuric acid, and a neutral solution of 1% ferric chloride. The development of a reddish-brown coloration was taken as a positive indication of glycosides.

2.7.3. Phenols

A few drops of 10% lead acetate solution were introduced to the fraction. The presence of phenols were detected by the formation of a white precipitate.

2.7.4. Phytosterols

To test for phytosterols, the fraction was mixed with 1 mL each of acetic anhydride and chloroform, followed by two drops of concentrated sulfuric acid. A color transition from pink to green or bluish-green indicated a positive result.

2.7.5. Proteins

A few drops of Biuret reagent were added to the fraction. The development of a violet or purplish color signaled the presence of proteins.

2.7.6. Saponins

The fraction was shaken vigorously with 5 mL of distilled water. The appearance and persistence of foam were indicative of saponins.

2.7.7. Tannins

Five drops of a neutral 10% ferric chloride solution were added to the fraction. A color change to gray or black suggested the presence of tannins.

2.7.8. Terpenoids

For terpenoid detection, the fraction was treated with 3 mL of concentrated sulfuric acid and 1 mL of chloroform. A reddish-brown interface between the layers signified a positive result for terpenoids.

2.8. Quantification of Flavonoid Content

The TFC was determined using a modified procedure adapted from Valentin *et al.* [6]. In this method, 1 mL of each fraction was transferred into separate 10 mL volumetric flasks and mixed with 5 mL of methanol. Afterward, 0.8 mL of a 5% sodium nitrite solution was added. The mixtures were allowed to incubate for 30 minutes, after which 0.8 mL of 10% aluminum chloride was introduced. Following an additional 30-minute incubation period, 4 mL of 2 M sodium hydroxide was added, and the mixtures were diluted to the 10 mL mark with methanol. The final mixtures were incubated for another 30 minutes before measuring absorbance at 510 nm using a UV-Vis spectrophotometer. A calibration curve was created using quercetin solutions at concentrations ranging from 0 µg/mL to 50 µg/mL, prepared and analyzed under identical conditions. TFC values were expressed as milligrams of quercetin equivalents per gram (mg QE/g) of dry fraction.

2.9. Evaluation of Antibacterial Activity

The plant fraction with the highest flavonoid content was tested for antibacterial activity against *Staphylococcus aureus* (BIOTECH 1582) and *Escherichia coli* (BIOTECH 1634) using the disk diffusion assay.

2.9.1. Preparation of Inoculum

The test microorganisms, originally acquired in sterile sodium chloride-peptone buffer, were inoculated onto freshly prepared Mueller-Hinton agar (MHA) plates and incubated at 37°C for 48 hours. After colony formation, the cultured microbes were transferred into sterile, screw-capped test tubes filled with a standard saline solution. The turbidity was adjusted to 0.5 MacFarland units, or 1.5×10^8 colony-forming units (CFU) per milliliter. The suspension was mixed thoroughly using a vortex mixer and stored at a cool temperature until use.

2.9.2. Disk Diffusion Assay

20 mL of freshly prepared, sterile MHA was left to solidify after pouring into separate Petri dishes. The test bacteria were then uniformly streaked across the surface using a sterile cotton swab. Three sterile paper discs, each measuring 6 mm in diameter, were then placed above the agar surface. Each disc received 20 µL of EAF at concentrations of 50 µg/mL, 75 µg/mL, and 100 µg/mL. The inoculated plates were incubated at 37°C for 36 hours before measuring the zones of inhibition (ZOI) using a digital Vernier caliper. The measurements were taken in millimeters, according to the criteria established by the Clinical and Laboratory Standards Institute (CLSI). Cefoxitin (30 µg/disc) served as the positive control, while 1% dimethyl sulfoxide (DMSO) was used as the negative control.

2.10. Statistical Analysis

The assay was performed in three separate trials, with the outcomes expressed as the mean ± standard deviation (SD). Statistical Package for Social Science (SPSS) version 20 was used to conduct a one-way analysis of variance (ANOVA) to identify significant differences in antibacterial activity between samples ($p < 0.05$).

3. Results and Discussion

3.1. Percentage Yield

Approximately 153.33 g of crude extract were recovered from 717.18 g of dried, ground leaves, yielding 21.38%. While there is no established criterion for the minimum yield required for further extraction and analysis, several studies have reported yields of at least 10% that are suitable for subsequent analysis and extraction [7, 8, 9]. Various factors, such as solvent type and concentration, extraction time, pH, and particle size of the plant material, can influence the amount of extract recovered [10]. The relatively high yield can be attributed to the use of ethanol, a polar solvent recognized for efficiently extracting polyphenolic compounds, including flavonoids, due to its polar nature caused by the presence of hydroxyl groups.

