



PHYTOCHEMICAL ANALYSIS AND BIOACTIVITY OF THEOBROMA CACAO LEAF EXTRACT: ANTIMICROBIAL, ANTIOXIDANT, AND CYTOTOXIC PROPERTIES

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ABSTRACT

Theobroma cacao leaves have traditionally been used for medicinal purposes in various cultures. This study investigated the phytochemical composition and antimicrobial, antioxidant, and cytotoxic properties of Theobroma cacao leaf extract. This study investigated the phytochemical composition and bioactivity of Theobroma cacao leaf extract and revealed its antimicrobial, antioxidant, and cytotoxic properties. The ethanolic extracts of T. cacao leaves were subjected to phytochemical screening, revealing the presence of tannins, flavonoids, steroids, and phenols. Thin-layer chromatography (TLC) analysis confirmed the presence of various bioactive compounds. The extract exhibited significant antibacterial activity against Escherichia coli and Staphylococcus aureus, with mean inhibition zones of 9.18 mm and 13.98 mm, respectively, suggesting its potential as a natural antimicrobial agent. Antioxidant properties were also observed, indicating the free radical-scavenging capacity of the extract. The cytotoxicity assessment using the Brine Shrimp Lethality Assay (BSLA) demonstrated a dose-dependent toxic effect, with the highest mortality rates observed at 250 µg/mL (67.5%) and 1000 µg/mL (62.5%). The Allium cepa test further confirmed the cytotoxic and genotoxic properties of the extract, as evidenced by the reduced mitotic indices and chromosomal aberrations in treated onion root tip cells. These findings highlight the therapeutic potential of T. cacao leaf extract and its bioactive constituents, warranting further investigation for pharmaceutical and nutraceutical applications while considering concentration-dependent cytotoxic effects.

Keywords: *Allium cepa test, Antimicrobial Activity, Antioxidant Potential, Cytotoxicity, Phytochemicals, Theobroma cacao*

INTRODUCTION

Theobroma cacao L., commonly known as the cocoa tree, is native to the Amazon basin and the surrounding regions of South and Central America. It is also cultivated in many tropical areas worldwide, particularly in West Africa, where most of the cocoa production occurs today. The cocoa tree belongs to the family Malvaceae and genus *Theobroma*, which means "food of the gods" in Greek, highlighting its importance and reverence among ancient civilizations.

Theobroma cacao is a small-to medium-sized evergreen tree that typically grows to a height of 4–8 m (13–26 feet), although under ideal conditions, it can grow taller. It has broad glossy green leaves that are oblong or lanceolate in shape. The flowers are small, white to pale pink, and grow directly on the trunks or larger branches. The fruit of the cocoa tree is a large, oval, or ellipsoid berry known as a cocoa pod. These pods can range in color from yellow to red or purple when ripe, depending on the variety. There were numerous seeds in each pod, which were cocoa beans. Sweet-tasting pulp surrounded the beans. Tiny midges primarily pollinate cocoa trees, although some degree of self-pollination may also occur. The flowers produce nectar to attract these insects, which aids pollen transfer between flowers. Cocoa trees thrive in tropical climates with relatively elevated temperatures of approximately 25-30°C or 77-86 °F and abundant rainfall is evenly distributed throughout the year. They prefer well-drained, fertile soils with a good organic matter content. Cocoa trees are typically grown from seeds, although vegetative propagation methods, such as grafting and budding, are also used in some cases to maintain desirable traits. They are often intercropped with shade trees such as bananas or plantains to provide the necessary shade and protection from direct sunlight. Cocoa trees are susceptible to a variety of pests and diseases, including fungal diseases such as black pod disease and witches' broom disease, as well as insect pests such as cocoa pod borers and cocoa mirids. These factors can significantly impact cocoa yields if they are not properly managed.

Wickramasuriya and Dunwell (2017). Cacao holds special economic interest, both as an everyday delight and confectionery ingredient, as well as for other uses. Cocoa butter and its seeds are particularly rich in polyphenols and flavonoids, making cacao a plant of economic interest to industries such as pharmaceuticals and cosmetics, and the consumption of cacao products can cover our everyday needs in antioxidants better than green tea, wine, soy, and blueberries. Indications that cocoa might contain chemicals that function as cardioprotective agents, enhance the immune system, slow down cancer, and suppress obesity have increased interest in studying the health benefits of chocolate beyond the scope of confectionery consumption. In addition, the high concentration of potassium in pod husks makes them ideal for soap production.

Cocoa leaves are the result of crop pruning to increase productivity and plant maintenance, such as maintaining the economic life of plants. These by-products from cocoa plantations are usually left to rot and have not been used optimally or only as compost, animal feed, etc.. It is well known that cocoa leaves contain phenolic compounds, which are a class of bioactive substances that serve as antioxidants

(Supriyanto et al. 2015). Cocoa leaves also contain theobromine, caffeine, anthocyanins, leucoanthocyanidin, and catechol, the amounts of which vary and are influenced by leaf age and plant age (Supriyanto et al., 2015). A similar study by Ingente and Anselmo (2024) demonstrated that *Ipomoea reptans* is capable of accumulating Pb in a hydroponic system while maintaining its physiological function. This suggests that certain plants possess inherent biochemical pathways that enable them to interact effectively with heavy metals, potentially attributable to bioactive compounds with chelating properties. Similarly, the bioactive compounds identified in the *Theobroma cacao* leaf extract may contribute to its antimicrobial, antioxidant, and cytotoxic properties.

