AVOCADO ETHANOL LEAF EXTRACT: EXPLORING THE EFFECTS OF BALM FORMULATION ON ANTIMICROBIAL ACTIVITY AGAINST SELECTED MICROORGANISMS

BY FAVOUR IMMUENTIYAN-OSA EHIJIE

PHA1808487

DEPARTMENT OF PHARMACEUTICAL CHEMISTRY,

FACULTY OF PHARMACY UNIVERSITY OF BENIN,

BENIN CITY.

NOVEMBER, 2025

AVOCADO ETHANOL LEAF EXTRACT: EXPLORING THE EFFECTS OF BALM FORMULATION ON ANTIMICROBIAL ACTIVITY AGAINST SELECTED MICROORGANISMS

BY FAVOUR IMMUENTIYAN-OSA EHIJIE

PHA1908487

SUPERVISED BY:

DR. IRENE O. OSEGHALE

A PROJECT SUBMITTED TO THE DEPARTMENT OF PHARMACEUTICAL CHEMISTRY IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF THE DOCTOR OF PHARMACY (PHARM.D) DEGREE OF THE UNIVERSITY OF BENIN, BENIN CITY, EDO STATE.

NOVEMBER, 2025

CERTIFICATION

This is to certify that this project was carried out by **EHIJIE FAVOUR IMMUENTIYAN-OSA** in the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Benin, Benin City, Edo State, Nigeria.

_____ _____

DR. I.O. OSEGHALE DATE

PROJECT SUPERVISOR

_____ _____

DR. V.O. IMIEJE DATE

HEAD OF DEPARTMENT

DEDICATION

This project is dedicated to God Almighty, whose grace, wisdom, and strength has sustained me through every challenge. All glory and honour belong to Him. This project is also dedicated to my mom, Mrs. Mary Ehijie for her selfless and sacrificial love throughout my academic journey.

ACKNOWLEDGEMENT

I am profoundly grateful to God Almighty for His infinite grace, wisdom, provision, protection and strength, which have guided me through this academic journey. His unwavering support has been my greatest source of strength and resilience.

My sincere appreciation goes to my loving parents, Mr. Fidelis Ehijie and Mrs. Mary Ehijie, for their unconditional love, sacrifices, and prayers. To my wonderful siblings, Blessing Ehijie, Bright Ehijie and Benefit Ehijie, thank you for being a constant source of joy and encouragement.

A special appreciation to Mr. Osunbor Osagie and Mrs. Goodness Osagie whose generosity and commitment have made my academic journey possible.

I also wish to express my deep sincere gratitude to my project supervisor, Dr. Irene O. Oseghale, for her patience, mentorship, and invaluable contributions toward the successful completion of this project. I also appreciate the entire staff of the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Benin, for their academic guidance and support throughout my studies. I also acknowledge Mr. Wilfred O. Aisagbonbuomwam of the Department of Pharmaceutical Microbiology for his assistance and support.

My heartfelt appreciation goes to my friends, Favour Momoh, Itohan Lawrence, Lesley Otaru, Naomi Ibadin, Stella Uwoghiren and Michelle Edemakhiota, my project sister for bestowing me with the gift of friendship through encouragements and academic assistance. I would also love to appreciate Dako Isreal, Ogieva Theophilus and my other reading partners which have never failed to assist me academically.

Finally, I express my gratitude to everyone who has contributed to my journey—whether through encouragement, prayers, or support in any form. This achievement is not mine alone but a reflection of the love, kindness, and support I have received. Thank you all!

TABLE OF CONTENT

Deleted[Remy Ogbeide]:

ABSTRACT

The leaves of *Persea americana* have been discovered to exert antimicrobial properties. The upsurging need for new antimicrobial formulations has driven the quest to formulate and test the compatibility of formulation excipients and avocado extract. The aim of this study is to determine and compare the antimicrobial activity of the ethanol avocado leaf extract of *Persea americana* and its balm formulation.

Fresh *Persea americana* leaves were harvested, dried, pulverized and macerated in ethanol for a week. Phytochemical screening was carried out on the extract, after which it was formulated into balms (0, 100, 200, and 400 mg/ml). The different balm concentrations were tested against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa*, using the ditch plate method. Ciprofloxacin was used as the positive control, and statistical significance was determined using ANOVA, with a significance level of $P \leq 0.05$.

The ethanol plant extract was found to contain alkaloids, saponins, phenolics, triterpenoids, and flavonoids. The extract with (0, 100 mg/ml) showed no activity on the tested organisms. The extracts at 200 and 400 mg/ml exhibited antimicrobial activity ranging from 19 – 27 mm with the 400 mg/ml extract showing greater activity against the tested organisms. The balm formulation exerted reduced antibacterial activity compared to crude extract. The balm formulation with (0, 100 mg/ml) showed no activity, while the 200 and 400 mg/ml showed lesser activity ranging from 8 – 20 mm.

Conclusively, the antimicrobial activity of the ethanol extract of avocado leaves was influenced by the balm formulation. This indicates that the interactions and compatibility of formulation excipients are crucial factors to consider when formulating herbal products.

CHAPTER ONE

1.0 INTRODUCTION

In early centuries, healers often relied on traditional remedies to manage a wide range of illnesses. These treatments frequently produced notable results and they eventually drew the attention of scholars and practitioners who began to examine them more closely. Most of these remedies came from various plant parts, yet their effectiveness was not supported by documented evidence or scientific investigation at the time (Balkrishna *et al.,* 2024).

The growing incidence of adverse reactions and side effects of synthetic medicines in pharmacy practice today, has led to an increase in the need to research for safer and more secure alternatives of unorthodox medicines. Today, many scientists and pharmacists have resorted to intensely exploring plants as a prospective source of treatments of different infections and diseases such as HIV, hepatitis, cancer etc. Many plants have been recognized by folk medicine for their ethno-medicinal benefits, some of which include; *Persea americana* (Avocado leaves).

1.2. *Persea americana* (AVOCADO LEAVES)

Persea americana Mill. (*Lauraceae*), commonly called avocado, originally comes from the Central Highlands of Mexico and Guatemala, extending into parts of Central and South America (Abraham *et al*. 2018). Today, the avocado tree is widely grown across tropical and subtropical regions because of its highly nutritious and flavour full fruit. The tree itself is medium to large, usually reaching 9 to 20 m in height, and is characterised by a vigorous growth pattern with a rounded, heavily branched canopy.

The leaves of *Persea americana* typically measure between 3.5 and 19 cm in width and 6 to 30 cm in length. Their shape ranges from narrow to broadly elliptical. Young leaves are soft, slightly hairy and often reddish, but as they mature, they become smooth, leathery and dark green. These simple, petiolate leaves may appear elliptical to lance-shaped and can have fine hairs, especially when young, with margins that remain entire. The species is bifacial, meaning the upper and lower leaf surfaces differ; the underside is paler, with more pronounced veins and a clearly visible midrib. The tips are generally acute, while the bases are also acute, supported by a pinnate reticulate venation pattern. Most of the leaf hairs are unicellular and found mainly on the lower surface. The leaf also contains simple crystals of various sizes along with small raphides. Stomata are present only on the underside and are anomocytic, usually bordered by about three to six subsidiary cells. A cross-sectional view of the petiole shows that the majority of hairs cluster along its upper surface (Kendir and Koroglu 2018).

A few common names of avocado leaf include; English: Alligator pear, Avocado pear, Spanish: Guatema, Nigerian: Pears or Èwà (Yoruba), Ube (Igbo).

According to the Centre for Invasive Species and Ecosystem Health (2018), *Persea americana* Mill. is classified within the kingdom Plantae. It falls under the spermatophytes and the angiosperms, which make up its division and super-division. The species is placed in the phylum Magnoliophyta and the class Magnoliopsida. It is further categorised under the order Laurales and the family Lauraceae, with its full scientific name identified as *Persea americana* (Mill).

FIGURE 1.1: Avocado growing in its natural habitat

1.2.1. PHYTOCHEMISTRY, ETHNO-MEDICINAL USES, AND PHARMACOLOGICAL PROFILE OF *Persea americana* LEAVES.

Deleted[Remy Ogbeide]:

Persea americana (Mill) is a tropical and subtropical fruit native to Mexico and Central America, although it is now grown and eaten widely around the world. It belongs to the *Lauraceae* family and the genus *Persea*, a group that includes more than 150 known species of evergreen trees. While the fruit is well known, various parts of the plant particularly the leaves have long been used in traditional medicine to manage different health conditions (Biasi-Garbin *et al.*, 2016).

The leaves of *Persea americana*, though often underestimated, have attracted increasing scientific attention because of their nutritional value, phytochemical composition, and potential therapeutic benefits. Traditionally, the leaves are brewed into teas used as diuretics, carminatives, antirheumatic agents, antidiarrheals, analgesics, and anti-inflammatory remedies. They are also used to encourage bile flow and to support the treatment of kidney problems, urinary stones, and bladder disorders. In addition, homemade alcoholic extracts of the leaves are applied topically for issues such as headaches, convulsions, and rheumatic pain (Biasi-Garbin *et al.*, 2016).

The biological activities seen in *Persea americana* are likely linked to the abundance of polyphenolic compounds found in its leaves. Earlier research supports this, noting that avocado leaves contain several important phytochemicals, including phenolic acids, flavonoids, and procyanidins (Jiménez *et al*., 2017).

Bioactive profiling of *Persea americana* leaves has shown that they contain a range of phytochemicals, including tannins, saponins, terpenoids, steroids, alkaloids, flavonoids and glycosides. These compounds are known to possess significant medicinal and pharmacological value for human health (Yasir *et al*., 2010).

The leaves of *Persea americana* are known to be nutritionally value packed and contain substantial amounts of oil, especially monounsaturated fatty acids like palmitic, palmitoleic, stearic, oleic, and linoleic acids. Studies have also identified carotenoids, vitamins, minerals, lecithin, β-sitosterol, polyphenols, and flavonoids within the leaves. Previous studies also highlight their antioxidant and antimicrobial properties (Ramdhan and Yusuf 2023).

