ANTIMICROBIAL ACTIVITIES OF THE METHANOL LEAF EXTRACT OF *Acalypha wilkesiana* MUELL.ARG (EUPHORBIACEAE) FORMULATED AS A TOOTH PASTE

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CERTIFICATION

We the undersigned do hereby bear witness to the certification of this project work entitled "Antimicrobial activities of the methanol leaf extract of *Acalypha wilkesiana M*uell Arg (Euphorbiaceae) formulated as a toothpaste" as research project work carried out by Tsetimi Omatsola Jackson and has been approved in partial fulfillment of the requirement for the award of the Doctor of Pharmacy (Pharm D) degree, Faculty of Pharmacy, University of Benin, Benin city.

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DEDICATION

This project work is dedicated to my maker, the Almighty God, who gave me the grace and strength I needed throughout the course of this pursuit and to my lovely parents Mr. and Mrs. Tsetimi for their all-round support.

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I want to express my profound gratitude and I also ascribe all glory and honor to God almighty for keeping me alive and sustaining me till the end of the program.

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ABSTRACT

Background: Oral hygiene is an important part of the body overall well-being, and should be treated with the utmost care to prevent dental problems. *Acalypha wilkesiana* Muell. Arg. (Euphorbiaceae), commonly known as copper leaf, is a tropical plant native to West Africa, with notable antimicrobial activities. This study was carried out to investigate the antimicrobial properties of the methanol leaf extract of Acalypha wilkesiana formulated as herbal toothpaste.

Method: Toothpaste was formulated using the leaf extract of *A. wilkesiana*. The extract was incorporated into a toothpaste base prepared using calcium carbonate, starch, glycerin, sodium lauryl sulfate, saccharine and peppermint oil. Sensory and physicochemical properties of the toothpaste were evaluated. Antimicrobial evaluation was by the Agar well diffusion method against *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, Klebsiella aerogenes, Candida albicans* and *Aspergillus niger* at concentrations of 100-500 mg/ml.

Results: The *A. wilkesiana* formulated toothpaste had a pleasant smell and was sweet to taste. It had good foaming abilities with a pH range of 7.4-7.8. The formulated herbal toothpaste had poor antibacterial activity but no antifungal activity against the clinical isolates at low concentrations. Significant activities were recorded at 500 mg/ml against all five (5) bacteria isolates, with *Bacillus subtilis* recording the highest zone of inhibition.

Conclusion: The formulated *A. wilkesiana* toothpaste showed significant antibacterial effects against microbes implicated in periodontal diseases and dental caries, hence serving as a potential alternative to orthodox toothpastes for maintaining oral hygiene.

Keywords: *Acalypha wilkesiana*, Euphorbiaceae, Antimicrobial, Toothpaste

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Herbs are defined as any plants that lack the woody tissue which are a characteristic of shrubs or trees (Al-Maweri *et al.*, 2020). Alot of herbs have shown beneficial effects as anti-inflammatory, antibacterial, antifungal, antiviral and analgesic agent. Herbs have had a favourable role in the dental field, such as with reducing oral microbial load and also in oral healthcare overall.

Dental plaque are biofilms that form on the surfaces of teeth and is composed of microorganisms, food particles, and molecules from saliva (Hihara *et al.*, 2021). Biofilm develops gradually and if not removed mechanically through actions such as tooth brushing or flossing, can lead to various dental health issues, which includes dental caries, gingivitis, and even periodontitis (Mazzoleni *et al.*, 2024).

Microorganisms living in the mouth have significance in altering the balance between health and sickness, and they include several hundred to thousands of diverse species. The mouth provides home to alot of bacteria, protozoa, fungi, and viruses, with each having its own distinct characteristic traits but they usually live in symbiotic relationship with the host (Xiao *et al.*, 2023).

Toothpastes are essential to everyday oral hygiene, and play a key role to maintaining excellent oral health (Braedan *et al.*, 2022). There are two main purposes of toothpastes, which includes: to function as an adjunctive antimicrobial agent in the removal of pathogenic dental biofilm, afterwards providing a protective barrier to the teeth, with this they provide increase resistance to future dental diseases (walsh *et al.*, 2019).

Acalypha wilkesiana also known as copperleaf and Jacob's coat is an erect or spreading evergreen shrub in the family of Euphorbiaceae (Sherifat *et al.,* 2021). The plant can grow up to 2 - 4 metres tall and can occasionally reach 6 metres. The genus Acalypha has about 450 - 462 species (Oso, 2020). *A. wilkesiana* is valued for its wide range of patterned varieties and is often grown as a fence line (Oso, 2020).

1.2 statement of the problem

Bacterial biofilms comprise of a highly structured communities of microorganisms encased in a self-produced extracellular polymeric substance (EPS) matrix. This matrix is primarily composed of polysaccharides, proteins, lipids, and extracellular DNA, which protect bacteria from antimicrobial agents and host immune defenses while strengthening the structure of the biofilm (Zhao *et al.*, 2023). Dental plaque present as a sticky film that coats teeth and contains microorganisms which includes bacteria. If plaque is not removed on a regular basis, it will harden and turn into tartar (calculus) (Chow, 2020). It is important in dental care to maintain optimal oral health and prevent disorders, this is promoted by various oral hygiene tools designed for thorough cleaning and plaque removal (Palanisamy, 2024).

1.3 Justification of study

Dental decay if left unaddressed may result in oral health problems, which includes infection and tooth loss with irreversible consequence (Lima and Buarque, 2019). Advanced dental decay may also lead to a decline in chewing efficiency and speech clarity through several mechanisms. This would result in malnutrition and altered mental well being of the affected individuals (Aly *et al.*, 2024). Therefore, there is a need to reduce the effect of cariogenic microorganisms in order to prevent certain diseases associated with dental decay (Chen *et al.*, 2020).

Several people are more sensitive to the chemicals found in regular toothpaste. Herbal toothpaste provides an alternative option for those seeking a more natural cleansing option (Bharwal *et al.*, 2024). Some of these toothpastes lack fluoride, which works to protect your enamel and strengthen the teeth. The aim of this study is to determine the antimicrobial activity of a herbal toothpaste formulated using the crude extract of the leaves of *Acalypha wilkesiana*, a known medicinal plant with antimicrobial effect.