According to Othman et al. (2007), the total polyphenols in old leaves showed a higher yield of 32.4%, while that of young leaves was 27.3%. Another study by Osman et al. (2004) showed that the total polyphenol content of old leaves was 28.4%. However, the percentage of young leaves was 19%. The total catechin content in the old and young leaves was 5.25% and 9.75%, respectively. The contents of antioxidants, flavonoids, and other bioactive compounds found in cocoa leaves can be extracted from plant tissues using an extraction method. The extraction method was chosen based on several factors such as the nature of the bioactive compound to be targeted and the sample of the material used.

Systemic Botany

Theobroma cacao, commonly known as the cacao tree or cacao, is a neotropical evergreen plant with a suggested origin from the tropical forests of Laradon at the southeastern borders of Mexico with Guatemala. Its seeds were spread all over the Mesoamerican pre-Columbian zone, from the shores of Costa Rica to the North and the shores of Ecuador in the South, from parrots and monkeys that ate the sweet flesh of the pods and spat the seeds. Later, the species was positively affected by Mayan cultivation and commerce (Khodorowsky and de Loisy, 2003). However, new evidence doubts the proposed area of origin and places the birthplace 'of cacao trees in the 18 northern areas of the Amazon Basin due to the huge biodiversity of the species in this area and recent archeological findings of 5.300 years old domesticated cacao traces, the most ancient of their kind, in Southeastern Ecuador (Zarrillo et al., 2018).

Perhaps the best explanation was provided by Mexican Dr. Jose Cuatrecasas in 1964. "It may be assumed that in early times a natural population of *Theobroma cacao* was spread throughout the central part of Amazonia- Guiana, westward and northward to the south of Mexico; that these populations developed into two different forms geographically separated by the Panama isthmus; and that these two original forms, when isolated, had sufficiently consistent characters to be recognized as subspecies." (Wrigley et al., 1985)

Botanically, the species was described in 1605 by various travelers under the name of Cacao fruits; however, its final characterization was performed in 1753 by Swedish botanist Carl Linnaeus when his book "Species Plantarum" was published. It is then that the cacao tree is first mentioned as *Theobroma cacao* and gets singled out from its relative species, *T. bicolor* and *T. grandiflora*, based on its leaf morphology (Wrigley et

al.,1985)

In modern systemic botany, the cacao tree belongs to the Malvaceae family, which combines it with other dicotyledonous angiosperm plants and in the same family as cotton. *The Theobroma genus* includes twenty-two species, of which *T. cacao* is cultivated around the world, while *T. bicolor* and *T. grandiflora* are known for being used to produce liqueur from the flesh of their pods, but not for producing cacao (Wrigley et al., 1985).

Theobroma cacao is a diploid plant with a genome of 20 chromosomes and is divided into two subspecies: *Theobroma cacao spp cacao*, which includes the Criollo botanical variety of Central and Southern America, and *Theobroma cacao spp sphaerocarpum*, which includes the rest of the distinct cacao populations around the globe and is mainly identified by the Forastero botanical variety (Wrigley et al., 1985).

Phytochemical Activity

According to Tiwari et al. (2011), phytochemicals are many plant-derived substances that are believed to provide much of the disease protection provided by diets high in fruits, vegetables, beans, cereals, and plant-based beverages such as tea and wine. Phytochemicals are classified into tannins, flavonoids, glycosides, saponins, alkaloids, triterpenoids, and sterols, based on their chemical structures. Leaves, stem bark, and roots are the most commonly used plant parts. The roots are dried, dissolved in water, and then taken orally to treat malaria and venereal diseases, as well as stomachache and premenstrual syndrome symptoms. The stem bark is also crushed, soaked in water, and taken orally to treat diarrhea, as well as applied to help treat abscesses and a variety of other skin lesions (Kabuka et al., 2022).

Antimicrobial Activity

Theobroma cacao (cacao) is a popularly known chocolate tree with high nutritional value and medicinal properties. In addition, cacao is used as a folk medicine (tribal), and it grows near seaways because it requires high humidity for its growth and as a medicine for many diseases. The leaf extracts of *Theobroma cacao* showed antimicrobial potential against the bacteria; thus, tannins and flavonoid-like secondary metabolites in the leaves function as antimicrobials. Although the different extracts exhibited a zone of inhibition, they can be used as substitutes for antibiotics that are commercially available in pharmacies or drugstores because of their level of effectiveness.

Antioxidant Activity

Antioxidants function as free-radical scavengers by terminating free-radical chain reactions and inhibiting other oxidation reactions. They alleviate health problems, such as cardiovascular disease, diabetes, and cancer caused by oxidative damage. Antioxidants may also enhance immune defenses, lowering the risk of cancer and infection (Tsang et al., 2011). They also have industrial uses, such as preservatives in food and cosmetics and in preventing the degradation of rubber and gasoline (Khaki & Fathiazad, 2012). The study plant, cocoa *Theobroma cacao L.*, is a small evergreen tree belonging

to the Sterculiaceae family. It is traditionally used to treat various disorders such as anemia, malaria, mental fatigue, tuberculosis, fever, and gout, as a worm expeller, and for wound healing (Enete & Amusa, 2010; Dillinger et al., 2000; Baharum et al., 2016). Hence, this study aimed to determine the antioxidant activities and anthelmintic properties of aqueous and ethanolic extracts of *Theobroma cacao* leaves. Scientific data are needed to create the confidence needed in the use of medicinal plants that have been used since ancient times to treat illnesses.