According to a study conducted by Kendir and Koroglu, 2018, *Persea americana* leaves have long been incorporated into traditional healing practices across different cultures. In Turkey, they are commonly used to help manage kidney stones and urinary tract infections. Beyond this, the leaves are taken as an infusion to relieve ailments such as diarrhoea, stomach discomfort, general body pain, headaches, wounds, fever, heart-related issues, osteoporosis, vomiting and sore throat. In parts of Mexico, they are also believed to ward off negative spiritual influences. Across the Caribbean, the leaves are prepared as a decoction to help control high blood pressure.

In Ecuador, fresh avocado leaves are consumed as an aqueous infusion or decoction to ease conditions like influenza, bronchitis, menstrual cramps, diabetes and rheumatism, and they are even applied externally as a hair tonic. In Nigeria, the leaves have been traditionally employed for their antimalarial effects (Kendir and Koroglu 2018).

1.1.2 PHARMACOLOGICAL ACTIONS OF *Persea americana* LEAVES

Different in vitro investigations have explored the biological potential of *Persea americana* leaves, revealing activities such as anticancer, anticholinesterase, antimicrobial, antioxidant, and antityrosinase effects. Beyond these laboratory findings, the leaves have also demonstrated a wide range of pharmacological actions, including analgesic, anti-inflammatory, anticonvulsant,

antihyperlipidemic, hypocholesterolemic, hypoglycemic, and blood-pressure-lowering properties (Kendir and Koroglu 2018).

The leaves of *Persea americana* have also been reported help reduce cholesterol levels and trigger uterine contractions. They are traditionally used to manage dysentery caused by helminths and amoebas, treat skin rashes, and address fungal or bacterial infections. Additionally, the leaves are used in the relief of asthma, high blood pressure, and typhoid fever. Beyond their medicinal value, the avocado tree itself can function as a natural air purifier when grown around living spaces (Tcheghebe *et al.*, 2016).

1.1.2.1 ANTIHYPERTENSIVE PROPERTY

A decoction prepared from *Persea americana* leaves has been shown to significantly lower both systolic and diastolic blood pressure in certain individuals (Selvia and Rahmataway 2023).

Additionally, studies report that the aqueous leaf extract can decrease total cholesterol and low-density lipoprotein levels, thereby helping to reduce the risk of hypertension and coronary heart disease (Arackal and Parameshwari 2017).

1.1.2.2 ANTIOXIDANT ACTIVITY

From a study carried out by Oboh *et al*, extracts from Persea americana leaves demonstrated the ability to neutralize hydroxyl radicals (OH) as well as 2,2-diphenyl-1-picrylhydrazyl (DPPH). These antioxidant effects were linked to the phenolic compounds naturally present in the leaf extract (Oboh *et al*., 2015).

1.1.2.3 ANALGESIC AND ANTI-INFLAMMATORY ACTIVITY

The anti-inflammatory effects of *Persea americana* leaves were evaluated in male albino rats using the carrageenan-induced paw oedema model. Researchers tested the methanolic extract alongside its petroleum ether, chloroform, and ethyl acetate fractions. The degree of paw swelling in rats that received these extracts before carrageenan injection was compared with that of untreated animals. All extracts and fractions demonstrated clear anti-inflammatory activity.

These same extracts were then assessed for their analgesic potential using the hot plate method. The methanolic extract and its fractions showed significant pain-relieving effects, confirming the plant's analgesic properties (Mahmoud *et al*., 2021).

1.1.2.4 HYPOGLYCAEMIC ACTIVITY

In the hypoglycaemic investigation conducted by Makopa *et al*., (2020), the non-polar extracts of *Persea americana* leaves produced a modest reduction in alpha-glucosidase activity. The dichloromethane extract, however, did not inhibit the enzyme. A noticeable decline in enzyme activity was recorded with hexane extract at concentrations ranging from 0 to 0.05 mg/ml, but when the concentration of the hexane extract increased, alpha-glucosidase activity also rose. Extracts with intermediate polarity, such as ethyl acetate and acetone, produced only minimal and statistically insignificant inhibition. Among all the tested extracts, the methanolic extract demonstrated the strongest ability to suppress alpha-glucosidase activity (Makopa *et al.*, 2020).

1.1.2.5 ANTICONVULSANT

Persea americana leaf extract has demonstrated anticonvulsant properties, this makes it beneficial in the management of both petit mal and grand mal epilepsy. The extract effectively

countered convulsions triggered by pentylene tetrazole (PTZ), an effect attributed to its ability to enhance GABA neurotransmission in the brain (Gupta *et al*., 2018).

1.1.2.6 SAFETY PROFILE

The toxicological effect of ethanol extracts of *Persea americana* leaves was investigated and discovered that no death occurred nor were there signs of toxicity in the rats after administration, but some histopathological and biochemical changes occurred at higher doses (Kamagate *et al.*, 2016).

1.1.4 ANTIBACTERIAL ACTIVITY OF *Persea americana* LEAVES

Persea americana leaf extract contains important bioactive compounds such as alkaloids, saponins, and flavonoids, which have been shown to inhibit the growth of various bacteria (Wijaya, 2020).

These compounds contribute significantly to the antimicrobial activity of the leaves. Flavonoids can damage bacterial proteins and disrupt the cell wall, tannins interfere with microbial adhesion and enzyme activity, saponins form complexes in the bacterial cell wall, and alkaloids affect peptidoglycan, ultimately leading to bacterial death (Yusuf *et al.,* 2023)

According to Boadi *et al*., methanolic leaves extract of *Persea americana* was observed to have the highest antimicrobial effects (greatest zone of inhibition) on organisms such as *Bacillus subtilis*, *Escherichia coli, Salmonella typhi, Staphylococcus aureus, Psuedomonas aeruginosa* in

comparison to other solvents (chloroform, ethyl acetate, ether) which had lesser antibacterial potency (Boadi *et al*., 2015).

Enterococcus faecalis, a bacterium often linked to failed root canal treatments, was inhibited by the ethanol extract of *Persea americana* leaves. The extract produced zones of inhibition measuring 8.99 mm, 10.73 mm, and 11.82 mm at concentrations of 25%, 50%, and 100%, respectively, compared with 10.53 mm observed in the positive control (Rival *et al*., 2020).

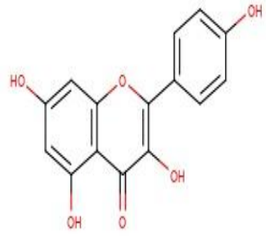
The ethanol extract of *Persea americana* leaves has been shown to exhibit antibacterial activity against *Klebsiella pneumoniae* and *Proteus mirabilis*. These results suggest that the extract can effectively inhibit the growth of these clinical isolates, highlighting its potential as a natural antimicrobial agent (Nasri *et al.,* 2024).

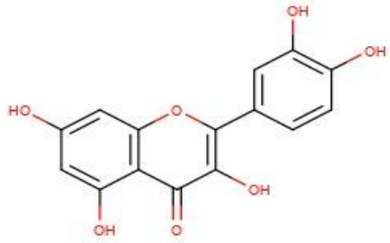
Avocado leaves, rich in saponins, alkaloids, flavonoids, polyphenols, and quercetin, are recognized for their antibacterial properties. An ethanol extract of *Persea americana* leaves (96 %) was formulated into a gel peel-off mask at concentrations of 0.1%, 0.15%, 0.2%, 0.25%, and 0.3% using an experimental laboratory method. Testing against Staphylococcus epidermidis with the Mueller-Hinton disc diffusion method revealed that the 0.3% concentration produced the largest zone of inhibition (Rival *et al.,* 2020).

Alcoholic extracts of *Persea americana* were also seen to possess significant antibacterial activity against *Salmonella typhi, Staphylococcus epidermidis, Klebsiella pneumoniae, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Shigella flexneri, Bacillus cereus, Enterococcus faecalis, and Bacillus subtilis* (Ningsih *et al.,* 2020).

Extracts from *Persea americana* leaves have been shown to impact bacterial biofilms. In a study examining their effect on *Klebsiella pneumoniae* strains carrying aminoglycoside resistance genes, the broth microdilution method was used. The results demonstrated that the leaf extract exhibited antibacterial activity even against aminoglycoside-resistant strains (Kizilyidirim *et al*., 2024).

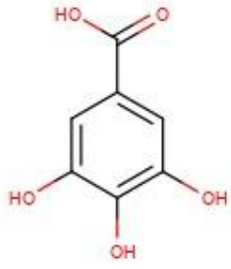
Some compounds that have been detected in the leaves of ***Persea americana*** which are believed to be responsible for their pharmacological activities are shown below.

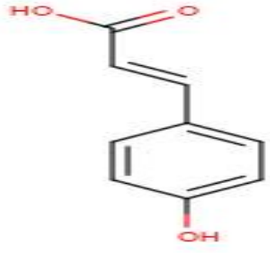




Quercetin

Kaempferol





p-Coumaric acid

Gallic acid

FIGURE 1.1: Some compounds detected in the leaves of *Persea americana*

1.2 *Apis mellifera* (BEESWAX)

Beeswax is a naturally occurring, fat-rich material mainly secreted by worker bees belonging to the species *Apis mellifera* (Linnaeus, 1758). Though *Apis mellifera* itself originates from an animal source, its trace floral compounds arise indirectly from the bees' foraging on nectar and pollen from blooming plants, including both angiosperms and certain gymnosperms (Crailsheim *et al*., 2019).

Consequently, the taxonomic placement of *Apis mellifera* is linked to the insect-based classification of its primary producer*, Apis mellifera*, commonly referred to as the Western or European honey bee, belongs to the order *Hymenoptera.,* a group that includes many key pollinators essential for plant fertility. *Apis mellifera* dominates global beeswax production for commercial purposes, while other closely related species, such as *Apis cerana* (Fabricius, 1793), yield smaller quantities (Ilyasov *et al.,* 2020).