1.4 Aims

The aim of this study is to determine the antimicrobial activity of a formulated herbal toothpaste containing the crude extract of the leaves of *Acalypha wilkesiana*.

1.5 Objectives

The objectives of this study include:

1. To provide a preliminary characterization of the leaves of *Acalypha wilkesiana.*
2. To investigate the phytochemical constituents of the leaves of *Acalypha wilkesiana*.

3. To formulate a herbal toothpaste using the crude methanol extract of the leaves of *Acalypha wilkesiana.*
4. To evaluate the antimicrobial activities of the leaves of *Acalypha wilkesiana* extract and the formulated herbal toothpaste.
5. To evaluate the physiochemical properties of the formulated herbal toothpaste.

LITERATURE REVIEW

1.6. Toothpaste

Toothpaste consists of a range of ingredients, one of which is fluoride that helps to protect the teeth against tooth decay and gum disease. Tooth paste is an important part of a daily dental care routine and the world dental federation recommends that people brush with it twice a day (Melo *et al.*, 2020). Toothpaste is not supposed to be swallowed due to the it's fluoride content, but is generally not very harmful if swallowed in small amounts, however, one should seek medical attention after swallowing an abnormally large amount due to it toxicity (Petrović *et al.*, 2023).

1.7. History of toothpaste

Egyptians are believed to have started using a paste to clean their teeth around 4000BC, before the invention of toothbrushes. Ancient Greeks and Romans are known to have used toothpastes, and the people of China first used toothpaste around 618 - 907AD (Kirtley and Kayla, 2022). Around 4,000 BC, ancient Egyptians first developed a dental cream which contained mainly of a mixture of Purnice powder and white vinegar, primarily with the aim to remove debris from teeth. The Romans were known to have added more abrasives to their dental powder mixture, for

example crushed bones and snail shells. Romans appear also to be the first to add flavors, most likely to help with bad breath and to make their paste more palatable. The flavorant added was myrrh (Kirtley and Kayla, 2022). Most common issues with ancient toothpastes were the high level of abrasive in it, poor taste and high cost, making it not affordable for the populace as toothpastes product are nowadays.

In the year 1960, the American dental association (ADA) recognizes fluoride additives as effective in fighting tooth decay. Earlier, in 1955, Crest a toothpaste brand introduced its fluoride formulation, which it claimed prevented the progression of tooth decay and increase remineralization of the tooth structure. This was later recognized by the American dental association (ADA) five years later. With the introduction of fluoride, toothpastes were now recommended by dentists to be used on a routine basis to prevent tooth decay (Kirtley and Kayla, 2022).

In recent times, manufacturers have gradually improved toothpaste formulations for better fluoride bioavailability, lower abrasivity, and better stain removal and breath freshening. Furthermore, toothpastes have become a multipurpose tool due to the incorporation of active ingredients in the hope to combat a variety of oral diseases and conditions and to provide cosmetic benefits. Worth mentioning here are anti-plaque agents which were largely introduced to control the formation of supragingival plaque and periodontal disease (Nachu *et al.*, 2022). It must be noted though that enzymes can still be found in some toothpastes nowadays. These enzymes include Lactoperpxidase and glucose oxidase, and are known for their antimicrobial activity. Supposedly, enzymes supports oral health by aiding in plaque removal (Paqué *et al.*, 2021).

The development of toothpastes, however, still has a long way to go. The biggest challenge yet to overcome is the generally short lived oral activity of the active agents and most importantly fluoride toxicity (Vieira, 2021).

1.8. TYPES OF TOOTHPASTE

1.8.1. Fluoride Toothpaste

Fluoride toothpaste is the most recommended type of toothpaste. Regular use of fluoride toothpaste provides effective protection against tooth decay, and ensures your teeth stay healthy and strong. It is suitable for all age groups, from young children to adults, and plays an important role in maintaining good oral health (Bracesbar, 2023).

1.8.2. Sensitive Teeth Toothpaste

This is for individuals that experience tooth sensitivity to hot, cold, sweet, or acidic foods. This type of toothpaste consists of ingredients that help desensitize the nerves in your teeth, thereby reducing sensitivity and discomfort. Regular use of sensitive teeth toothpaste can prevent tooth sensitivity issues and permit you to enjoy your favorite meals without pain or discomfort (Bracesbar, 2023).

1.8.3. Whitening Toothpaste

Whitening toothpaste removes the surface stains on teeth and brightens it. It consists of mild abrasives and chemical agents that carefully polishes and whiten the teeth over time. While whitening toothpaste can improve the appearance of your teeth, it is not as effective as

professional teeth whitening treatments for significant discoloration. It is important to use whitening toothpaste as directed and use sparingly to prevent enamel wearing (Bracesbar, 2023).

1.8.4. Gel Toothpaste

Gel toothpaste has a unique consistency, which is often more translucent than traditional paste. It comes in different flavors and is a favoured choice for those who prefer a gel-like texture and taste. Gel toothpaste offers same benefits as regular toothpaste, which includes cleaning the teeth and protecting against cavities. Some people find gel toothpaste more refreshing and enjoyable to use, which enhances their overall brushing experience (Bracesbar, 2023).

1.8.5. Natural Toothpaste

Natural toothpaste is formulated with nature-derived ingredients and is free from artificial additives like fluoride, preservatives, and artificial flavors. It is great for individuals who prefer a more environmentally friendly and free from synthetic chemicals for their oral care. While natural toothpaste may not consist of fluoride, it can still offer help removing plaque and maintaining oral hygiene. Some natural toothpaste options consist of alternative ingredients like baking soda, essential oils, and herbal extracts that contribute to oral health (Bracesbar, 2023).

1.8.6. Children's Toothpaste

Children's toothpastes are particularly formulated for young kids who are learning to brush their teeth. It usually comes in delicious flavors and has a lower fluoride content to prevent accidental ingestion. Children's toothpaste encourages good oral habits and makes brushing enjoyable for kids at an early age. The appealing flavors and kid-friendly design help create a beneficial link with oral care, which encourages children to brush regularly (Bracesbar, 2023).