According to Liu et al. (2018) and Behera (2019), the use of antioxidants from natural ingredients is due to the presence of phenolic and polyphenolic compounds such as flavonoids and proanthocyanidins. Antioxidant activity was positively correlated with flavonoid, polyphenol, and anthocyanidin contents.

Research Objectives

1. To identify the phytochemical composition of *Theobroma cacao* leaf extract and determine the types and concentrations of bioactive compounds present.
2. To evaluate the antimicrobial properties of the leaf extract against specific bacterial strains, including *Staphylococcus aureus* and *Escherichia coli*.
3. To assess the antioxidant potential of the extract and its effectiveness as a free radical scavenger, contributing to its health benefits.
4. To investigate the cytotoxic effects of the extract using methods such as the Brine Shrimp Lethality Assay and *Allium cepa* test, with a focus on dose-dependent responses.

METHODOLOGY

A. Collection and Preparation of Samples of Ethanol Extracts

Handpicked samples of fresh cocoa leaves *Theobroma cacao* L. were collected at Barangay Poblacion Norte, Maddela, Quirino. The leaves of *Theobroma cacao* L. were washed, air-dried until crisp, and powdered before extraction with 95% ethanol at room temperature for 72 h. This process was repeated three times, and the combined ethanol fractions were evaporated under reduced pressure using a rotary evaporator at 55°C.

B. Phytochemical Analysis

Qualitative phytochemical analysis was conducted using the standard procedure of Sofowara (1993), as cited and modified by Jacob and David (2016). The results were determined based on the color and intensity of the reaction and interpreted as + if the chemical was present in a traceable amount, and - if the chemical was absent. The following analyses were conducted:

- a. Test for Tannins. Two drops of FeCl₃ (5%) were added to 1 mL of extract. Each extract produced a dirty-green precipitate.

- b. Test for Saponins using frothing test. In a test tube, 2 mL of each extract was shaken vigorously for 2 min. Frothing was also observed, indicating the presence of saponins.
- c. Test for Phlobatannins. Five milliliters of distilled water were added to 5 cm³ of each extract and boiled for 2 min with 5 mL of HCl (1%). No visible reaction was observed, indicating the absence of phlobatannins.
- d. Test for Flavonoids. The extract (2 mL) was heated in a water bath with 10 cm³ ethyl acetate and cooled. The layers were separated, and the color of the NH₃ layer was noted (red coloration).
- e. Test for Alkaloids. In each test tube, 1 mL HCl (1%) was added to 3 mL of the extract. A few drops of Mayer's reagent were then added to each extract. The presence of alkaloids was indicated by the formation of a creamy white precipitate.

C. Cytotoxicity Test using Brine Shrimp Lethality Assay Preparation of Brine Shrimp Nauplii

Brine shrimp eggs were obtained from Central Natural Sciences, Saint Mary's University, Bayombong, Nueva Vizcaya, Philippines. Brine shrimp eggs were hatched in artificial seawater prepared as described by Mclaughlin and Rogers (1998). Brine shrimp eggs were added to artificial seawater where 30 g of salt was diluted per liter of water in a glass chamber and kept under constant aeration and illumination. After 48 h of incubation, brine shrimp nauplii were attracted to one side of the vessel using a light source and collected using a pipette.

Cytotoxic Lethality Assay

The cytotoxic property of *Theobroma cacao* ethanol extract was monitored using the brine shrimp lethality test described by Mclaughlin and Roger (1998), with modifications. Thirty newly hatched nauplii were placed in an Elisa well with ten (10) nauplii per well. Triplicates were performed for each treatment concentration. The setup containing extracts of the fungal isolates at different concentrations was left uncovered under a lamp. The number of dead nauplii was determined after 24 h. Observations of live and dead nauplii were compared to the standard. Live brine shrimps were observed to be actively and constantly moving, whereas dead nauplii were observed to be non-motile and floating. The number of dead nauplii was identified using a stereomicroscope. Percent mortality was documented, and the LC₅₀ was determined through Probit Analysis.

D. Thin Layer Chromatography

The crude extract of the sample was purified and diluted with 3 ml of chloroform and set on a marked TLC (chromatographic paper) at three different points and dipped inside a mixture solution of 7 ml of Ethyl Acetate to determine the chromatographic flow rate of the sample through the solvent phase of the chromatographic layer and to determine the distance traveled through the solvent phase medium and the flow rate. (R_{f1} = 0.4898, R_{f2} = 4082 and R_{f3} = 0.3878). The major use of this technique is to identify compounds based on their flow rate values and to monitor organic reactions.

E. Spotting the Sample

Obtaining a capillary spotter may be required to make the spotter by stretching a softened pipette. The spotter was placed into the diluted sample to analyze and withdraw liquid into the spotter through capillary action.

F. Development of TLC Plate

Obtain a TLC chamber with a lid and TLC plate, touching the plate only on the back or edges but not on the white surface. The liquid level must be below the pencil line where the samples are spotted, or the compounds must dissolve in the pool of eluent instead of traveling up the plate. Cap the chamber delicately while keeping it vertically, and do not touch it again until the TLC is complete.

G. Visualization

The plate was removed from the chamber and allowed to dry, and the separated compounds were visualized under UV light or by spraying the plate with a chemical reagent.