Apis mellifera produces a complex mixture of chemicals through specialized wax glands located in its abdomen. (Fratini *et al.,* 2016; Bogdanov, 2016).

Juvenile worker bees, between age 12 to 18 days, after emerging from their cells, release this wax in liquid form; it quickly solidifies upon exposure to air. They use it to build the honeycomb's precise hexagonal cells, which serve as nurseries for developing larvae and secure storage for honey and pollen (Bogdanov, 2016).

These sturdy wax compartments not only protect the brood but also hold vital food reserves, while the overall structure provides essential support for the bustling colony of bees at work. Beeswax is a food grade ingredient that comes out white after the manufacturing process. It turns yellow with time (yellow beeswax), due to propolis and pollen extracts. Its distinct odor is a combination of honey, bee-secretions, propolis and pollen remnants. The wax crystalline

structure is storage atmosphere dependent; upon its formation, the more organized the crystal structure is found, as well as higher rigidity and strength. One of beeswax most noticeable characteristics is its hardness. It exhibits more rigidity at lower temperatures. Its physical property changes when heated—molten wax shrinks by approximately 10 %. At 30–35 °C, it is flexible like rubber. Water is not a solvent for beeswax, but it amalgamates with other organic solvents e.g acetone, benzene etc. (Bogdanov, 2016).

Based on the classical system by Linnaeus (1758) and the modernized classification by Ilyasov *et al*., (2020), *Apis mellifera* is classified under the Kingdom Animalia, Order *Hymenoptera,* Family Apidae, Subfamily Apinae, Tribe Apini, Subtribe Apina, and belongs to the Genus *Apis* and Species *mellifera*. It is commonly known as the yellow or white beeswax bee.

FIGURE 1.2: Picture of *Apis mellifera*

1.2.2. PHYTOCHEMICAL CONSTITUENTS, ETHNOMEDICINAL USES AND PHARMACOLOGICAL USES OF *Apis mellifera*

Apis mellifera has a long and rich history of traditional use across many cultures for skin and wound-related applications. For centuries, traditional healers used *Apis mellifera* in salves and ointments to soothe bruises, minor burns, cracked heels, and superficial wounds (Dumitru *et al.,* 2022).

It was valued for creating a protective film on the skin helping to retain moisture and shield the area from external irritants. Contemporary reviews confirm that ancient records (such as in Egyptian, Chinese and Ayurvedic medicine) describe *Apis mellifera* based formulations for inflammation, wound healing and skin barrier repair (Fratini *et al*., 2016). In addition, ethnomedicinal texts note its use as a topical occlusive vehicle, often mixed with herbal extracts, oils or honey, to enhance the therapeutic effect. The wax helped to hold the herbal medicine on the skin longer and support the healing process. (Arredondo-Ochoa *et al*., 2018).

Deleted[Remy Ogbeide]:

Modern investigations have confirmed multiple pharmacological actions of beeswax and its derivatives, particularly in topical and dermatological contexts. Barrier and skin-protective effect: Beeswax forms a hydrophobic film over the skin surface, reducing transepidermal water loss, supporting skin barrier integrity and providing a mechanical protective layer. This is why it's frequently used in lip balms, hand creams, body salves, and wound-care formulations. (Nong *et al.,* 2023).

Some fraction of *Apis mellifera* contains trace phenolic and flavonoid compounds (often from plant resins or propolis contamination) which contribute to antioxidant and mild anti-

Deleted[Remy Ogbeide]:

inflammatory actions supporting its use in soothing irritated skin or damaged barrier. (Dumitru *et al.,* 2022).

Besides active roles, beeswax is pharmaceutically useful as a formulation ingredient, it provides structure, defines melting point, influences release of actives in semi-solid topical dosage forms (like balms), and enhances product stability and spreadability in topical preparations. (Arredondo-Ochoa *et al*., 2018).

Beeswax is chemically complex contains different phytoconstituents including long-chain fatty acid esters, wax esters, long n-alkane chains which contribute to its hydrophobic character and structural stability and free fatty such as 'palmitic and oleic acid' (Dumitru *et al*., 2022); Ledjanac *et al*.,2024**);** Ertürk *et al.,* (2024).

Deleted[Remy Ogbeide]:

Apis mellifera reportedly contains trace sterols, plant-derived pigments and aromatic compounds that influence colour and may add to antioxidant effects. Remnants from *Apis mellifera* processing contain detectable levels of phenolic compounds and flavonoids, which contribute to their observed antibacterial activity (Dumitru *et al.,* 2022; Peron *et al.,* 2023*).*

Deleted[Remy Ogbeide]:

1.2.3 ANTIBACTERIAL PROPERTIES OF *Apis mellifera*

Studies have shown that *Apis mellifera* possesses notable antibacterial properties, which are largely linked to minor bioactive compounds such as phenols, flavonoids, and small amounts of resin-derived materials. Although these compounds occur in limited quantities, they play a key role in the antimicrobial effectiveness of beeswax (Dumitru *et al.,* 2022; Peron *et al.,* 2023).

According to Fratini *et al*., (2016*), Apis mellifera* and extracts obtained from it are capable of suppressing the growth of Gram-positive bacteria, particularly *Staphylococcus aureus*, a common cause of skin and wound infections. Their study suggests that this antibacterial effect is linked to trace amounts of phenolic residues and propolis-like compounds that remain in raw, unrefined beeswax.

Supporting this observation, Peron *et al.,* (2023) reported that by-products from *Apis mellifera* processing exhibit strong antibacterial effects against several notable pathogens, including *Escherichia coli*, *Salmonella spp.*, and *Listeria monocytogenes*. These authors associate the antimicrobial action with the presence of flavonoids, aromatic acids, and antioxidant molecules capable of disrupting bacterial membranes and interfering with microbial survival.

Further evidence from Ertürk *et al*., (2024) demonstrates that natural *Apis mellifera* can inhibit both Gram-positive and Gram-negative bacteria, with noticeable effects against organisms such as *Staphylococcus aureus* and *Klebsiella pneumoniae*. Their findings also reveal that natural *Apis mellifera* is more potent than synthetic counterparts because it retains a higher concentration of bioactive compounds that contribute to antimicrobial activity.

Additionally, Dumitru *et al.,* (2022) explain that the antibacterial potential of *Apis mellifera* can be enhanced when it is used together with other bee-derived substances like honey or propolis. These combinations increase the overall content of phenolic and flavonoid compounds, leading to a synergistic antimicrobial effect that is stronger than *Apis mellifera* alone.

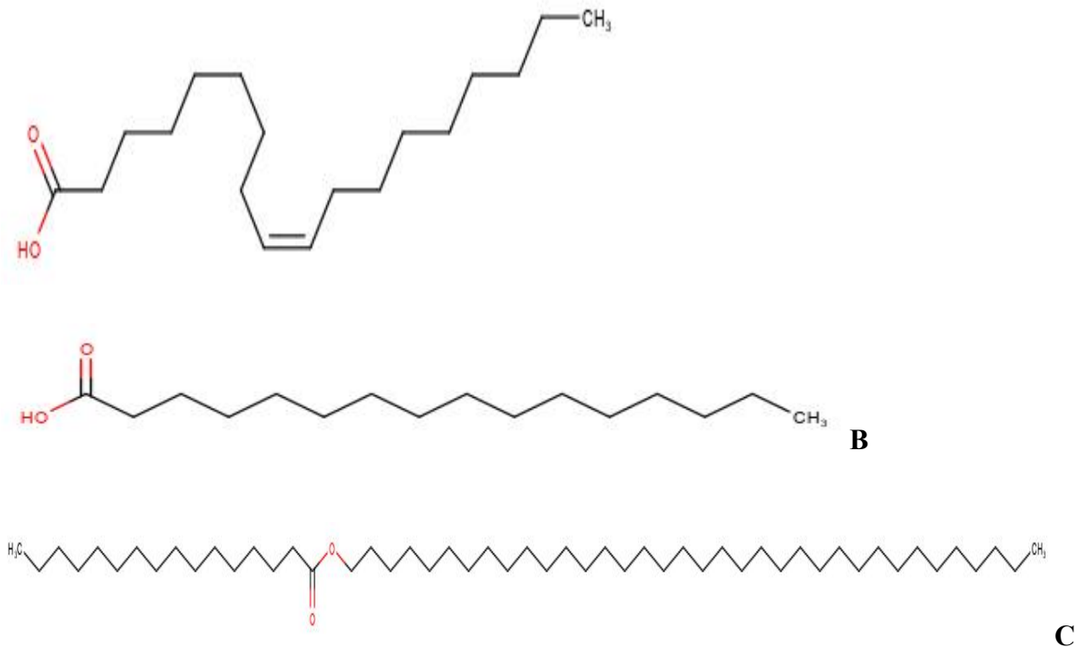


FIGURE 1.2: Compounds detected in structures of *Apis mellifera*

A – Oleic acid **B –** Palmitic acid **C –** Myricyl palmitate

1.7 PROBLEM STATEMENT

Antibiotics have significantly lowered mortality from infectious diseases and have made high-risk medical procedures safer. However, infections remain widespread in developing nations, with pathogens such as *E. coli* and *K. pneumoniae* continuing to cause serious illness. Overuse and misuse of antibiotics contribute to growing resistance, sparking interest in plant-based alternatives. Global data indicate that approximately one in six infections no longer respond to conventional antibiotics, leading to poorer health outcomes and higher healthcare costs (Ahmed, 2024; Naylor, 2022; Pancu *et al*., 2021).

Bacteria resist antibiotics through target changes, enzyme production, efflux pumps, biofilms and gene transfer making treatment even harder. Slow antibiotic development is linked to scientific and financial challenges. These limitations drive interest in repurposed drugs and plant-derived antimicrobial. Plants produce metabolites with strong antibacterial effects and recent reviews highlight new advances and formulation challenges. Many plant compounds also boost antibiotic activity as adjuvants (Woo *et al*., 2023; Angelini *et al*., 2024; Khameneh *et al*., 2019).