1.9. Concept of oral hygiene

As with other regions of the body, the mouth is filled with bacteria, which are mostly harmless. However, without adequate oral hygiene, bacteria can attain levels that might lead to oral infections, such as tooth decay and gum disease (Gizaw *et al.*, 2024). The mouth is the point of entry to the digestive and respiratory tracts, and some of these bacteria can induce infection there. Recent studies have shown a correlation between oral hygiene and a risk of infection affecting the respiratory tract like pneumonia, chronic obstructive pulmonary disease (COPD) and lung cancer (Dong *et al.*, 2022). Good oral hygiene, such as daily brushing and flossing, can help with keeping bacteria under control. This will in turn prevent poor oral hygiene associated health problems (Gizaw *et al.*, 2024).

1.10. Herbal and natural toothpaste

Herbal toothpaste is a formulation of natural ingredients that provides long-term and full-scale oral care to gums and teeth. It consists of Herbal extract and essential oils that combat successfully against bacteria (Chiba, 2023). The herbal paste not only cleans teeth but also provides long-lasting freshness in breath. The use of herbal paste is a great option for everyone especially for those who are coping with toothaches, bleeding gums and bad breath. Various dental and gum disease are highly curable with the use of herbal toothpaste (Chiba, 2023). Herbal toothpaste is free from dyes or artificial flavorings. Herbal toothpaste is artificial colors and flavors free in its composition which can be a huge appeal (Bharwal *et al.*, 2025). Herbal toothpastes should consist of mint, neem, clove and many other herbs. Clove is abundant in calcium and vitamin C, and gives relief from oral pain. Basil leaves serves as a disinfectant and

eliminates the germs present in mouth. Mint, another special ingredient of the herbal-toothpastes not only regulates the bad breath problem but also eradicate the harmful bacteria. Mint oils are actually the most effective ingredient to kill germs in the mouth. These ingredients fights regularly with the bacteria and defends against tooth decay (Bharwal *et al.*, 2025). Additional benefits are fighting of mouth ulcers, stomach ulcers and whiten of teeth.

1.11. Oral microorganisms

The human oral cavity supports a rich and intricate microbial community, frequently called the oral microbiome (Rajasekaran *et al.*, 2024). The ecosystem of the oral cavity offers both hard and mucous attachment areas (i.e. tongue, lips, cheeks, palate, and teeth); it is regularly moistened by saliva and in certain areas by the gingival fluid thereby providing nutrients to the microorganisms inhabiting the mouth. The surface of the tooth encourages the development and maturation of a complex biofilm (Santacroce *et al.*, 2023). This dynamic ecosystem covers a diverse array of bacterial phyla, including *Actinobacteria, Bacteroidetes, Chlamydia, Euryarchaeota, Fusobacteria, Firmicutes, Proteobacteria, Spirochaetes, and Tenericutes*. Comprised of these microorganisms, the oral microbiome's Organization and processes are closely connected to various physiological processes. Disruptions in this fragile equilibrium can lead to oral dysbiosis, which can precipitate a spectrum of oral ailments like dental caries, gingivitis and periodontitis (Rajasekaran *et al.*, 2024).

Consisting of over 700 known bacterial species, the oral microbiome is the second widest array microbiota within the human body (Deo and Deshmukh, 2019). The mouth is a thriving landscape for hundreds of species of bacteria since saliva is mostly water and rich nutrients circulates through the mouth each day. Saliva performs a key function in the preservation of oral

health, impaired salivary flow (hyposalivation) that may occur as a result of xerogenic medication, illness and radiotherapy to the head and neck region can increase the risk to Oral infection (Faruque *et al.*, 2022).

Tooth decay, gingivitis and periodonitis are the most prevalent dental and oral infection, which occurs around different age group population and in different parts of the world. In the progression of these diseases, various organisms such as *Streptococcus mutans* have a profound effect (Fang *et al.*, 2024). This organism plays a crucial role in carbohydrates fermentation producing lactic acids which leads to the demineralization of the enamel. This bacterium results in the development of tooth decay and easy absorption of carbohydrates by the production of extracellular polysaccharides that is a base component of dental plaques (Bloch et al., 2024). Today, in toothpastes, a diverse array of chemical agents such as flouride, which are mainly antimicrobial agents have been combined to form a direct inhibitory effect on plaque formation (Walsh *et al*, 2019).

1.12. The family: Euphorbiaceae

Euphorbiaceae family exhibit great diversity with mostly monoecious herbs, shrubs, and trees and at times succulent and cactus-like members**.** The Euphorbiaceae family of plants include more than 228 plant genera accepted according to Plants of the World Online by Royal Botanic Garden Kew (Royal Botanic Gardens, Kew, 2023) and greater than 7000 species of plants according to the Global Biodiversity Information Facility (Global Biodiversity Information Facility, 2023). These plants can be annuals or perennials and normally produce some type of latex.

The leaves of these plants are oppositive (sometimes alternatives) and have stipules which may be modified into spines or glands. The flowers assemble in an inflorescence called cyathium, and at times they can be inconspicuous flowers that lack a corolla. Sometimes the cyathium has numerous masculine flowers and only one feminine flower. Their Androecium contain one or several stamens, and gynoecium with a small group of styles and carpels. The fruits are typically found in capsules, and they separate with ease from each other (Jiménez-González *et al.*, 2023).

1.12.1. The genus: *Acalypha*

Acalypha, featuring approximately 500 species, is the third most extensive genus in the family Euphorbiaceae, after *Euphorbia* and *Croton* (Montero-Muñoz *et al.*, 2020). Acalypha falls within the subfamily Acalyphoideae, the most varied in the Euphorbiaceae (Montero-Muñoz *et al.*, 2020).

Acalypha is a large, monophyletic genus distribution which grows primarily in tropical and subtropical regions but stretches into temperate areas in eastern Asia and eastern North America, and some weedy species have taken root in Europe (Roy et al., 2020). Their physical structure comprises of annual and perennial herbs, sub shrubs, shrubs and small trees (Levin *et al.*, 2022).

The flowers, like those of all Euphorbiaceae, are consistently unisexual, with plants being monoecious or rarely dioecious, but inflorescence position and sexuality are both display significant variability. Three species are commonly cultivated as ornamentals, this includes; *Acalypha herzogiana*, *Acalypha hispida*, and *Acalypha wilkesiana* (Levin *et al.*, 2022).

Examples of species of *Acalypha* found in some parts of the world includes:

Acalypha abingdonii Seberg

Acalypha acapulcensis Fernald

Acalypha accedens Müll.Arg.