H. Allium Cepa Test

Preparation of Onion Bulbs

Healthy onion bulbs were selected and the dry outer layers were removed without damaging the root primordia. The onion bulbs were then placed in a beaker filled with distilled water, ensuring that only the base of the bulb was submerged. This promoted root growth. The roots were allowed to grow for 2-3 days until they reach approximately 1-2 cm in length.

Exposure to Test Substances

Prepare test solutions with various concentrations of the test substance, such as 125, 250, 500, 1000, and 2000 µg/mL. Distilled water was replaced with the test solutions and the onion bulbs for 24-48 hours. A negative control (distilled water) and a positive control (ethanolic extract) were used. After exposure, the root tips (approximately 1-2 cm) were cut using a scalpel or a sharp knife. The root tips were rinsed with distilled water to remove residual test solutions.

Fixation

The root tips were placed in Carnoy's fixative solution for approximately 24 h. This step preserves cellular structures. After fixation, root tips were rinsed with distilled water. The fixed root tips in 1N HCl at 60°C for 5 min. This step softens the cell wall and enhances staining.

Staining

The root tips were transferred to a staining solution of aceto-orcein under a microscope. The stained root tips were rinsed with distilled water to remove the excess stain. Stained root tips (approximately 1-2 mm from the tip) were cut and placed on a microscope slide. Then, a drop of water or mounting medium was added and gently covered with a cover slip. Apply gentle pressure to the coverslip to squash the root tip and spread the cells into a single layer.

Microscopic Examination

The slides were observed under a microscope at high magnification, and the number of cells in various stages of mitosis (prophase, metaphase, anaphase, and telophase) and interphase.

I. Antibacterial Property of Theobroma Cacao

The antibacterial properties of the ethanol extracts of *Theobroma cacao* were determined using the disc diffusion method of Bauer et al. (1996) with modifications. Measurement of the zone of inhibition was the basis for assessing antibacterial properties.

J. Source of Test Organism

Bacterial strains of *S. aureus* and *E. coli* were obtained from the bacterial culture collection of Central Natural Sciences, Saint Mary's University, Bayombong, and Nueva Vizcaya.

K. Antibacterial Assay

The antibacterial properties of the *Theobroma cacao* ethanolic extracts were assessed using the procedure described by Bauer et al. (1996). Following the aseptic technique, the assay was performed inside a laminar flow chamber containing an efficient particulate air (HEPA) filter to avoid contaminants that may affect the process.

L. Preparation of Mueller Hinton Agar (MHA)

38 g of Mueller Hinton agar (MHA) was dispensed in a clean sterile Erlenmeyer flask with one (1) liter of distilled water. The mixture was heated until it was homogenized. It was then sterilized in an autoclave for 15 min at 121 °C and 15 psi. After sterilization, the dishes were allowed to cool and then plated on sterilized petri dishes.

M. Disc Diffusion Assay

An antibacterial assay was performed using the disc diffusion method described by Bauer et al. (1996). This involved the use of filter paper discs as carriers of antimicrobial agents. Circular sterilized discs with a diameter (6) mm were cut from Whatman no. 1 filter

paper and impregnated with liquid treatments. Cultures of *E. coli* and *S. aureus* were spread thoroughly onto MHA plates using a sterile cotton swab under aseptic conditions. The impregnated discs were placed equidistantly on the surface of the medium. The plates were incubated at 37 °C and turned upside down to prevent contamination. The zone of inhibition of each paper disc was observed and recorded every eight (8) hours within a 24-hour incubation period. The zone of inhibition was measured using a calibrated digital Vernier caliper.

RESULTS AND DISCUSSION

Phytochemical Screening of *Theobroma Cacao*

The plant extract of cocoa contains many phytochemicals, and physiologically active compounds have been reported. Kim et al. (2011), reported that selected procyanidins present in cocoa inhibited tumorigenesis, tumor growth, and angiogenesis.

Table 1. Phytochemicals in the extracts of the leaves of *Theobroma cacao L.*

	Tannins	Flavonoids	Steroids	Phenols	Saponins	Terpenoids
Ethanollic extract	+	+	+	+	-	-
+ indicates presence of phytochemicals; – indicates absence of phytochemicals						

Phytochemical analyses showed that biochemical constituents, such as tannins, flavonoids, steroids, and phenols were present in the ethanolic extracts of *Theobroma cacao* leaves, whereas Terpenoids and Saponins were not present in the ethanolic extracts, as shown in Table 1. This may be because ethanol is a better extractant than ethanol.

Studies have shown that plants contain a large variety of plant chemicals or phytochemicals with antioxidant activity, with phenols and flavonoids contributing the most. In addition, compounds such as tannins, saponins, alkaloids, and flavonoids have been suggested to be involved in the antimicrobial activity (Palombo, 2006). The presence of these phytochemically active compounds is known to have beneficial medicinal properties for pharmaceutical and therapeutic purposes.

Saponins and flavonoids were present in all extracts. Saponins are mild detergents with therapeutic effects, such as anticancer, hypercholesterolemic, and antioxidant effects. Flavonoids are also known for a wide range of biological activities, including antimicrobial, analgesic, antiallergic, cytostatic, and anti-inflammatory activities. In addition, tannins exhibit antiviral, antibacterial, and anticancer properties. They can also inhibit HIV replication, sensitivity, and diuretics.