1.8 JUSTIFICATION OF STUDY

There is an upsurging need for accessible and newer antimicrobial agents due to increasing antibiotic resistance. Plant-based medicines offer a promising alternative because they contain diverse bioactive compounds and are widely available. *Persea americana* is abundant, inexpensive, and traditionally used, but its potential in formulated topical products remains underexplored. This study is justified as it provides scientific evidence for the plant's

antimicrobial activity and supports the development of a natural antimicrobial balm that could serve as a safe, affordable option for managing skin infections such as boils, cellulitis etc.

1.9 AIM OF STUDY

To evaluate the antibacterial activity of the ethanol extract of *Persea americana* (avocado) leaves and assess its compatibility and performance when incorporated into a topical balm formulation.

1.10 OBJECTIVES OF THE STUDY

1. To determine the phytochemical constituents of the ethanol extract of *Persea americana* leaves.
2. To evaluate the antibacterial activity of the ethanol leaf extract against selected bacterial pathogens (*Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae*).
3. To assess the antimicrobial activity of the formulated balm and compare its effectiveness with that of the ethanol extract and standard antibiotics.
4. To provide recommendations for further optimization or development of plant-based topical antimicrobial formulations.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1. MATERIALS

Leaves of *Persea americana*, Sabourand dextrose agar and Mueller hinton agar (Titan Biotech), Mylar agar (Titan Biotech), Yellow Beeswax (Jules), Cocoa Butter (Tulip Ltd), Olive oil (Goya), Vitamin E (Pure life), Pepper mint oil (Silver line).

2.2. EQUIPMENTS

Water bath (Stuart®), Rotary evaporator (Stuart®), Glass rod, Foil paper, Maceration jar, 250ml beaker, Porcelain dish, Measuring cylinder, Spatula, Masking tape, Sample tubes, Oven (Gallenkamp, England), Electronic weighing balance (M-METLAR M4 11R), Autoclave (Gallenkamp, England), Dispensing container, White handkerchief, Whatmann No 1 filter paper, Tissue paper, Micropipette, Test tubes, Loop, Petri dishes, EDTA bottle, Cork borer, Incubator (Gallenkamp, England), Metre rule.

2.3. REAGENTS AND SOLVENTS

Ethanol (GH-TECH), Tween-80 diluent (Titan Biotech), Distilled water.

2.4. METHODOLOGY

2.4.1. PLANT COLLECTION AND PREPARATION OF ETHANOL EXTRACT

Persea americana leaves were collected from Obagie community in Ikpoba Okha local government area of Edo State. Identification and authentication of the plant was carried out by Prof. H.A. Akinnibosun of the Department of Plant Biology and Biotechnology, Faculty of Life

Sciences, University of Benin, Benin City, where the herbarium specimens were deposited and herbarium number and UBH-P408 was issued. The plant was dried under a shade for a sufficient period of time and afterwards pulverized using a mechanical grinder. 500 g of the powder was weighed with the aid of an electrical weighing balance (M-METLAR M4 11LR) and extracted using 1,800ml of ethanol in a glass jar. The content was manually stirred twice daily with the aid of a glass rod at room temperature, and after 7 days, the content was filtered with the aid of a white handkerchief, and Whatmann No 1 filter paper. A rotary evaporator was used to concentrate the extract at 40°C, and the ethanol solvent was recovered under reduced pressure, yielding an ethanol extract which was transferred into a clean sample bottle. The weight of the concentrated extract was determined using an electronic balance, and its percentage yield was further calculated using the formula below:

$$Percentage\ Yield = \frac{\text{Weight of extract obtained}}{\text{Weight of powdered sample}} \text{ x } 100\%$$

2.4.2. PHYTOCHEMICAL SCREENING OF ETHANOL EXTRACT

Simple chemical tests were performed on the ethanol extract following standard procedures to detect its phytochemical constituents. Approximately 5 g of the ethanol extract was dissolved in 5 ml of ethanol. The solution was filtered with Whatmann no 1 filter paper. The filtrate obtained was used to carry out various phytochemical tests. All tests were performed in triplicate to ensure reliability and accuracy. (Sofowora, 1982; Harborne, 1998; Evans 2002).

2.4.2.1 TEST FOR ALKALOIDS

To test for alkaloids, 2 ml of the ethanol extract was placed into four separate test tubes, and a few drops of Wagner's, Mayer's, Hager's, and Dragendorff's reagents were added to each tube, respectively. The tubes were gently shaken and left to stand for five minutes. The appearance of a reddish-brown precipitate with Wagner's reagent, a cream-colored precipitate with Mayer's or Hager's reagents, or an orange-brown precipitate with Dragendorff's reagent indicates the presence of alkaloids (Sofowora, 1982; Harborne, 1998; Evans 2002).

2.4.2.2 TEST FOR CARBOHYDRATES (MOLISCH'S TEST)

To test for carbohydrates, 2 ml of the extract was combined with two drops of Molisch's reagent, after which concentrated sulfuric acid was carefully added down the side of the test tube to form a distinct layer. The appearance of a violet or purple ring at the interface between the two layers indicates the presence of carbohydrates (Sofowora, 1982; Harborne, 1998; Evans 2002).

2.4.2.4 TEST FOR REDUCING SUGARS (FEHLING'S TEST)

To test for reducing sugars, 2 ml of the extract was mixed with 2 ml each of Fehling's solutions A and B in a test tube and heated in a boiling water bath for five minutes. The formation of a brick-red precipitate indicates the presence of reducing sugars (Sofowora, 1982; Harborne, 1998; Evans 2002).

2.4.2.5 TEST FOR SAPONINS (FROTHING TEST)

The presence of saponins was tested using the froth test. In this method, 2 ml of the extract was diluted with 10 ml of distilled water in a test tube and shaken vigorously for 30 seconds. The

mixture was then left to stand for 10 minutes and observed for the formation of a stable layer of froth, which indicates the presence of saponins (Sofowora, 1982; Harborne, 1998; Evans 2002).

2.4.2.6 TEST FOR STEROIDS AND TRITERPENES (LIEBERMANN–BURCHARD TEST)

To test for steroids and triterpenes, 2 ml of the extract was combined with 2 ml of acetic anhydride, followed by the careful addition of 1 ml of concentrated sulfuric acid down the side of the test tube. The development of a blue-green color indicates the presence of steroids, while a red or pink color signifies the presence of triterpenes (Sofowora, 1982; Harborne, 1998; Evans 2002).

2.4.2.7 TEST FOR PHENOL COMPOUNDS (FERRIC CHLORIDE TEST)

Phenol compounds were tested by adding 2 ml of 10% ferric chloride solution to 2 ml of the extract. The appearance of a deep blue or green color indicated the presence of phenolic compounds, confirming that these compounds were successfully extracted. (Sofowora, 1982; Harborne, 1998; Evans 2002).

2.4.2.8 TEST FOR TANNINS

To detect tannins, 2 ml of the extract was mixed with 2 ml of distilled water, boiled for five minutes, and then filtered. Two drops of 10% ferric chloride were added to the filtrate, and the mixture was observed for a color change. The appearance of a blue-black or greenish color indicated the presence of tannins in the extract (Sofowora, 1982; Harborne, 1998; Evans 2002).

2.4.2.9 TEST FOR FLAVONOIDS (ALKALINE REAGENT TEST)

Flavonoids were tested by combining 2 ml of the extract with 2 ml of 10% sodium hydroxide solution. The development of a deep yellow color, which fades upon acidification with dilute hydrochloric acid, indicates the presence of flavonoids (Sofowora, 1982; Harborne, 1998; Evans 2002).

2.4.2.10 TEST FOR DEOXY SUGARS (KELLER–KILLIANI TEST)

The Keller–Killiani test was performed to detect deoxy sugars linked to cardiac glycosides. In this test, 2 ml of the extract was mixed with 2 ml of glacial acetic acid containing a trace of ferric chloride, followed by the careful addition of concentrated sulfuric acid down the side of the test tube. The formation of a brown ring at the interface confirmed the presence of deoxy sugars (Sofowora, 1982; Harborne, 1998; Evans 2002).

2.4.2.12 TEST FOR PROTEINS (XANTHOPROTEIC TEST)

To 2 ml of the extract solution, 1ml of concentrated nitric acid was added and formation of a dark green precipitate indicated the presence of proteins (Sofowora, 1982; Harborne, 1998; Evans 2002).

2.4.3. PREPARATION OF BALM USING ETHANOL EXTRACT

The crude extract was formulated into a balm using several excipients such as beeswax, cocoa butter, olive oil, vitamin E and pepper mint oil. The butters were melted together over a hot water bath, after which the ethanol extract was incorporated into the melted butters and the oils

were added concurrently. Different concentrations of the ethanol extract were incorporated to get different balm concentrations (0, 100, 200 and 400 mg/ml). The mixtures were poured into containers and allowed to solidify at room temperature.

2.4.5. ANTIMICROBIAL ASSAY

Test microorganisms include clinical isolates of *Pseudomonas aeruginosa, Bacillus subtilis, Escherichia coli, Staphylococcus aureus and Klebsiella pneumoniae*. These were obtained from the University of Benin Teaching Hospital.