Acalypha acmophylla Hemsl.

Acalypha acrogyna Pax

Acalypha acuminata Benth.

Acalypha adenostachya Müll.Arg.

Acalypha aliena Brandegee (Cardiel et al., 2020).

1.12.2. The specie: *Acalypha wilkesiana*

Acalypha wilkesiana falls under the "spurge family" (Euphorbiaceae) of the genus *Acalypha*, and is often called copper leaf or fire dragon in the South Pacific islands. *Acalypha wilkesiana* have large leaves which are spotted with bronze, cream, yellow and red coloration (Kadiri and Ossai, 2023).

The leaves are elliptic to broad ovate having a roughly serrated margin, extending up to 20 cm long and 10 to 15 cm wide, the plant is highly regarded because of its wide range of multicolored varieties and is often cultivated for hedging. (Oso, 2020).

1.12.3. Plant taxonomy

Kingdom: Plantae

Phylum: Streptophyta

Class: Equisetopsida

Subclass: Magnoliidae

Order: Malpighiales

Family: Euphorbiaceae

Genus: *Acalypha*

Species: *Acalypha wilkesiana*

Botanical name: *Acalypha wilkesiana* Müll.Arg (Royal Botanic Gardens, Kew, 2021).

Figure 1: photograph of *Acalypha wilkesiana* leaves

1.12.4. Phytochemical constituents of *Acalypha wilkesiana*

The biochemical analysis for both the ethanolic and aqueous leaf extracts of *A. wilkesiana* (Ify *et al.*, 2021) identified the presence of some secondary metabolites comprising tannins, flavonoids, anthocyanins/betacyanins, alkaloids, glycosides, Phenols, acids and reducing sugars. Saponins and phlobatannins were identified to be seen only in the ethanolic extract. Glycosides and terpenoids were detected only in the aqueous extract.

Also, High performance liquid chromatography (HPLC) analysis was successful in identifying five classes of alkaloidal compounds, which includes; quinolinamine, benzenesulfonamide, allylamine, benzamide and indolizine (Odion *et al.*, 2024). High performance liquid chromatography (HPLC) analysis revealed that *Acalypha wilkesiana* leaf is high in flavonoids, carotenoids, and phytosterols, however, levels were low in simple terpenes (Omage and Azeke, 2019).

1.12.5. Reported biological activities of *Acalypha wilkesiana*

The biological activities of *Acalypha wilkesiana* are as follows:

Antibacterial and Antifungal Activities

Mansur *et al.* (2024) reported the assessment of the plant extracts of *Acalypha wilkesiana* which were assessed for their effectiveness to inhibit the growth of the following bacteria; *Staphylococcus aureus, Staphylococcus saprophyticus, Enterococcus faecalis, Escherichia coli,* and *Pseudomonas aeruginosa*. Agar diffusion was employed for the antibacterial activities, and

Mueller Hinton Agar (MHA) minimum inhibitory concentration (MIC) was evaluated with the agar dilution method. With a MIC of 2.5 mg/mL against *Pseudomonas aeruginosa* and *Enterococcus faecalis* and 5.0 mg/mL against *Staphylococcus aureus*. Hence, *Acalypha wilkesiana* leaf extract had a broad therapeutic spectrum antibacterial activity.

Sherifat *et al.* (2021) reported that the ethanolic extract of *Acalypha wilkesiana* showed the highest activity against *Trichophyton rubrum* and *Candida albicans*. There was also activity against *Malassezia furfur* which affirms suitability for Pityriasis vesicolor and seborrheic dermatitis. This shows the antifungal property of this plant

Anti-diabetic activity

Isirima and Uahomo. (2023) reported in their study which showed that the aqueous leaves extract of *Acalypha wilkesiana* exhibited blood sugar-lowering effects in a dose-dependent manner. Its antidiabetic activity is similar to that of glibenclamide and hence utilize a similar mode of action to cause hypoglycemia in alloxan-induced diabetic albino rats. Also, the aqueous leaves extract of *Acalypha wilkesiana* regenerated alloxan-induced injury to the pancreatic and spleenic tissues of albino rats. This indicated that the aqueous leaves extract of *Acalypha wilkesiana* possesses protective and healing capabilities against pancreas and spleen injury. This supports its Ethnomedicinal application in the management of High sugar blood level.

Anti-cancerous activity

Halimah *et al.* (2021) reported that the ethanol extract, along with its water, ethyl acetate, butanol, and n-hexane fractions of *Acalypha wilkesiana* have antiproliferative activity against

cervical cancer cells HeLa with the most notable results from ethyl acetate fraction. Based on the IC50 value, it can be established that the ethyl acetate fraction of the leaves has a marked cytotoxic effect against human cervical cancer cells and has low toxicity to normal cells .

Anti-inflammatory activity

Rats administered 500 mg/kg of A. wilkesiana extract demonstrated a substantial decline in inflammation. (Abere *et al.*, 2024).

Neuroprotective activity

Abhishek *et al.* (2024) reported in a study were scopolamine and diazepam were given centrally to induce significant memory impairment in mice, and extracted *Acalypha wilkesiana* (250 and 500 mg/ kg) was given orally and used for treatment. The extracted *Acalypha wilkesiana* (250 and 500 mg/ kg) treated animals showed significant increased time spending and improved cognitive function in an elevated plus maze test.

1.13. Antimicrobial investigation

An antimicrobial agent is one that eradicates or curtails the growth of microorganisms. A plant is believed to have antimicrobial effect if it neutralizes or slows the growth of microorganisms and they are grouped according to:

1. The microorganisms that are mainly active against bacteria are called Antibacterials, those that are mainly active against fungi are called Antifungals, those that act against viruses and protozoa are called Antivirals and Antiprotozoan respectively.
2. The extent of their action: Microbicidal (those that eradicate microbes) while Microbiostatic are those that simply suppress growth of microbes.

1.13.1. Methods of antimicrobial evaluation

The ongoing pursuit for new and exceptionally good antimicrobial compounds remains an enduring quest motivated by the critical issue of antimicrobial resistance, which lessen the impact of traditional antibiotics. To evaluate potential antimicrobial compounds, both new and established, researchers use a variety of methods of antimicrobial assay techniques. The following antimicrobial assay methods are commonly employed for the identification and evaluation of antimicrobial activity.