Thin Layer Chromatography

The table provides results from various chemical tests used to identify different classes of compounds in the *Theobroma cacao* leaf extract. The preliminary test for essential oils in *Theobroma cacao* leaf extract contained essential oils. These volatile compounds often contribute to the aroma of plants and exhibit various biological activities. Second, the Vanillin sulfuric acid test indicated the presence of phenolic compounds in the extract. Phenols are known to have antioxidant properties and potential health benefits. Third, Naphthol-sulfuric Acid, the absence of a positive reaction, suggests that sugars are not present in significant amounts in *T. cacao* leaf extract. Fourth, ethanolic potassium hydroxide does not contain anthraquinones, coumarins, or anthrones, which are known for their laxative properties (anthraquinones), anticoagulant effects (coumarins), or other biological activities. The fifth is potassium ferricyanide ferric chloride. This indicated the presence of tannins, flavonoids, and phenolic compounds in the extract. These compounds are known to have antioxidant and anti-inflammatory properties. The presence of alkaloids in Dragendorff's reagent suggests that the extract contains nitrogen-containing compounds, which can have various pharmacological effects, such as analgesic or stimulatory properties. Moreover, the presence of flavonoids and steroids was confirmed in antimony (III) chloride. Flavonoids are known for their antioxidant activity, whereas steroids can have a range of biological effects, including anti-inflammatory actions. The absence of a positive reaction indicates that anthraquinones are not present in the extract, corroborating the result from the methanolic potassium hydroxide test and lastly, the Ninhydrin test suggests that free amino acids are not present in significant amounts in the *Theobroma cacao* leaf extract

Distinct spots were observed on the TLC plate after development, indicating the presence of multiple compounds in *T. cacao* leaf extract. The retention factor (R_f) of the separated compounds was also calculated. Spot 1: $R_f = 0.25$, Spot 2: $R_f = 0.45$, Spot 3: $R_f = 0.65$, and Spot 4: $R_f = 0.85$. The spots were visualized under UV light at 254 and 365 nm. Additional visualization was performed by spraying with iodine vapor, which revealed more distinct spots, indicating different phytochemicals.

The different R_f values suggest the presence of various compounds in *T. cacao* leaf extract. Each spot represents a distinct compound, separated based on its affinity towards the stationary and mobile phases. The chosen solvent system effectively separated the compounds, as indicated by clear and distinct spots. Adjusting the solvent ratios can further optimize the separation if required. The isolated compounds may include flavonoids, alkaloids, terpenoids, and other bioactive constituents known to be present in *Theobroma cacao*. Further analyses, such as co-TLC with standards or spectroscopic methods, are required to definitively identify these compounds. The isolated compounds were subjected to bioassays to evaluate their biological activity. Understanding the bioactive components helps assess the therapeutic potential and safety of *Theobroma cacao* leaves.

Table 2. Phytochemical Screening of *Theobroma Cacao* Leaves

REAGENT	COMPOUND TESTED	RESULT
1. Preliminary Test	Essential Oils	+
2. Vanillin Sulfuric Acid	Higher Alcohols, Steroids, Triterpenes, Essential Oils, Phenols, Fatty Acid	+ phenols
3. Naphthol-sulfuric Acid	Sugars	-
4. Methanolic potassium hydroxide (KOH-MetOH)	Anthraquinones, Coumarins Anthrones	- - -
5. Potassium Ferricyanide-ferric chloride	Tannins, Flavonoids, Phenols	+
6. Dragendorff's Reagent	Alkaloids	+
7. Antimony (III) Chloride	Flavonoids, Steroids	+
8. Magnesium Acetate	Anthraquinones	-
9. Ninhydrin	Amino Acids	-

Cytotoxic Property on *Theobroma Cacao* using Brine Shrimp Lethality Assay

The brine shrimp lethality assay (BSLA) has been used routinely in the primary screening of crude extracts and isolated compounds to assess the toxicity of brine shrimp, which could also indicate the possible cytotoxic properties of the test materials (McLaughlin *et al.*, 1991).

The data from the Brine Shrimp Lethality Assay (BSLA) are provided below, showing the number of surviving larvae at different concentrations of *Theobroma cacao* leaf extract.

Table 3. Cytotoxicity test results of *Theobroma cocoa leaf* extract on brine shrimp lethality test after 24 h.

Treatment	Number of Larvae	Concentration (µg/mL)				
		2000	1000	500	250	125
1	10	5	4	5	8	8
2	10	6	4	4	5	7
3	10	8	5	3	7	7
4	10	8	2	5	6	8

Table 4. Average mortality percentage across all treatments

Concentration (µg/mL)	Average Mortality (%)
2000	32.5%
1000	62.5%
500	57.5%
250	67.5%
125	35%
Control	25%

The mortality percentage generally increased with the extract concentration, indicating dose-dependent toxicity. The highest mortality rates were observed at 250 µg/mL (67.5%) and 1000 µg/mL (62.5%). *Theobroma cacao* leaf extract showed significant toxicity at higher concentrations, as evidenced by higher mortality rates. The mortality at the lowest concentration (125 µg/mL) was still higher than that of the control, suggesting that even low concentrations have some toxic effects. The mortality of the control group was relatively low (25%), indicating that most of the mortality in the test groups was due to toxicity of the extract rather than environmental factors. Based on these results, further studies should focus on concentrations around 250 µg/mL to 1000 µg/mL where significant toxic effects are observed, to better understand the lethal dose and mechanism of action. The Brine Shrimp Lethality Test demonstrated that *Theobroma cacao* leaf extract had a dose-dependent toxic effect on *Artemia salina* larvae. The extract exhibited significant cytotoxicity at concentrations of 250 µg/mL or higher. These results highlight the potential of *Theobroma cacao* leaf extract, which contains bioactive compounds with

cytotoxic properties, warranting further investigation to identify these compounds and explore their therapeutic applications or potential risks.