2.4.5.1. PROCEDURE FOR ETHANOL EXTRACT (DITCH PLATE METHOD).

Approximately 2 g of the crude extract was weighed into a porcelain dish and 4 ml of 20 % Tween-80 diluent was incorporated to obtain a concentration of 500 mg/ml. The content was triturated to obtain a consistent homogenous liquid, and afterwards 0.2 ml, 0.4 ml and 0.8 ml respectively were pipetted from the 500 mg/ml to give different concentrations of 100 mg/ml, 200 mg/ml and 400 mg/ml respectively. The stock solutions were transferred into an already cleaned and sterilized sample tubes. The same procedure was also repeated for the balm formulation. An aseptic area was created, around which subsequent activities were carried out. Fresh agar plates were prepared, using Mueller Hinton Agar (MHA) as the growth medium for the bacteria isolates, and Sabouraud Dextrose Agar (SDA) as the growth medium for the fungi isolates. About 38 g of MHA was dissolved in 1000 ml of distilled water, sterilized in an autoclave, and allowed to cool. Afterwards, 30 ml of the prepared agar was poured into three petri dishes and was allowed to set. Also, 65 g of SDA was dissolved in about 1000 ml of distilled water, sterilized in an autoclave, and allowed to cool. Thereafter, 30 ml of the prepared agar was poured into three petri dishes and was allowed to set. With the aid of a sterile knife, a ditch (well) was created and the ethanol extract was added to each ditch created in the petri

dishes. Each petri dish was labelled A, B and C for 100 mg/ml, 200 mg/ml and 400 mg/ml respectively. The bacteria *Pseudomonas aeruginosa* was obtained from its colony using a loop, and dissolved in a test tube containing 1 ml of distilled water. With the aid of a swab stick, a little quantity was taken from the test tube and used to streak a straight line on the surface of the ditched fresh agar medium (MHA) in each petri dish. The same was repeated with *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli,* and *Klebsiella pneumoniae*. The six plates were labelled using abbreviations peculiar to each organism: BS for *Bacillus subtilis*, EC for *Escherichia coli,* KB for *Klebsiella pneumoniae*, PA for *Pseudomonas aeruginosa* and SA for *Staphylococcus aureus*.

The same method was carried out for the control drugs i.e Ciprofloxacin (4mg) for bacteria using the ditched MHA. The control drug was introduced into each petri dish, about 0.2 ml of the drug was introduced into the wells on the agar plates. Thereafter, they were inoculated with the same strains of pathogenic bacteria and fungi used previously for the ethanol extracts. The plates were incubated at 37°C for 24 hours, after which the inhibitory zone diameter (IZD) around each well was measured with the aid of a metre rule, and the results recorded.

2.4.5.2. PROCEDURE FOR BALM ASSAY (DITCH PLATE METHOD).

Approximately 0.5 g of the formulated balm (100mg/ml, 400mg/ml and the control) was weighed into a porcelain dish and 2 ml of 20 % Tween-80 diluent was incorporated to obtain a concentration of 250 mg/ml. Also, approximately 0.5 g of the commercial control and 200 mg/ml balm formulation was weighed and dissolved in 3 ml of 20 % Tween-80 diluent. The content was triturated to obtain a consistent homogenous liquid. The stock solutions were transferred into an already cleaned and sterilized sample tubes. An aseptic area was created, around which subsequent activities were carried out. Fresh agar plates were prepared, using Mueller Hinton

Agar (MHA) as the growth medium for the bacteria isolates. About 38 g of MHA was dissolved in 1000 ml of distilled water, sterilized in an autoclave, and allowed to cool. Afterwards, 30 ml of the prepared agar was poured into five petri dishes and was allowed to set. With the aid of a sterile knife, a ditch (well) was created and the balm stock solutions were added to each ditch created in the petri dishes. Each petri dish was labelled A, B, C, D and E for the control balm (devoid of the ethanol extract), commercial balm, 100 mg/ml, 200 mg/ml and 400 mg/ml respectively. The bacteria *Pseudomonas aeruginosa* was obtained from its colony using a loop, and dissolved in a test tube containing 1 ml of distilled water. With the aid of a swab stick, a little quantity was taken from the test tube and used to streak a straight line on the surface of the ditched fresh agar medium (MHA) in each petri dish. The same was repeated with *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli,* and *Klebsiella pneumoniae*. The five plates were labelled using abbreviations peculiar to each organism: BS for *Bacillus subtilis*, EC for *Escherichia coli,* KB for *Klebsiella pneumoniae*, PA for *Pseudomonas aeruginosa*, SA for *Staphylococcus aureus*. The plates were incubated at 37°C for 24 hours, after which the inhibitory zone diameter (IZD) around each well was measured with the aid of a metre rule, and the results recorded.

CHAPTER THREE

3.0 RESULTS

3.1. PERCENTAGE YIELD OF THE EXTRACT

A percentage yield of 10.56 % was obtained after the extraction of 500 g of *Persea americana* leaves, in 1,800 ml of ethanol extract to obtain a 52.8 g extract.

3.2. ORGANOLEPTIC PROPERTIES OF THE BALM FORMULATION

Colour: Dark Green

Odour: Strong Characteristic Smell

Texture: Smooth

Temperature: Solid at room temperature, melts when warmed

3.3. pH OF THE ETHANOL EXTRACT AND BALM FORMULATION

Table 3.1: pH of the ethanol extract and balm formulation

CONCENTRATIONS	pH VALUE
0 mg/ml Balm	5.11
100 mg/ml Balm	5.50
200 mg/ml Balm	5.19
400 mg/ml Balm	5.48
Ethanol Extract	6.01

FIGURE 3.1: Different concentrations of the formulated balm

3.4. RESULTS OBTAINED FROM PHYTOCHEMICAL SCREENING.

Table 3.2: Phytochemicals detected in the ethanol extract of *Persea americana*.

TESTS	RESULTS
Alkaloids	+
Carbohydrates	+
Reducing sugars	+
Saponins	+
Deoxysugars	+
Tannins	+
Steroidal saponins	+
Phenol	+
Flavonoids	+
Triterpenoids	+
Proteins	+

KEY: +: Positive

-: Negative

3.5. RESULTS OBTAINED FROM ANTIMICROBIAL SCREENING

Table 3.3: Antimicrobial activity of different concentrations of ethanol extract of *Persea americana* leaves

CONCENTRATION / **MICROORGANISM**	**100 mg/ml**	**200 mg/ml**	**400 mg/ml**	**Ciprofloxacin 4 mg/ml**
Pseudomonas aeruginosa	NZ	19 mm	24 mm	32 mm
Escherichia coli	NZ	20 mm	22 mm	30 mm
Klebsiella pnuemoniae	NZ	NZ	26 mm	34 mm
Bacillus subtilis	NZ	19 mm	21 mm	30 mm
Staphylococcus aureus	NZ	26 mm	27 mm	36 mm

KEY:

NZ – No zone

mm – Milimetres

Table 3.4: Antimicrobial activity of balm formulation and control

CONCENTRATION **MICROORGANISM**	**100 mg/ml**	**200 mg/ml**	**400 mg/ml**	**Control Balm**	**Ciprofloxacin 4 mg/ml**
Pseudomonas aeruginosa	NZ	10 mm	12 mm	NZ	32 mm
Escherichia coli	NZ	NZ	19 mm	NZ	30 mm
Klebsiella pnuemoniae	NZ	18.5 mm	20 mm	NZ	34 mm
Bacillus subtilis	NZ	08 mm	9.5 mm	NZ	30 mm
Staphylococcus aureus	NZ	16 mm	18 mm	NZ	36 mm

KEY:

NZ – No zone

mm – Milimetre

CHAPTER FOUR

4.0 DISCUSSION

The percentage yield after extracting with ethanol was 10.56 % with a total weight of 52.8 g of the ethanol extract. The weight obtained was sufficient for the whole procedure.

From the observed results of the phytochemistry screening, the ethanol extract was found to contain all the phytochemical constituents it was tested for viz alkaloids, carbohydrates, reducing sugars, saponins, deoxysugars, tannins, steroidal saponins, phenol, flavonoids, triterpenoids and proteins. This result was observed because ethanol is a medium-polarity, protic solvent capable of dissolving a vast range of compounds. The vast polarity of ethanol allows it to extract non-polar and polar constituents such as phenol, flavonoids, tannins, carbohydrates and proteins as well as moderately non-polar constituents like triterpenoids and steroidal saponins. This could be attributed to its hydrogen bonding effects and its ability to penetrate plant cells (Bitwell *et al.*, 2023; Arya *et al.,* 2025; Tourabi *et al.*, 2025).

Ethanol's intermediate polarity index (about 5.2) and dielectric constant around 24–25, enables it to extract hydrophilic compounds e.g., sugars, phenolic glycosides and also amphiphilic or slightly lipophilic compounds e.g., terpenoids, saponins (Bitwell *et al.*, 2023; Arya *et al.,* 2025; Tourabi *et al.*, 2025).

The balm had a relative dark green colour with a characteristic smell of the ethanol extract. It had a creamy texture was observed to melt when warmed due to the volatile nature of the excipients incorporated into the extract.

The pH values observed from the results implied that the extract had a higher pH than the different balm formulations. Hence, the excipients/incorporation of the ethanol extract into the balm caused a slight decrease in pH of the balm.

4.1 DISCUSSIONS FOR THE ETHANOL EXTRACT MICROBIAL ASSAY

The antimicrobial assay showed that *Bacillus subtilis* had no inhibition at 100 mg/ml of the ethanol extract but became susceptible at 200 mg/ml and 400 mg/ml, where it recorded inhibition zones of 19 mm and 21 mm, although these remained lower than the 30 mm produced by ciprofloxacin. *Staphylococcus aureus* followed a similar pattern, showing no inhibition at 100 mg/ml but demonstrating strong activity at 200 mg/ml and 400 mg/ml, with zones of 26 mm and 27 mm, compared to 36 mm for the control. For *Escherichia coli*, inhibition was only seen at the higher concentrations, measuring 20 mm and 22 mm, while the control gave 30 mm. *Pseudomonas aeruginosa* was also resistant at 100 mg/ml but showed moderate to good susceptibility at 200 mg/ml and 400 mg/ml, with inhibition zones of 19 mm and 24 mm, compared to 32 mm for ciprofloxacin. *Klebsiella pneumoniae* showed no inhibition at 100 mg/ml and 200 mg/ml but demonstrated susceptibility at 400 mg/ml with a zone of 26 mm, which was lower than the 34 mm produced by the control drug.