Agar diffusion based screening of antimicrobial activity

Agar diffusion based assays, includes the disk diffusion, well diffusion, agar plug, and agar spot assays. They are commonly used and affordable techniques in antimicrobial research for assessing the antimicrobial activity of test compounds. These methods depends on the diffusion of antimicrobial agents from paper discs, wells, or plugs into the agar medium, and thereby inhibiting the growth of the test microorganism inoculated on the agar surface . By evaluating the inhibited growth area which represents the area where microbial growth is halted or hindered by the agent, researchers can compare the effectiveness of the test compound against the specific microorganism under review (Hossain, 2024).

Cross streak method

The cross-streak method is a customized version of agar diffusion-based assays. In comparison to the earlier described assays where the antimicrobial agent is placed on the agar medium, this method involves streaking the test microbe and the indicator microbe(s) overlapping each other

on the agar surface. If the test microorganism yields an antimicrobial agent, it penetrates into the agar medium leading to growth inhibition for the other microorganisms at the point of overlap. In addition to its function in evaluating antimicrobial activity, this method is also utilized to study antagonism between microbial cells (Hossain, 2024).

Co-culture assay

The co-culture assay involves growing two microbes simultaneously in a shared environment to enable direct interaction between them like in cross-streak method. The growth of the indicator organism is then assessed and examined in comparison to a standard to verify the inhibitory activity of the test microbe. This method is frequently used to evaluate the antimicrobial effects of probiotic lactic acid bacteria against pathogens (Hossain, 2024).

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Consumables and reagents:

Nutrient agar, Nutrient broth, Sabouraud's agar, Dimethyl sulphoxide, Alcohol (absolute ethanol), Methanol, chloroform, calcium carbonate (SRL Pharma GmbH product, India), , glycerin (anhui Elite Industrial Co., Ltd, China), distilled water, sodium lauryl sulfate (SLS), peppermint, saccharine, Ciprofloxacin, Ketoconazole, Bacteria incubator, Pasteur's pipettes, Fehling's solution, Dragendorff reagent, Meyer's reagent, Hager's reagent, Wagner's reagent, swab stick, paper masking tapes, syringes, cotton wool, nose mask, latex gloves and Aluminium foil.

The microbiological strains used included: *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Candida albicans, Aspergillus niger, Pseudomonas aeruginosa* and *Klebsiella aerogenes*.

2.1.2 Glasswares and equipment

Mortar, pestle, conical flask, measuring cylinder, micro pipette, mechanical blender, electric blender, autoclave (Health Team Instruments, England), pH meter (Oaklon pH meter model 1100), water bath (Electronic thermostat water tank, model no; HH-W21-CR411), analytical

weighing balance (Ohaus corp. pine brook,NJ, USA), Petri dishes, cotton wool, masking tape, aluminum foil. Maker sterile wire loop, refrigerator, incubator (ESCO-Isotherm, Singapore), hot air oven (Springfield Instrument, England), stirring rod and dispensing bottle.

2.2 Methods

2.2.1 Plant collection and identification

The plant material *Acalypha wilkesiana* was collected inside the environs of Akanu Ibiam hostel (Hall 4), University of Benin, Edo state, Nigeria. The plant material *Acalypha wilkesiana* was correctly identified by Professor Henry Akinnibosun, a plant taxonomist from the Department of Plant Biology and Biotechnology, Faculty of Life Science, University of Benin, Benin City, with the voucher number UBH-A660.

2.2.2 Extraction of active ingredients

The fresh leaves of *Acalypha wilkesiana* were washed with water and air dried for five days until they were crispy dried. The leaves were then pulverized using mechanical blender and the particle size further reduced using an electric blender.

A 400g quantity of the powdered plant material was extracted using the Soxhlet extraction method with 8 cycles of 1.5 litres of methanol. The final filtrate was then transferred into a beaker and concentrated in the water bath at 45°C until all the solvents have evaporated leaving behind the extract. The percentage yield was calculated using the equation;

Percentage yield = (Weight of extract in grams/ initial weight of plant material) x 100

2.3 Phytochemical tests

The following phytochemical tests were carried out based on procedures outlined by Harbourne (1998) and Evans (2009). This was carried out to identify the presence of active phytochemical constituents.

2.3.1 General test for glycosides

Molisch Test

The methanol extract of *Acalypha wilkesiana* (2ml) was mixed with 2ml of 10% alcoholic solution of α-naphthol in a test tube. The test tube was inclined at angle 45 degree and 2ml of concentrated sulphuric acid was cautiously poured down the sides of the tube to form a layer below the extract without mixing. A deep violet ring formed at the interface of the two liquids indicated the presence of sugars.

Fehling's Test

Reducing sugars such as glucose, fructose and lactose will reduce Fehling's solution from deep blue to green, yellow or red colouration due to the formation of red precipitate of cuprous oxide, non-reducing sugars such as sucrose and polysaccharides such as starch will reduce Fehling's solution only when they are hydrolysed with acids on boiling. But the acid must be neutralized before testing with Fehling's solution.

The methanol extract of *Acalypha wilkesiana* (2ml) and a mixture of fehlings A and B were boiled in separate test tubes, 2 ml of the extract was added with Fehling's solution (A and B) and the result was noted.

To the remaining extract, 5 ml dilute H_2SO_4 was added and boiled gently for 5 minutes and filtered. The filtrate was made slightly alkaline with $NaHCO_3$, Fehling's solution that has already been boiled was added. Any change in color from blue to green, yellow or red indicated a positive reduction test.

2.3.2 Test for saponins

Frothing test

The methanol extract of *Acalypha wilkesiana* (0.5g) was put in a test tube and 4ml distilled water was added to it. The extract was diluted with water and shaken vigorously. Thick and persistent frothing was taken as evidence for the presence of saponins.

2.3.3 Tests for anthraquinone derivatives

The methanol extract of *Acalypha wilkesiana* (0.5g) was shaken with 15ml of chloroform to extract. It was filtered and 5 ml of extract was shaken with 5 ml of dilute ammonia. A pink colour indicated the presence of anthraquinone glycosides in the extract.