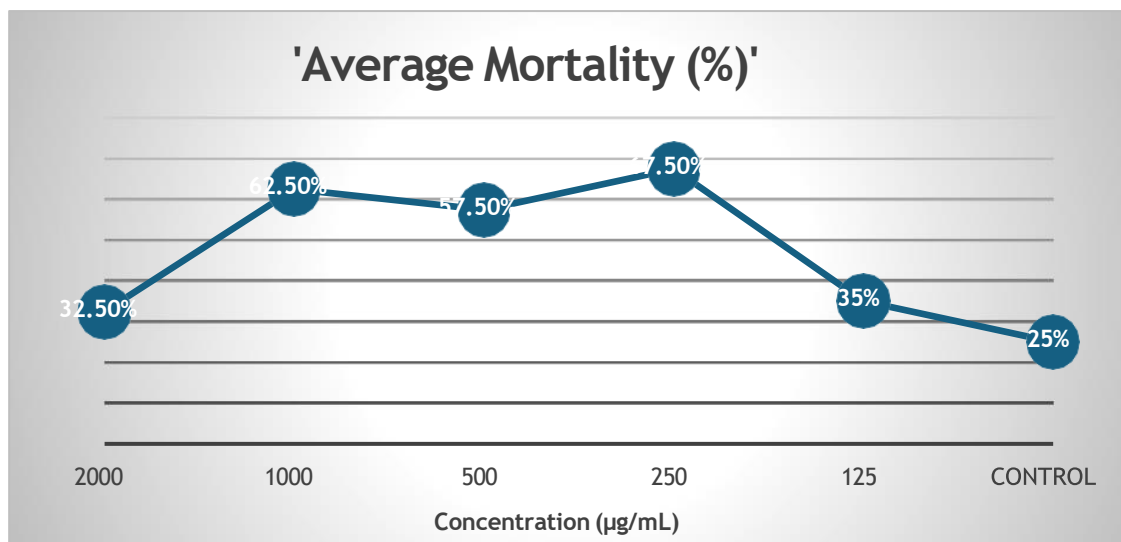


Figure 4. Mortality test of brine shrimp against different concentrations of *Theobroma Cacao*

Allium Cepa Test on *Theobroma Cacao*

The antimutagenic activity of the cocoa seed extract was determined using the *Allium cepa* root meristem model, commonly known as the Allium assay. Root meristematic cells have been used to screen drugs with antimutagenic activity (Fiskesjö, 1985). Cell division in the meristematic region is similar to cancer cell division in humans. Therefore, these meristematic cells can be used to screen for drugs with potential antimutagenic activity. The Allium assay is a rapid, sensitive, and reproducible bioassay for detecting cytotoxicity and genotoxicity. Root growth inhibition and antimutagenic effects indicated genotoxicity. The good genotoxic assay performance of *Allium cepa* as a plant system has been attributed to the easily studied karyotype of plants [$2n = 16$] and the ability to correlate the outcomes of assays with those of mammalian cells during toxicity evaluations. The *A. cepa* species, commonly known as the onion, is characterized by homogenous meristematic cells, and a large number of chromosomes are ideal for use in bioassays (Shachi, 2012).

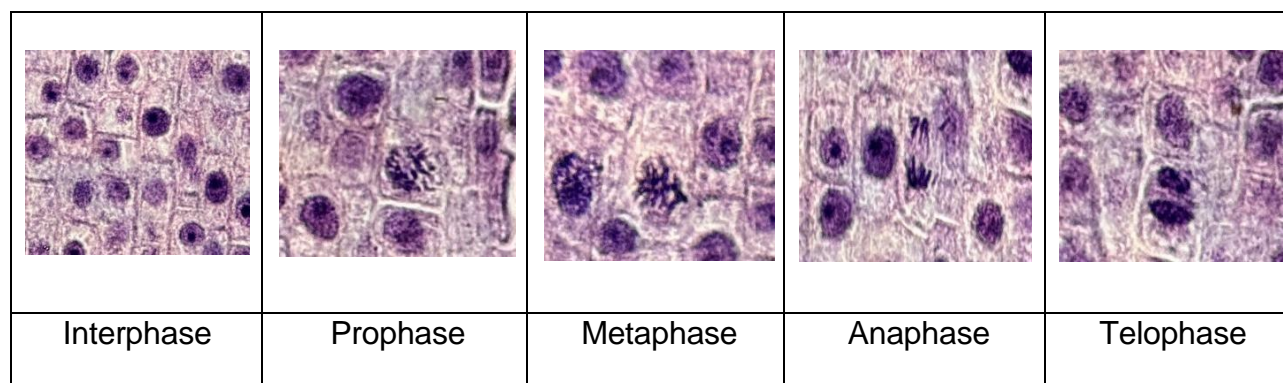


Figure 5. Distinct stages of Mitotic Division Phases under the light microscope on *Theobroma Cacao*

The mitotic index (MI) indicates that the frequency of cell division is an important parameter for examining the cytotoxicity of agents. A decreased mitotic index, which may reflect a disturbance in growth, could be indicative of the presence of cytotoxic agents in the environment. Chromosome aberration refers to an atypical number of chromosomes or changes in the chromosomal structure of cells exposed to physical or chemical agents. Diverse types of chromosomal aberrations, such as chromosomal bridges and breaks, losses, and C-mitosis, can be evaluated using the *Allium cepa* test.