The extract was most effective against *Staphylococcus aureus* at higher concentrations, while *Klebsiella pneumoniae* was the least responsive, requiring 400 mg/ml to show activity. These variations can be linked to differences in bacterial cell wall structure. Gram-positive bacteria such as *S. aureus* and *B. subtilis* have a thick, permeable peptidoglycan layer and lack an outer membrane, making them more accessible to the extract. In contrast, gram-negative bacteria like *E. coli*, *P. aeruginosa* and *K. pneumoniae* possess an outer membrane rich in lipopolysaccharides

that act as a barrier and contain selective porins, limiting the entry of active components (Panawala, 2017).

Although, the ethanol extract showed measurable antimicrobial activity, its effects were consistently lower than those of ciprofloxacin, this may be due to the highly purified, standardized and targeted nature of the established drug. This study only assessed three concentrations hence, further work is recommended to determine the concentration at which the extract produces its maximal antimicrobial effect.

4.2 DISCUSSIONS FOR THE BALM FORMULATION MICROBIAL ASSAY

From the results obtained in the antimicrobial assay of the ethanol extract balm formulation samples against the test organism, *Bacillus subtilis*, it was observed that the balm exhibited a zone of inhibition of 8 mm and 9.5 mm, for balm concentration 200 mg/ml and 400 mg/ml respectively indicating very mild antibacterial activity. The 100 mg/ml balm concentration exhibited no zone of inhibition just like the ethanol extract. The balm formulation, however, did not significantly demonstrate enhanced microbial activity when compared to the ethanol extract. These findings therefore suggest the presence of compounds within the ethanol extract that can inhibit the growth of this gram-positive bacterium, while balm formulation possesses reduced antibacterial properties compared to the ethanol extract. This may be due to the lack of a synergistic effect between the excipients of the balm formulation and the ethanol extract and a possible indication that the combination of the mechanisms of the excipient and the extract may have interfered with each other's activity.

Against *Staphylococcus aureus*, the ethanol extract balm formulation of 100 mg/ml showed no zone of inhibition while that of 200 mg/ml demonstrated a zone of inhibition of 16 mm and that

of 400 mg/ml 18 mm. These results indicate moderate antibacterial activity of the extract balm formulation at 200 mg/ml and 400 mg/ml. Compared to the results of the extract, the balm formulation also showed reduced antibacterial effects which could be as a result of interaction between the excipients resulting in reduced activity.

With the bacterium *Pseudomonas aeruginosa*, the balm demonstrated no zone of inhibition at 100 mg/ml. At concentrations of 200 mg/ml and 400 mg/ml exhibited zones of 10 mm and 12 mm respectively indicating reduced activity compared to ethanol extract.

The antibacterial activity of the balm against the organism *Escherichia coli*, interestingly demonstrated no activity at 100 mg/ml and 200 mg/ml, while that of 400 mg/ml, exhibited zone of inhibition of 19 mm.

That of *Klebsiella pnuemoniae,* surprisingly showed no activity only at 100 mg/mL, but showed zones of inhibition at 200 mg/ml and 400 mg/ml of 18.5 mm and 20 mm respectively. Compared to the activity of the extract which exhibited a larger zone of inhibition only at 400 mg/ml, the balm showed activity at lower concentration. This suggests that the balm formulation may have increased the extract's solubility, delivery or permeability leading to enhanced antibacterial effects. Also, the balm might contain other ingredients that have similar effects thus exerting synergistic effects.

From the results of the control balm (the balm formulated without the extract), no zone of inhibition was observed at any of the three concentrations for any of the test organisms.

Notably, from the overall results of the balm assay, combining the extract with other excipients did not significantly potentiate activity in comparison to the results of just the ethanol extract. This may be due to the competitive binding of the fatty acids from the excipients on the binding

sites of the active ingredients in the formulation. Some of the excipients may have interacted with the active ingredient preventing it from interacting with the microbes.

Also, the excipients could alter the pH of the formulation reducing the activity of the extract in the balm formulation. Hence, the ethanol extract would be more effective in treating skin conditions and infections such as cellulitis, boils, folliculitis etc. Although the balm has more clinical applications there is a need to optimize the formulation parameters to maximize the release of active compounds in the final product.

4.3 PHYTOCHEMICAL CONSTITUENTS OF THE EXTRACT

The antibacterial activity of the ethanol extract and balm formulation can be attributed to these compounds present in the individual constituents of the ethanol extract and balm.

Fatty acids present in *Persea americana* leaves, beeswax, olive oil and other constituents exhibit antimicrobial activity, particularly against bacteria. Mechanisms of its antimicrobial action include membrane disruption, pH disruption and protein denaturation (Obukhova and Murzina, 2024).

Fatty acid-derived esters also show notable antimicrobial activity against specific bacteria and fungi. They can inhibit key enzymes within microbial cells or disrupt the fluidity and permeability of microbial cell membranes. Huang *et al.,* (2010) reported that selected n-6, n-7, and n-9 fatty acids and their esters demonstrated strong antimicrobial effects against oral microorganisms.The esters Hexadecanoic acid, methyl ester etc.

Terpenes found in *Persea americana* leaves can exhibit antimicrobial effects through mechanisms such as disrupting microbial membranes, denaturing proteins, and inhibiting enzymes. One example is caryophyllene oxide, a terpene present in the ethanol extract of *P.*

americana (Guimarães *et al*., 2019). In their study, Guimarães *et al.*, (2019) evaluated the antibacterial activity of 33 free terpenes commonly found in essential oils, and 16 of these compounds showed antimicrobial activity during the initial screening.

Vitamin E, although not directly antimicrobial, it can support antimicrobial defense indirectly through its antioxidant properties by neutralizing free radicals and protecting cells from oxidative damage caused by infections (Divyadharsini *et al*., 2023). In a study conducted by Divyadharsini *et al*., (2023), vitamin E was tested against Staphylococcus aureus, Staphylococcus mutans, Enterococcus faecalis, and Candida albicans, and the results demonstrated significant antimicrobial activity.

Alcohols are widely recognized for their effectiveness against a broad spectrum of microorganisms, including both bacteria and fungi (ChlorhexidineFacts.com). They have the ability to denature proteins within microbial cells, as well as disrupt the lipid layer of microbial cell membrane, ultimately leading to cell lysis. Though alcohols are not active against spores, they are very 47 bactericidal against vegetative forms of bacteria (Rio-Carbajo and Vidal-Cortes, 2019).

Previous studies have examined the antimicrobial activity of oleanolic acid, a triterpenoid, against *Streptococcus* strains including *S. mitis*, *S. mutans*, *S. salivarius*, and *S. sanguis*. The hydroxy and carboxy groups in triterpenes were identified as key contributors to the antimicrobial effects of oleanolic acid (Cunha et al., 2010). Additionally, research has shown that the tested pentacyclic triterpenoids can influence peptidoglycan structure, alter gene expression, and inhibit biofilm formation (Huang *et al*., 2015; Park *et al*., 2015).

Phenols are multi-target agents and they exert their antibacterial properties by disruption of cell membranes and increased permeability, protein or enzyme inactivation and metabolic interference, generation of oxidative stress and metal chelation, anti-biofilm and anti-quorum sensing activity. Gallic acid is a prominent compound present in phenol compounds. It is a 3,4,5-trihydroxybenzoic acid found as a free or as esters in plants. Gallic acid exhibits bacteriostatic and bactericidal activity via rapid killing in some strains, inhibition of biofilm formation and synergy with antibiotics. It has been found to inhibit the growth several microbes such as *Streptococcus pyogenes, S. mutans, Staphylococcus aureus, Bacillus subtilis, Escherichia coli* etc.

Menthol alongside menthone are key monoterpenes in peppermint oil that display antibacterial (and anti-biofilm or quorum sensing) effects, primarily through membrane disruption**,** alteration of lipid structure**,** and interference with virulence/biofilm formation. In the balm formulation these compounds deliver antibacterial benefit for topical applications, provided formulation and safety factors (concentration, vehicle, stability, skin compatibility) are well managed and their mechanism is complementary to other antimicrobials and makes them valuable for skin or topical applications.

Previous studies, showed the GC-MS analysis of the ethanol extract of *P. americana* leaves contained major compounds viz phytols, 13-Octadecenal, hexadecenoic acid, methyl esters, linoleic acid ethyl ester, caryophyllene, germacrene D, 2,4-di-tert-butylphenol, α-Tocopherol to mention a few.

Caryophyllene is a tricyclic sesquiterpene, a terpene with 15 carbons, derived from three isoprene units. It has a bicyclic structure - a nine-membered ring fused to a cyclo-butane ring

containing one double bond in the cyclo-butane ring and another in the nine-membered ring. Caryophyllene is a non-polar and hydrophobic hydrocarbon. Due to its structure and bulky ring system, there may be competition for space in the excipient used in formulating the balm (beeswax). These bulky features it possesses due to fused rings in its structure may cause stearic hinderance and also interact with the hydrophobic portions of beeswax through van der Waals forces. This explains why the balm may have altered or reduced antibacterial activity compared to the ethanol extract.

Hexadecanoic acid also known as palmitic acid was another compound observed in the GC-MS analysis of the ethanol extract possessing antibacterial activity. It is a saturated fatty acid ($C_{16}H_{32}O_2$) and a straight chain hydrocarbon with a terminal carboxylic acid group. Its long hydrophobic chain gives lipophilicity, enabling interactions with waxes and oils (excipients in the balm). The long chain can interact with lipid matrices, potentially competing with other compounds for space in hydrophobic environments especially since they may have similar structures.

2,4-di-tert-butylphenol ($C_{14}H_{22}O$), another compound detected in the GC-MS analysis, can be described as a substituted phenol. It possesses two bulky tert-butyl groups at positions 2 and 4. These tert-butyl groups provide stearic hinderance preventing the phenolic hydroxyl group from oxidation. Bulky groups limit access to the ring, making it less reactive toward electrophilic aromatic substitution, steric bulk can also interfere with binding of other compounds in hydrophobic matrices like waxes, affecting molecular packing. This leads reduced reaction rate or prevention of the reaction altogether. Stearic hinderance cause bulky groups (large atoms or group of atoms attached to a molecule's core) to block or hinder reacting species from getting

close enough to interact with each other thus causing a steric barrier. This causes competitive binding between this compound and that of beeswax and other excipients of the balm, thus causing reduced antibacterial effects and antagonized effects on the ethanol extract.