3.2. Qualitative Phytochemical Analysis

The results of the phytochemical analysis for each of the three leaf fractions are displayed in **Table 1**. Rodríguez De Luna *et al*. [11] classify flavonoids into two groups: aglycones, which have higher solubility in polar solvents, and glycosides, such as flavones and flavanols, which readily dissolves nonpolar solvents such as chloroform, dichloromethane, and alcohols. The presence of both types of flavonoids helps explain why all three fractions tested positive for flavonoids and glycosides, despite flavonoids being polyphenolic compounds generally considered polar. Similarly, tannins, which are also polyphenolic, are more effectively extracted using polar solvents such as ethanol. Introducing small quantities of acid into the solvent helps to inhibit the oxidation of the phenolic groups [12]. Yuliana *et al.* [13] further explained that tannins contain aromatic groups, which are nonpolar, suggesting that less polar solvents such as acetone may be more effective for extraction. Although their study found hexane to be the least efficient solvent for extracting phenolic compounds, trace amounts of tannins were still detected, which accounts for the positive result in the HF. It is also noteworthy that the HF tested negative for terpenoids, which are generally nonpolar [14]. This is likely due to the plant containing relatively polar terpenoids, which were not fully extracted by hexane and instead were more effectively extracted into the AF and EAF, both of which are more polar than hexane. As expected, only the HF tested positive for phytosterols and saponins. Phytosterols are plant-derived compounds with a steroid structure and aliphatic side chains, making them highly soluble in nonpolar substances such as oils or margarine [15]. In contrast, saponins consist of hydrophilic sugar chains attached to a hydrophobic sapogenin backbone, giving them amphipathic properties that allow them to dissolve in both polar and nonpolar solvents [16]. Overall, the results emphasize the importance of solvent selection in ensuring the efficiency and reliability of the extraction process for isolating the

desired plant compounds, as influenced by the solvent's dielectric constant. This constant indicates a solvent's ability to dissolve molecules through its polarization effect [12]. Since the compounds targeted in this study are generally polar, the use of polar solvents is expected to enhance the extraction yield.

Table 1 Results of phytochemical analysis of cacao leaf fractions.

Phytochemical	Sample		
	AF	HF	EAF
Flavonoids	+	+	+
Glycosides	+	+	+
Phenols	+	+	+
Phytosterols	-	+	-
Proteins	-	-	-
Saponins	-	+	-
Tannins	+	+	+
Terpenoids	+	-	+

(+): presence; (-): absence

3.3. Results of TFC Analysis

Table 2 presents the results of the total flavonoid content (TFC) analysis for the three leaf fractions. The AF exhibited the lowest TFC at 145.00 mg QE/g, followed by the HF with 207.29 mg QE/g. The EAF showed the highest TFC at 581.67 mg QE/g and was subsequently selected for further evaluation of its antibacterial activity. Cacao is well known for its nutritional richness and versatility, primarily due to its abundant phytochemical content. These include alkaloids such as caffeine and theobromine, which contribute to its stimulant effects; carotenoids, known for their properties against oxidative stress; and flavonoids such as catechins and epicatechins, which exhibit anti-inflammatory and anticancer activities [17]. Blumberg *et al.* [18] reported that cocoa contains the highest flavanol content among commonly consumed dietary sources. These studies support the observed results indicating that relatively high flavonoid content is achievable in the three tested fractions, with concentrations exceeding 100 mg QE/g. Interestingly, the HF demonstrated a higher TFC than the AF, which was unexpected considering the polarity of water and its presumed efficiency in extracting polar compounds such as flavonoids. Sillero *et al.* [19] previously noted that the low extraction efficiency of water for flavonoids may be attributed to physicochemical incompatibilities between water and certain flavonoid structures. Specifically, their study showed that water yielded the lowest flavonoid extraction from *Larix decidua* bark. Chaves *et al.* [20] suggested that flavonoid extraction using water can be improved by acidifying the solvent or employing advanced techniques such as pressurized liquid extraction, which typically uses a water-ethanol mixture. They also reported that combining water with organic solvents generally enhances the extraction of polar flavonoids compared to water alone. These findings support the hypothesis that less polar flavonoids, such as flavones and flavanols, may be present in cacao leaves, possibly explaining the higher TFC observed in the hexane fraction. Furthermore, the EAF exhibited the highest TFC among the three fractions. This result is consistent with those reported by Zubaydah *et al*. [21], who investigated the TFC and antioxidant activity of cacao peel. Similarly, Oyeleke *et al.* [22] observed comparable outcomes in their study on the anti-inflammatory activity of cacao bark. According to Acquaviva *et al.* [23], ethyl acetate, due to its intermediate polarity, is effective at extracting phenolic compounds across a wide polarity range. Their findings also indicated that compounds such as caffeic acid, known to be present in cacao, are soluble in relatively nonpolar solvents such as dichloromethane and ethyl acetate. Collectively, the current findings, supported by existing literature, suggest that semipolar phenolic and flavonoid compounds are predominant in cacao. This likely accounts for the higher TFC measured in both the EAF and HF compared to the AF.