Table 5. Mitotic indices across different concentrations of test substances and controls.

Treatment	Total Cells Observed	Cells in Mitosis	Mitotic Index (%)
Control	30	7	23.33%
Extract 125 µg/mL	30	3	10%
Extract 250 µg/mL	30	7	23.33%
Extract 500 µg/mL	30	5	16.67%
Extract 1000 µg/mL	30	4	13.33%
Extract 2000 µg/mL	30	4	13.33%

As shown in the data the control group has a mitotic index of 23.33%, indicating the normal rate of cell division in untreated onion root tips. The mitotic index dropped to 10%, suggesting significant inhibition of cell division at this concentration, indicating cytotoxic effects. The mitotic index remained at 23.33%, similar to that of the control, indicating no significant cytotoxic effect at this concentration. The mitotic index was 16.67%, showing a moderate reduction in cell division compared with the control, indicating a cytotoxic effect. The mitotic index further decreased to 13.33%, suggesting an increased

cytotoxicity at this higher concentration. The mitotic index remained at 13.33%, similar to that at 1000 µg/mL, indicating that the cytotoxic effect plateaued at higher concentrations. There was a clear trend of decreasing mitotic index with increasing concentrations of the extract, with significant inhibition observed at ≥ 125 µg/mL. The most substantial decrease in the mitotic index occurred between the control and the 125 µg/mL concentration. Concentrations of 500 µg/mL and above showed a consistent cytotoxic effect, with reduced mitotic indices compared to the control. The *Allium cepa* root tip assay data indicated that *Theobroma cacao* leaf extract had a dose-dependent cytotoxic effect on cell division. The significant reduction in the mitotic index at higher concentrations suggests that the extract inhibits cell proliferation, making it potentially useful for further studies on its cytotoxic properties and potential applications in cancer research or other areas where the inhibition of cell division is desirable.

Results on the Antibacterial Properties of Ethanol Extracts of Theobroma Cacao

Theobroma Cacao species were screened for their antibacterial activities against *E. coli* and *S. aureus* using the disc diffusion method. This species was found to suppress the growth of *E. coli*, and *S. aureus* exhibited inhibitory activity against the tested bacteria.

The results revealed that after 24 h of incubation, ethanol extracts of *Theobroma cacao* had a zone of inhibition with a mean diameter of 9.18 mm and 13.98 mm against *E. coli* and *S. aureus*, respectively. However, the results differ from those exhibited by the 95% ethanol 7.83 mm. The highest zone of inhibition was recorded for meropenem with a mean diameter zone of 30.85 mm which showed a significant difference among other treatments at a 5% level of significance.

Table 6. Zone of inhibition exhibited by the ethanolic extract of *Theobroma Cacao* against *E. coli* and *S. aureus* after 24 h.

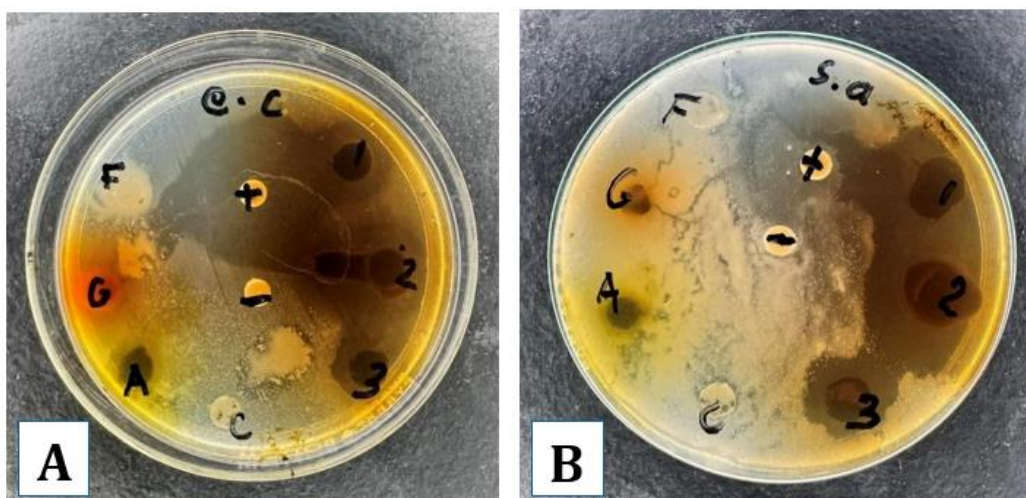
Treatments	Zone of inhibition (mm)	
	<i>E. coli</i>	<i>S. aureus</i>
Meropenem (+)	30.85 mm	30.85 mm
Clindamycin (+)	17.61 mm	17.61 mm
95% Ethanol (-)	7.83 mm	7.59 mm
<i>Theobroma Cacao</i>	9.18 mm	13.98 mm

Furthermore, the ethanolic extract of *Theobroma cacao* showed a mean diameter of 13.98 mm, which was not significantly different that from of 95% ethanol. In contrast, meropenem was highly effective against both *E. coli* and *S. aureus*, showing the largest zones of inhibition among the treatments. It demonstrated a strong antibacterial effect, and clindamycin was effective against both *E. coli* and *S. aureus*, but less effective than

meropenem. However, it still exhibited significant antibacterial properties. Ethanol (95 %) exhibited minimal antibacterial activity against both the types of bacteria. The zones of inhibition were the smallest, indicating that it is not very effective as an antibacterial agent in this context, showing in *E. coli* with a diameter of 7.825 mm and in *S. aureus*: 7.59 mm. Finally, *Theobroma Cacao* showed that *E. coli* has a diameter of 9.18 mm and *S. aureus* 13.98 mm.