Phytol is an acyclic diterpene alcohol long hydrocarbon chain with one terminal hydroxyl group (-OH)**.** It is a hydrophobic molecule with the hydroxyl group conferring slight polarity at one end. The bulky chain and flexible structure of phytol, allows it to interact with lipid membranes or waxes**.** Thus, allowing hydrophobic interactions occur and compete for space with other lipophilic molecules, particularly in lipid-rich matrices.

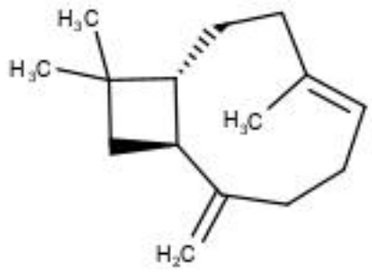


FIGURE 4.1: Caryophyllene

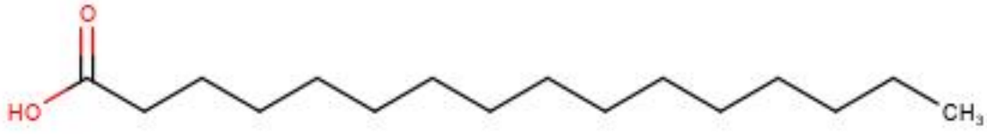


FIGURE 4.2: Structure of Hexandecanoic acid

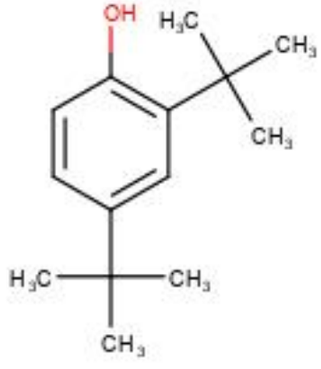


FIGURE 4.3: Structure of 2,4-di-tert-butylphenol.

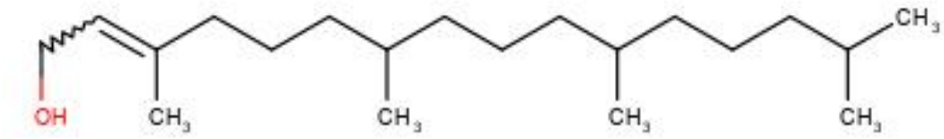


FIGURE 4.4: Structure of phytol

4.4 LIMITATIONS OF STUDY

Some limitations to this study must be acknowledged. Ethanol was used as the sole extraction solvent, which, while effective in extracting a wide range of phytoconstituents, may not provide a complete chemical profile of the plant material. Future research should compare extracts using solvents of different polarities to achieve a more comprehensible understanding of the phytochemical composition and antimicrobial potential.

Also, the antimicrobial evaluation in this study was limited to in vitro assays. Subsequent studies could involve testing the balm on suitable in vivo models such as animal or human skin with appropriate ethical approval to assess the antimicrobial performance and local skin tolerance.

Detailed physicochemical characterization of the balm (spreadability, viscosity and stability), should be performed. Additionally, parameters such as microbial load reduction at treated sites, irritation potential and formulation stability under recommended conditions should be closely monitored.

Finally, the potential interactions between the extract and excipient within the balm formulation warrant further investigation. Studies focusing on release kinetics and long-term stability would provide valuable insights into the formulation’s performance and consistency over time.

CHAPTER FIVE

5.0 CONCLUSION

The ethanol extract of *Persea americana* leaves contains biochemically active compounds such as tannins, flavonoids, saponins, phenolic compounds, and steroidal saponins, which contribute to its antibacterial properties. The ethanol extract delivers potent, dose dependent antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*, particularly at higher concentrations (19 – 27 mm).

The formulated balm containing the extract also exhibited moderate antibacterial activity (8 – 20 mm), its lower zone of inhibition highlights the fact that excipients can modulate the extract's antimicrobial efficacy.

Hence, formulators must be mindful of the excipients used as they can significantly influence the final antimicrobial effects.

5.2 RECOMMENDATIONS

1. Future research should explore different extraction solvents to obtain a wider range of phytochemicals and possibly enhance the antimicrobial activity of the extract.
2. The balm formulation should be further refined by improving its stability, release profile, and interactions between the extract and excipients to enhance its overall antimicrobial performance.
3. In vivo studies using suitable animal or human skin models are recommended to evaluate the balm's real-life effectiveness, safety, and potential to cause irritation.

4. Additional antimicrobial testing, including minimum inhibitory concentration or minimum bactericidal concentration determinations and evaluation against a broader range of bacterial and fungal strains, should be carried out to strengthen the scientific reliability of the findings.

REFERENCES

Abraham, J.D., Abraham, J. and Takrama, J.F., 2018. Morphological characteristics of avocado *(Persea americana Mill.) in Ghana*. African Journal of Plant Science, 12(4), pp.88–97.

Adewole, S.O. and Ayodele, O.A. (2023) 'Antioxidant, antimicrobial and other pharmaceutical potentials of palmitic acid (n- hexadecanoic acid) isolated from algal sources', South Asian Research Journal of Pharmaceutical Sciences, 14, pp. 125- 127.

Ahmed, S. K. (2024) Antimicrobial resistance: Impacts, challenges, and future directions. Trends in microbiology. Advance online publication.

Ambrose, A. and Simmons, D. (2019) 'Polypharmacological properties and therapeutic potential of β- caryophyllene', Journal of Medicinal Food, 22(12), pp. 1213- 1223.

Angelini, P., 2024. Plant-derived antimicrobials and their crucial role in combating antimicrobial resistance. Antibiotics, 13(8), p.746.

Arackal J.J., and Dr. Parameshwari S. (2017). Health benefits and uses of avocado. World Journal of Pharmaceutical Research, Volume 6, Issue 17, 392-399.

Arredondo-Ochoa, T., García-Almendárez, B.E., Amaro-Reyes, A., Rivera-Pastrana, D.M., Gutiérrez-López, G.F., Martín-Belloso, O. and Regalado-González, C., 2017. Design and characterization of corn starch edible films including beeswax and natural antimicrobials. Food Bioprocess Technology, 10(1), pp.103–114.

Arslan, I., and Buyukgebiz, O. (2021). Phytochemical analysis of avocado leaves. Journal of Food Biochemistry, *45*(6), e13741.

Arya, P., Kumari, S., Danquah, J., Mensah, E., Liu, Y. and Zhang, Q. (2025). Effects of solvent polarity on phytochemical extraction in medicinal plants. Scientific Reports, 15(4), pp. 1–12.

Ashraf, A., and Rathod, V. K. (2016). Extraction of bioactive compounds from plant materials. Ultrasonics Sonochemistry*, 29*, 312–321.

Balkrishna A., Sharma N., Srivastava D., Kukreti A., Srivastava S. and Arya V., 2024. Exploring the Safety, Efficacy, and Bioactivity of Herbal Medicines: Bridging Traditional Wisdom and Modern Science in Healthcare. Future Integrative Medicine, 3(1): 35-49.

Bitwell, C., O'Connor, J., Mensink, P. and Harris, L. (2023). A review of modern and conventional extraction techniques and their influence on phytochemical recovery. Trends in Food Science and Technology, 138, pp. 240–259.

Blair, J. M. A., Webber, M. A., Baylay, A. J., Ogbolu, D. O. and Piddock, L. J. V. (2015) Molecular mechanisms of antibiotic resistance. *Nature Reviews Microbiology*, 13(1), pp. 42–51.

Boadi N.O., Saah S.A., Mensah J.K., Badu M., Addai-Arhinand S. and Mensah M.B. (2015). Phytoconstituents, antimicrobial and antioxidant properties of the leaves of *Persea americana* Mill cultivated in Ghana. Journal of Medicinal Plants Research.

Bogdanov S., 2016. Beeswax: Production, properties, composition and control. In: Beeswax Book. Bee Product Science.

Bouarab-Chibane, L., Forquet, V., Godreuil, S., Bolla, J.M., Djediat, C., Soula, G., Ouazzani, J., 2019. Antibacterial Properties of Polyphenols: Characterization and QSAR (structure–activity relationship) studies. Frontiers in Microbiology, 10, 829.

Brodschneider, R. and Crailsheim, K., 2019. Nutrition and health in honey bees. *Apidologie*, 41(3), pp.278–294.

Centre for Invasive Species and Ecosystem Health (2018), https://www.invasive.org/browse/subinfo.cfm?sub=14162

ChlorhexidineFacts.com, Other Antimicrobials: Alcohol, https://www.chlorhexidinefacts.com/alcohol.html.

Cunha, W.R., de Matos, G.X., Souza, M.G.M., Tozatti, M.G., Andrade e Silva, M.L., Martins, C.H.G., da Silva, R. and da Silva Filho, A.A., 2010. Evaluation of the antibacterial activity of the methylene chloride extract of *Miconia ligustroides*, isolated triterpene acids, and ursolic acid derivatives. Pharmaceutical Biology, 48(2), pp.166–169.

Dey, A., and De, J. N. (2015). Antimicrobial properties of plant terpenoids. Phytotherapy Research, 29(6), 914–927.

Divyadharsini V, TN Uma Maheswari, Rajeshkumar S (2023), Assessment of Antimicrobial Activity of Lycopene, Vitamin E, and Lycopene-Vitamin E combination against

Staphylococcus aureus, Streptococcus mutans, Enterococcus faecalis, and *Candida albicans*: An In Vitro Study, Cureus, 15(7):e42419.

Dumitru, C.D., Neacşu, I.A., Grumezescu, A.M. and Andronescu, E., 2022. Bee-derived products: Chemical composition and applications in skin tissue engineering. Pharmaceutics, 14(4), p.750. doi:10.3390/pharmaceutics14040750.