Table 2 Results of total flavonoid content analysis of cacao leaf fractions**.**

Sample	TFC (mg QE/g)
AF	145.00
HF	207.29
EAF	581.67

3.4. Results of Antibacterial Assay

As shown in **Table 3**, the antibacterial activity of the EAF was evaluated against *E coli* and *S aureus* at concentrations of 50 µg/mL, 75 µg/mL, and 100 µg/mL using the disk diffusion assay. The ZOI for *E. coli* was recorded as 6.47 ± 0.02 mm at 50 µg/mL, 6.69 ± 0.01 mm at 75 µg/mL, and 6.83 ± 0.00 mm at 100 µg/mL. Similarly, for *S. aureus*, the ZOI measured 6.34 ± 0.02 mm at 50 µg/mL, 6.43 ± 0.01 mm at 75 µg/mL, and 6.84 ± 0.00 at 100 µg/mL. Although a slight concentration-dependent increase in the ZOI was observed, all values remained below the thresholds defined by CLSI guidelines [24]. According to these guidelines, members of the Enterobacterales, such as *E coli*, are considered resistant when the ZOI is ≤14 mm, while *S aureus* is classified as resistant when the ZOI is ≤21 mm, and, based on testing with 30 µg of cefoxitin—the same standard used in this study. In contrast, cefoxitin exhibited significant activity against *E coli* and *S aureus*, with ZOI of 20.46 ± 0.08 mm and 31.45 ± 0.03 mm, respectively. The negative control (1% DMSO) exhibited no ZOI, confirming that the solvent had no effect on bacterial growth.

It is noteworthy that the EAF exhibited minimal antibacterial activity, despite having a high TFC. Two possible explanations may account for these findings. First, the flavonoids extracted from cacao leaves may be present in inactive forms, such as glycosylated derivatives or compounds bound to other molecules. Shamsudin and colleagues [25] reviewed various structural modifications of flavonoids and their effects on antibacterial activity. The study highlighted that the antibacterial activity of certain isoflavonoids, such as puerarin and genistin, was significantly reduced when glycosylation occurred at the C7 and C8 positions, compared to the aglycone counterpart, daidzein. The review also noted that antitubercular flavonoids such as luteolin and quercetin lost their activity when their hydroxyl groups were methylated or glycosylated. The results of the antibacterial assay support earlier observations that non-polar flavonoid glycosides were predominantly present in the fractions, which also explained the higher TFC measured in the HF and EAF compared to the AF. To address this issue, further isolation of flavonoids combined with spectroscopic analysis is essential to elucidate their specific structures and evaluate their potential biological activity. In addition, emerging *in silico* approaches that employ computational tools to predict the pharmacological properties of compounds provide a more efficient means of gaining insights into their characteristics. However, a key limitation of these methods is that traditional *in vitro* or *in vivo* analysis is still needed to confirm the predictions generated through *in silico* analyses.

Table 3 Results of the disk diffusion assay of cacao leaf fraction.

Sample	Concentration	ZOI (mm)	
		E coli	*S aureus*
EAF	100 µg/mL	6.83 ± 0.00	6.84 ± 0.00
	75 µg/mL	6.69 ± 0.01	6.43 ± 0.01
	50 µg/mL	6.47 ± 0.02	6.34 ± 0.02
Cefoxitin	30 µg	20.46 ± 0.08	31.45 ± 0.03
DMSO	1%	-	-

Second, since the EAF is a solvent-based fraction, it likely contains various compounds in addition to flavonoids, which may result in antagonistic interactions that reduce its antibacterial activity. This possibility was recently explored by Vaou *et al.* [2] in a study that examined various pharmacological interactions involving plant-derived compounds and their impact on antibacterial activity. An example highlighted is an extract containing magnolol, a polyphenolic compound derived from *Magnolia officinalis*, whose biological activity was initially reduced by the presence of antagonistic compounds. These antagonists were later removed through chromatographic techniques, resulting in the restoration of the extract's activity. Another example discussed is the inhibition of the antiplasmodial effects of artemisinin by the flavone casticin at a 1:3 ratio. The study concluded that antagonism between plant compounds can

occur when they compete for the same binding site or when their combined effects diminish one another. To identify and characterize such interactions, methods such as culturing samples and constructing dose-response curves are commonly employed.

4. Conclusions

The overall findings highlight the importance of selecting appropriate extraction methods to effectively isolate desired compounds, along with conducting structural analyses to theoretically evaluate their potential bioactivity. Although cacao nibs are well-established as rich sources of phytochemicals, this study also promotes the investigation of other plant parts, which may possess a broader diversity of compounds or novel constituents absent in the nibs. To maximize the antibacterial potential of cacao leaves, future research should explore their combinations with commercially available antimicrobial agents to determine whether synergistic or additive effects exist—an approach that may aid in addressing microbial resistance. Additionally, further studies are encouraged to explore other potential biological activities within the solvent fractions of cacao leaves, given the presence of compounds beyond flavonoids.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper's content.

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