2.3.4. Tests for cardiac glycosides

Salkowski's Test

The methanol extract of *Acalypha wilkesiana* (0.5g) is dissolved in 2ml chloroform and concentrated H_2SO_4 was added carefully to form a layer. A reddish brown colouration at the interface indicates the presence of a steroidal nucleus (aglycone of steroidal glycoside)

2.3.5. Test for cyanogenetic glycosides

The methanol extract of *Acalypha wilkesiana* (0.1g) was placed in three test tubes labeled A, B and C. Extract in test tubes A and B were mixed with little water and sodium picrate test paper placed in each of the test tubes. The test tubes were stoppered immediately and tube B was placed in boiling water for 5 min while tubes A and C were kept at room temperature. After 30 min, changes in colour of the sodium picrate test papers were noted.

2.3.6. Test for tannins

General Test for Tannins

The methanol extract of *Acalypha wilkesiana* was put in a test tube and 5ml distilled water was added to it. A few drops of aqueous ferric chloride was added. A blue black precipitate indicated the presence of gallitannins and ellagitannins, while a yellow precipitate indicated the presence of condensed tannins.

2.3.7. Test for alkaloids

Alkaloids are naturally occurring organic compound that have a cyclic nitrogenous nucleus exhibiting basic properties. They react with a number of substances to give characteristic

precipitates. It is important to note that only aqueous solutions or extract can be tested directly with alkaloidal reagents which include Mayer's, Wagner's, Hager's and Dragendorff's reagents. When non-polar or semi-polar solvents are used for extraction, it is removed by evaporation and the dried extract is re-dissolved in 1% H_2SO_4 to extract the alkaloidal salts present in the sample.

Extraction and detection of alkaloids

Test

The 2ml of extract + 5ml of Dragendorff's reagent

The 2ml of extract + 5ml of Wagner's reagent

The 2ml of extract + 5ml of Hager's reagent

The 2ml of extract + 5ml of Mayer's reagent

A reference test using Quinine salt in place of the extract for each of the four alkaloidal reagents was carried out to serve as control.

Using methanol as solvent

Powdered sample of leaves of *Acalypha wilkesiana* (5g) was boiled with 50ml of methanol in a beaker over a water bath for 15 minutes. It was filtered and the filtrate evaporated to dryness in an evaporating dish over a water bath. The residue was dissolved in 5ml of 1% H_2SO_4 and filtered. The filtrate was then used to test for the presence of alkaloid using alkaloid reagents and reference was made to the blank test (control).

2.4 Preparation of the Herbal toothpaste

The herbal toothpaste was formulated using the methanol extract of A.wilkensiana leaves as the active ingredients and other non- bioactive ingredients as given below:

Table 1: Composition of the formulated toothpaste

Ingredients	**Material used**	**Quantity**
API	Acalypha wilkesiana	5g
Abrasive	Calcium carbonate	38g
Humectants	Glycerin	28 ml
Water	Distilled water	17 ml
Foaming agents	Sodium Lauryl sulphate	1.5g
Flavourant	Peppermint oil	2 ml
Sweetner	Saccharine	1.5g
Binder	Starch	7g

The dry gum method was used. 38 grams of Calcium carbonate was put in a mortar and triturated with a pestle vigorously to get a fine powder. 7 grams of starch was dispersed in 14 ml of water in a porcelain dish and heated over a water bath. This mixture was stirred until a mucilage was formed, after which 28ml of glycerin was added to it and was stirred further for 5 mins. This was

then added to the calcium carbonate already in the mortar and was triturated vigorously until a fine paste was seen. 1.5 gram sodium Lauryl sulfate dissolved in a porcelain dish with 2ml water over a water bath, 1.5 gram of saccharine dissolve in 1ml of water and 2ml peppemint oil was added to the Preparation. Lastly, 5 grams of the methanol extract of *Acalypha wilkesiana* was added to the Preparation and this was further triturated until a fine mix. The resulting mixture was then transferred into a dispensing Jar.

2.5 Physiochemical evaluation of the toothpaste

2.5.1 Evaluation of organoleptic properties of the toothpastes

The organoleptic properties such as taste, color, and the texture of the formulated herbal toothpaste, the toothpaste base and the commercial herbal toothpaste were evaluated using the respective sense organs (Sevagaperumal and Periyasamy, 2024).

2.5.2 Evaluation of foaming ability of the toothpastes

A 5g quantity of the toothpaste was accurately weighed and placed in different 100ml glass beakers. To this, 10ml volume of water was added and the beaker was covered with foil and allowed to stand for 30 minutes. This operation was carried out to disperse the toothpastes in water. The contents of the beakers were stirred with a glass rod and slurries were transferred to a 250ml graduated measuring cylinders. During this transfer, care was taken to ensure that no foam was produced and no lump paste went into the measuring cylinder. The content of cylinder was

adjusted to 50ml volume by adding sufficient water and the contents had to be maintained at 30°C. The contents were stirred with a glass rod to ensure a uniform suspension. As soon as the temperature of the content reached 30°C, the cylinders were stoppered and shaken 12 times. The cylinders were allowed to stand for 5 minutes and the volume of foam with water (V_2) and water only (V_1) was noted for all samples. (Sevagaperumal and Periyasamy, 2024). Triplicate determination was done for this examination.

Determination of foaming power:

Foaming power = $V_2 - V_1$

V_2 - volume in ml of foam with water.

V_1 - volume in ml of water only.

2.5.3 Evaluation of pH of the toothpastes

A 5g quantity of the toothpaste was accurately weighed and placed in a 150ml glass beaker and 45ml volume of freshly boiled, cooled water was added. It was stirred well to make a thorough suspension. The pH was determined using a pH meter (Oaklon pH meter model 1100). The pH meter was set to neutral (7.0) at room temperature and the electrode was immersed into toothpaste suspension. This process was repeated for all the toothpaste suspension three replicate determination were made (Oluwasina *et al.*, 2019).

2.6 Antimicrobial assay

2.6.1 Microbial isolation

Clinical isolates of five bacteria comparising three gram negative bacteria (*Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella aerogenes*) and two gram positive bacteria (*Bacillus subtillis* and *Staphylococcus aurues*) were used for the antibacterial assay. Two fungi (*Candida albicans* and *Aspergillus niger*) were used for the antifungal assay. The organisms were obtained from the department of pharmaceutical microbiology and Biotechnology laboratory, Faculty of Pharmacy, University of Benin, Benin city, Nigeria. The purity of the culture was confirmed by conventional cultural, morphological and biochemical methods prior to use. The microbial culture were maintained in nutrient agar and Sabouraud Dextrose agar for bacteria and fungi respectively at 4^{o}C.