Farjana, A., and Shahriar, M. (2019). Topical herbal formulations: Preparation and evaluation. International Journal of Pharmacy and Pharmaceutical Sciences, 11*, 45–52.*

Flemming, H. C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A. and Kjelleberg, S. (2016) Biofilms: The functional form of bacterial life. Nature Reviews Microbiology, 14(9), pp. 563–575.

Fratini F, Cilia G, Turchi B, Felicioli A: Beeswax: A minireview of its antimicrobial activity and its application in medicine. Pac Journal of Tropical Medicine. 2016 Sep;9(9):839-43. Epub 2016 Jul 26.

Gupta S.K., Singhal P., Singh A., Chauhan A., Kumar B. (2018). Nutritional and Pharmaceutical Benefits of Avocado Plants. Journal of Advanced Scientific Research, 9 (2): 04-11.

Hoang, N.M.H. (2024) 'Applications of tert- butyl- phenolic antioxidants in environmental and health contexts', Toxics, 12(12), 869.

Huang Chifu B, Brian George, and Jeffery L Ebersole (2010), Antimicrobial activity of n-6, n-7, and n 9 fatty acids and their esters for oral microorganisms, Archives of Oral Biology, 55(8):555 560.

Huang Lirong, Heng Luo, Qiji Li, Daoping Wang, Jianxin Zhang, Xiaojiang Hao, & Xiaosheng Yang (2015), Pentacyclic triterpene derivatives possessing polyhydroxyl ring A inhibit gram positive bacteria growth by regulating metabolism and virulence genes expression, European Journal of Medicinal Chemistry, 95:64-75.

Hutchings, M. I., Truman, A. W. and Wilkinson, B. (2019) Antibiotics past, present and future. Current Opinion in Microbiology, 51, pp. 72–80.

Ilyasov R.A., Kutuzov A.E., Lee J.H., Nikolenko A.G., (2020). Phylogeny and classification of the honey bee subspecies: Integration of molecular and morphological data. Entomological Review. 100(3):293–307.

Islam, M.T., Ullah, M.A., Hakim, A., Parvez, M., Sturman, D. and Ashraf, M.A. (2018) 'Phytol: a review of biomedical activities', Life Sciences, 196, pp. 140- 149.

Jesus, D., Oliveira, F. E., Higa, K. C., Junqueira, J. C., Jorge, O. C., Back-Brito, G. N. and Oliveira, L. D. (2015) *Persea americana* glycolic extract: In vitro study of antimicrobial activity against *Candida albicans* biofilm and cytotoxicity evaluation. The Scientific World Journal, 2015, Article ID 531972.

Kamagate, M., Kouame, N.M., Koffi, E., Kadja, A.B., Camille, K., Yao, N.A.R., Balayssac, E., Daubrey-Potey, T., N'zoué, K.S. and Die-Kacou, H.M., 2016. Acute toxicity and hypoglycaemic activity of the leaf extracts of *Persea americana* Mill. (Lauraceae) in Wistar rats. African Journal of Pharmacy and Pharmacology, 10(33), pp.690–698.

Kendir G. and Koroglu A., (2018), Evaluation of Avocado (*Persea americana* Mill.) leaves in terms of public health, Marmara Pharmaceutical Journal, 22(3):347-356.

Keyvani-Ghamsar, S., 2023. An update on the potential mechanism of gallic acid as an antibacterial and anticancer agent. Food Science & Nutrition. (Review; full text available via PubMed Central).

Khameneh, B., Iranshahy, M., Soheili, V. and Fazly Bazzaz, B.S., 2019**.** Review on plant antimicrobials: a mechanistic viewpoint. Antimicrobial Resistance & Infection Control, 8(1), p.118.

Kızılyıldırım, S., Kandemir, T., Kendir, G., Muhammed, M.T., Köroğlu, A. and Özoğul, F., 2024. Antibacterial activity of avocado extract *(Persea americana Mill*.) against aminoglycoside-resistant *Klebsiella pneumoniae* strains. Food Bioscience, 60, p.104523.

Lanka Panawala, 2017. Differences between gram-positive and gram-negative bacteria. Microbiology Blogs.

Ledjanac, S., Hoxha, F., Jasnić, N., Tasić, A., Jovanović, M., Blagojević, S., Plavša, N. and Tosti, T.B., 2024**.** The influence of the chemical composition of beeswax foundation sheets on their acceptability by the bee colony. Molecules, 29(23), 5489.

Mahmoud A.H., Samy M.N., Wanas A.S., Kamel M.S., (2021). Gas chromatography-mass spectromrtry profiling and analgesic, anti-inflammatory, antipyretic, and antihyperglycemic potentials of *Persea americana* Mill. (Lauraceae). Biochemistry Research International. (2):1-10.

Makopa M, Mangiza B, Banda B, Mozirandi W, Mombeshora M, & Mukanganyama S (2020), Antibacterial, Antifungal, and Antidiabetic Effects of Leaf Extracts from *Persea americana* Mill. (Lauraceae), Biochemistry Research International, 1-10.

Mouanga-Ndzime, Y., Bisseye, C., Longo-Pendy, N. M., Bignoumba, M., Dikoumba, A. C., Onanga, R. *et al.* (2024) Trends in *Escherichia coli* and *Klebsiella pneumoniae* urinary tract infections and antibiotic resistance over a five-year period. *Antibiotics*.

Munita, J. M. and Arias, C. A. (2016) Mechanisms of antibiotic resistance. Microbiology Spectrum, 4(2).

Nasri, N., Satria, D., Kaban, V. E., Tania, C. G., Rani, Z. (2024). Antibacterial Potential of Ethanolic Extract of Avocado Leaves *(Persea americana Mill.)* against Clinical Isolate of *Klebsiella pneumoniae* and *Proteus mirabilis*. Trends in Sciences, 21 (7), 7821.

Naylor, N.R., *et al*., 2022**.** Economic costs of antimicrobial resistance: a global analysis. *The Lancet*, 399(10327), pp.629–640.

Nong, Y., 2023**.** A review of the use of beeswax in skincare. Journal of Cosmetic Dermatology, 22(8), pp.2166–2173.

O'Neill, J. (2016) Tackling drug-resistant infections globally*:* Final report and recommendations. Review on Antimicrobial Resistance.

Oboh G., Odubanjo., V.O, Bello F., Ademosun A.O, Oyelele S.I, Nwanna E.E., Ademiluyi A.O. (2015). Aqueous extracts of avocado pear (*Persea americana*) leaves and seeds exhibit anticholinesterase and antioxidant activities in vitro. J Basic Clin Physiol Pharmacol.

Oboh, G., and Rocha, J. (2021). Bioactive constituents of avocado leaf extract. BMC Complementary Medicine, 21*, 65.*

Obukhova, E.S. and Murzina, S.A., 2024**.** Mechanisms of the antimicrobial action of fatty acids: A review. Applied Biochemistry and Microbiology, 60, pp.1035–1043.

Otaigbe, I. I. (2023) Drivers of inappropriate antibiotic use in low- and middle-income countries. JAC-Antimicrobial Resistance, 5(3), dlad062.

Pancu, D. F., Scurtu, A., Macasoi, I. G., Marti, D., Mioc, M., Soica, C., Coricovac, D., Horhat, D., Poenaru, M. and Dehelean, C. (2021) Antibiotics: Conventional therapy and natural compounds with antibacterial activity. Antibiotics, 10(4), 401.

Park Soon-Nang, Sug-Joon Ahn, and Joong-Ki Kook (2015), Oleanolic acid and ursolic acid inhibit peptidoglycan biosynthesis in *Streptococcus mutans* UA159, Brazilian Journal of Microbiology, 46:613-617.

Ramdhan B and Yusuf A.L., (2023), Formulation and Evaluation of Avocado Leaf Extract (*Persea americana* Mill.) Cream Based on Various Stearic Acid Concentrations, Ad-Dawaa Journal of Pharmacy, 1(2):78-86.

Rio-Carbajo L. Del and P. Vidal-Cortes (2019), Types of antiseptics, presentations, and rules of use, Medicina Intensiva (Engl Ed.), 43(1):7-12.

Selvia A. and Rahmawawaty S. (2023). The Effect of Giving Avocado (*Persea americana)* Leaf Decoction on Blood Pressure in Patients with Hypertension: A Review Article.

Singh, P., and Yadav, R. (2020). MIC and MBC determination methods. Journal of Analytical Science and Technology, 11*, 33.*

Torrelles, J. B. (2024) Global trends in antimicrobial resistance: Clinical and public health implications. Clinical Microbiology Reviews, 37(1), pp. 1–29.

Tourabi, M., El Idrissi, M., Benali, T., Choukri, M., Fikri-Benbrahim, K. and Elachouri, M. (2025). Optimization of extraction processes and solvent polarity to maximize phytochemical recovery. Journal of Applied Natural Products, 11(2), pp. 55–72.

Ventola, C. L. (2015) The antibiotic resistance crisis: Causes and threats. *P and T*, 40(4), pp. 277–283.

Wijaya, I., 2020. The potential of avocado leaves (*Persea americana* Mill.) as antibacterial. Jurnal Ilmiah Kesehatan Sandi Husada, 12(2), pp.695–701.

World Health Organization (2025) Global antibiotic resistance surveillance report 2025. Geneva: World Health Organization.

Yasir M, Das S, and Kharya MD (2010), The phytochemical and pharmacological profile of *Persea americana* Mill, Pharmacognosy Reviews, 4(7):77-84.

Yusuf F., Ulla R., and Simbolon M. (2023), Antibacterial Test of Avocado Leaf Ethanol Extract (*Persea americana* Mill.) on *Staphylococcus aureus* bacteria, International Journal of Health, Engineering and Technology, 2(4):132-137.

Zhao, F., Wang, P., Lucardi, R.D., Su, Z. and Li, S. (2020) ‘Natural sources and bioactivities of 2,4- Di- tert- butylphenol and its analogs’, Toxins, 12(1), 63.