Thus, *Theobroma cacao* exhibited moderate antibacterial activity. It is more effective against *S. aureus* than against *E. coli*, but overall, its inhibitory effect is less than that of meropenem and clindamycin. Overall, meropenem was the most effective treatment for both *E. coli* and *S. aureus* infections. Clindamycin is also effective, but less potent than meropenem. Moreover, 95% ethanol showed minimal antibacterial activity against both the bacteria. *Theobroma Cacao* has a moderate effect and is particularly more effective against *S. aureus* than *E. coli*.

Figure 6. Zones of inhibition of *Theobroma Cacao* (A) *E. coli* and (B) *S. aureus* on Mueller Hinton Agar after 24 h of incubation. Legend: (1) Meropenem (+), (2) Clindamycin (+), (3) 95% Ethanol, (4) *Theobroma Cacao*



This study indicates that meropenem is the preferred treatment for infections caused by these bacteria, owing to its superior inhibitory effect. Therefore, clindamycin may be a secondary treatment option. Ninety-five percent ethanol is not suitable for antibacterial purposes in this context, while *Theobroma Cacao* shows potential, especially against *S. aureus*.

According to Supriyanto et al., (2014) reported that it is well-known that cocoa leaves contain phenolic compounds, a class of bioactive substances that also serves as an antioxidant. Cocoa leaves also contain theobromine, caffeine, anthocyanins, leuco anthocyanins, and catechol, the amount of which varies and is influenced by leaf age and plant age. As suggested by Othman et al. (2007), the total polyphenols in old leaves showed a higher yield of 32.4%, while that in young leaves was 27.3%. Another study by Osman et al. (2004) showed that the total polyphenol content in old leaves was 28.4%.

The value of in the younger leaves was 19%. The total catechin content in the old and young leaves was 5.25% and 9.75%, respectively. The content of antioxidants, flavonoids, and other bioactive compounds found in cocoa leaves can be extracted from plant tissues by extraction method.

Another study by Chowdhury et al. (2008) suggested that this was due to the development of partial or complete resistance of microorganisms against the test samples, which might be due to the indiscriminate use of antibacterial agents. However, the results of this study showed that the extracts used can inhibit the growth of gram-positive and gram-negative bacteria, as well as fungi.

Conclusions

A study of *Theobroma cacao* leaf extract has yielded significant findings regarding its phytochemical composition and biological activities. The extract contained various bioactive compounds, including tannins, flavonoids, steroids, and phenols. It exhibited notable antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* as well as antioxidant properties. Brine Shrimp Lethality Assay and *Allium cepa* test revealed dose-dependent cytotoxic and genotoxic effects. These results highlight the potential therapeutic applications of the *T. cacao* leaf extract in antimicrobial, antioxidant, and cytotoxic treatments. However, the observed concentration-dependent cytotoxicity emphasizes the need for careful dosage consideration in future applications. Although this study provides comprehensive evidence for the bioactive properties of the extract, further research is necessary to fully elucidate its mechanisms of action and potential pharmaceutical and nutraceutical applications.

Recommendations

Based on these conclusions, the following recommendations can be made:

1. Further research is needed to investigate the specific mechanisms of action of the antibacterial, antioxidant, and cytotoxic properties of *Theobroma cacao* leaf extract.
2. In-depth toxicological studies were performed to determine the safe dosage ranges for potential therapeutic applications, considering the observed concentration-dependent cytotoxicity.
3. Explore the development of pharmaceutical formulations utilizing *T. cacao* leaf extract for antimicrobial treatment, particularly against *Escherichia coli* and *Staphylococcus aureus*.
4. Investigate the potential use of the extract in antioxidant-based nutraceutical products, considering the appropriate dosage levels.
5. Clinical trials to assess the efficacy and safety of *T. cacao* leaf extract in human subjects for various therapeutic applications.
6. The individual bioactive compounds identified in the extract (tannins, flavonoids, steroids, and phenols) were used to determine their specific contributions to the observed biological activities.
7. The potential synergistic effects of the various bioactive compounds present in the extract were investigated.

8. To explore sustainable methods for harvesting and processing *T. cacao* leaves to ensure a consistent supply of the extract for future research and potential commercial applications.

Compliance with Ethical Standards

This study was conducted in compliance with all the applicable ethical standards. This study did not involve human or animal subjects. The ethical guidelines for the collection and handling of *Theobroma cacao* plant materials were strictly followed to ensure responsible and sustainable sourcing. All experimental procedures, including phytochemical extraction, antimicrobial testing, antioxidant assays, and cytotoxicity assessments, adhered to institutional and international research guidelines. The authors declare no conflicts of interest, and all data presented are original and have not been published elsewhere.”

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