2.6.2 Preparation of inoculum

An overnight culture was used for the preparation of microbial suspension with a turbidity equivalent to that of 0.5 Meforlard's standard.

2.6.3 Agar well diffusion method

The media was prepared and strerilized at 121^{o}C for 15 minutes. A total of 30ml Nutrient Agar was seeded with the bacterial culture and allowed to solidify and on each plate, wells of 10mm in diameter were made. The open wells were filled with different concentrations of the extracts and herbal formulation ranging from 100mg/ml to 500mg/ml and incubated at 37^{o}C for 24 hours. For antifungal assay, Sabouraud Dextrose agar was used in place of Nutrient agar and the medium incubated at 28^{o}C for 2 days. All tests were carried out in triplicates. The inhibition zone

diameter were measured and compared with Ciprofloxacin and Ketoconazole for antibacterial and antifungal assays respectively (Collins *et al.*, 1976)

CHAPTER THREE

RESULTS

3.1 Percentage yield

Table 2: Percentage yield of Methanol extract of *Acalypha wilkesiana* leaves

Plant material	Weight of plant material (g)	Weight of extract obtained (g)	Percentage yield (%)
Leaves of *Acalypha wilkesiana*	400	45	11.25

3.2 Phytochemical screening of *Acalypha wilkesiana* leaves extract

Table 3: Test for carbohydrates

Test	Observation	Inference
Molish test 2ml Methanol extract+ 2ml of 10% of alcoholic solution of alpha naphthol + 3ml of conc	Violet ring formed at the	Carbohydrate present

H_2SO_4	interface	
Fehlings test 2ml Methanol extract + 1ml of fehlings solution A + 1ml of fehlings solution B + heat	Reddish brown colouration formed	Reducing sugar present

Table 4: Test for saponins

Test	Observation	Inference
2ml Methanol extract + 5ml water + shake vigorously	Persistent frothing	Saponin present

Table 5: Test for anthraquinone

Test	Observation	Inference
2ml Methanol extract + 10ml chloroform + heat + filter + cool + 1ml dilute ammonia	Reddish colour observed at the upper layer	Anthraquinone derivatives present

Table 6: Test for cardiac glycosides

Test	Observation	Inference
Salkowski's test 2ml Methanol extract + chloroform + 1ml conc H_2SO_4	Greenish colour observed	Steroidal nucleus absent

Table 7: Test for cyanogenetic glycosides

Test	Observation	Inference
Test for cyanogenetic glycosides (for test tube A, B and C)	Yellow colour of test paper retained	Cyanogenetic glycosides absent

Table 8: Test for alkaloids

For reference test

Test	Observation	Inference

Quinine salt + Mayers reagent	Creamy precipitate formed	Alkaloid present
Quinine salt + Dragendorf reagent	Orange red precipitate formed	Alkaloid present
Quinine salt + Wagner reagent	Brown precipitate formed	Alkaloid present
Quinine salt + Hager reagent	Yellow precipitate formed	Alkaloid present

For test sample

Test	Observation	Inference
2ml Methanol extract + Dragendorf reagent	Orange red precipitate formed	Alkaloid present
2ml Methanol extract + Wagner reagent	Brown precipitate formed	Alkaloid present
2ml Methanol extract + Hager reagent	Yellow precipitate formed	Alkaloid present
2ml Methanol extract + Mayer reagent	Orange red precipitate formed	Alkaloid absent

Table 9: Test for Tannins

Test	Observation	Inference
General test for phenolic compounds with the extract and ferric chloride	Blue - black precipitate formed	Phenolic compound present

3.3 physiochemical evaluation of the herbal toothpaste formulation containing *Acalypha wilkesiana* leaves methanol extract

Table 10: physical evaluation and PH of formulation

PARAMETERS	OBSERVATION
Colour	Brown
Texture	Smooth
Taste	Sweet
Smell	Pleasant
Appearance	Paste-like
Foaming ability (centimeters)	6
pH	7.4 - 7.8

3.4 Antimicrobial evaluation of *Acalypha wilkesiana* leaves methanol extract

Table 11: Antimicrobial activity of the extract

Organism	Diameter of zones of inhibition (mm)						
	Crude extracts (mg/ml)					Cp (ug/ml)	Ket (ug/ml)
	100	200	300	400	500	10	10
E. coli	G	G	G	14±0.43	18±0.43	30±0.10	ND
P. aeruginosa	G	G	G	13±0.22	17±0.12	31±0.24	ND
K. aerogenes	G	G	G	15±0.13	19± 0.33	29±0.42	ND
S. aureus	G	G	G	15±0.52	18± 0.35	32±0.22	ND
B. subtillis	G	G	16±0.44	18±0 36	21±0.24	35±0.31	ND
C. albicans	G	G	G	G	G	ND	25±0.21
A. niger	G	G	G	G	G	ND	23±0.32

Values are expressed as mean ± SEM

Key:

G - no inhibition zone

ND - Not determined

CP - ciprofloxacin

Ket - ketoconazole

Table 12: Antimicrobial activity of the formulation containing the extract

Organism	Diameter of zones of inhibition (mm)						
	Herbal paste formulation (mg/ml)					Cp (ug/ml)	Ket (ug/ml)
	100	200	300	400	500	10	10
E. coli	G	G	G	G	13±0.42	29±0.13	ND
P. aeruginosa	G	G	G	G	12±0.52	28±0.21	ND
K. aerogenes	G	G	G	G	11±0.22	30±0.31	ND
S. aureus	G	G	G	G	15± 0.33	29±0.22	ND
B. subtillis	G	G	G	15±0.24	17±0.34	31±0.52	ND

C. albicans	G	G	G	G	G	ND	26±0.42
A. niger	G	G	G	G	G	ND	24±0.22

Values are expressed as mean ± SEM

Key:

G - no inhibition zone

ND - Not determined

CP - ciprofloxacin

Ket - ketoconazole

CHAPTER FOUR

DISCUSSION

Proper care of the teeth and the oral cavity plays a critical role in maintaining the overall health of an individual. Technological advancement in modern medicine has resulted in the development of toothpaste with therapeutic effects against bacteria, fungi and protozoa. Due to the negative effects of orthodox medicine, herbal medicine are gradually gaining prominence in health care.

For herbs to be used, extraction is the first critical step. In this study, 11.25% yield was obtained using methanol as a solvent for extraction. This is a slightly higher than yields reported by Jacob *et al*. (2023) with a yield of 10.92%. Variations in extraction yield may be as a result from differences in solvent polarity, plant material preparation (e.g., particle size and drying), extraction time, and plant chemotype (Fatmawati *et a*l., 2020). A high extraction yield indicates that *A.wilkesiana* leaves contains abundant extractable compounds under our conditions, which is promising for obtaining sufficient bioactive material.

Phytochemical evaluation of *Acalypha wilkesiana* methanol leaf extract

Secondary metabolite are phytochemical constituents of plants that confer their pharmacolgical activities. The Phytochemical constituents of the methanol extract of *Acalypha wilkesiana* leaves are anthraquinones, tannins, saponins and alkaloids. This outcome is comparable to those of some past studies (Sulaiman *et al.*, 2023; Bedona *et al*., 2024). Research indicates that plant-derived compounds including tannins, phenols, flavonoids, saponins, and alkaloids may

contribute to significant antimicrobial effects in plant extracts (Lin *et al.*, 2019). In a study carried out by Tandon *et al.* (2025), phytochemical constituents such as alkaloids, tannins and saponins from plants were responsible for the inhibition of oral pathogens such as *Streptococcus mutans* and *Streptococcus oralis*. Yan *et al*. (2021) stated that alkaloids exhibited its antibacterial effect through bacteria cell wall destruction and increased membrane permeability.

Physiochemical properties of the formulated toothpaste containing *Acalypha wilkesiana* leaf extract

With regards to the physical and organoleptic evaluation of the herbal toothpaste. The smooth texture of the formulation would prevent gum damage, as sharp edged particles are known for causing this. This has been reported by various findings on formulated herbal toothpaste (Shukla and Kumari, 2019). The pleasant smell and taste is as a result of the flavoring agent (peppermint oil) and sweetener (saccharine) respectively used in the formulation. Foaming ability which is crucial to the cleansing ability of toothpaste is affected by the presence of surfactant (sodium Lauryl sulfate). The presence of the extract also increased the foaming ability of the formulated toothpaste. Saponins present in the extract may also be responsible for this because of the frothing properties it has. The height of the foam of the toothpaste upon evaluation was consistently above 5.8 cm on the triplicate trials carried out and this was an indication of stable foam.

The pH of the toothpaste should neither promote demineralization of dental hard tissue nor facilitate the acidogenic breakdown of retained food debris in the oral cavity. The demineralization of the enamel of tooth occurs at a pH of below 5.5 (Muhammad and Ahmed, 2022). The basic pH of 7.4 - 7.8 of the toothpaste formulation will aid to prevent this. The

inclusion of the secondary plant metabolite alkaloid may render the oral pH alkaline, as it can form salt with acids present in the mouth (Oluwasina *et al.*, 2023).

Antimicrobial activity of the crude *Acalypha wilkesiana* leaf extract

The results from the antimicrobial assay of the extract showed that it has antibacterial activity but no antifungal activity. The crude extract showed moderate antibacterial activity at high concentration. No inhibition was seen below concentration of 300 mg/ml, but at 300 mg/ml zone of inhibition was seen for *Bacillus subtillis* and for concentration above 400 mg/ml zones of inhibition were seen for the other organism tested. Zones of inhibition of the gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtillis*) ranging from 15 to 21 mm compared to inhibition zones of gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa* and *Klebsialia aerogenes*) ranging from 14 to 19mm showed that the extract had more effect against gram positive bacteria in contrast to gram negative ones. This finding is agreeable with the work of Bashir and Usman (2025) who noticed similar findings when working with *Acalypha wilkesiana* methanol leaf extract. The antimicrobial effect of the extract can be related to the phytochemical constituents such as Tannins, alkaloids and saponins which are known to have an antimicrobial activities in plant extract (Oluwasina *et al*., 2023).

The assay also revealed a better activity of the standard drug (ketoconazole and ciprofloxacin) to the extract. The zones of inhibition respectively ranged from 23 to 25mm for ketoconazole and 29 to 35 mm for ciprofloxacin. It should be noted that ketoconazole and ciprofloxacin are standardized pharmaceutical agents containing purified and validated active ingredients with targeted activity against specific microorganisms. In contrast, crude plant extracts remain unpurified and lack specificity in their antimicrobial action (Oluwasina *et al*., 2023).

Antimicrobial activity of the formulated toothpaste containing Acalypha wilkesiana leaf extract

The antimicrobial assay of the formulated toothpaste indicated that it has antibacterial activity but no antifungal activity. It was also observed that the crude plant extract had more antibacterial activity than the formulated toothpaste. The concentration of the extract that started showing inhibition was from 300 mg/ml with zones of inhibition ranges from 14 to 21 mm, while that of the formulated toothpaste started at 400 mg /ml with zones of inhibition ranging from 11 to 17 mm. In comparison to the crude extract, the toothpaste formulation showed more activity against gram positive bacteria when compared to gram negative bacteria. Also, the standard drug had more antimicrobial activity when compared to the formulated toothpaste.

The antibacterial activity of the formulated herbal toothpaste can be related to the crude extract, because of the antibacterial activities it has too. Also, Its activity can also be linked to the other components of the formulated toothpaste. Sabri *et al*. (2023) suggest that sodium Lauryl sulfate has antibacterial effect and could also be combined with other antibacterial agent for synergistic effect.

CONCLUSION

Leaves of *Acalypha wilkesiana* contains various classes of phytochemicals such as Alkaloid, Tannins and anthraquinones. This study shows that incorporating herbal antimicrobial agents into formulated toothpaste such as the methanol extract of *Acalypha wilkesiana* provides antibacterial protection against microbes involved in the development of tooth decay and periodontal disease in vitro. The use of the *A. wilkesiana* extract as a constituent in toothpaste formulation will aid in maintaining oral hygiene to prevent tooth decay and periodontal diseases.

RECOMMENDATION

The toothpaste formulation of *Acalypha wilkesiana* can be used as an antibacterial agent, but the dose of the extract should be increased. Also further experiments can be conducted to study the toxicological profile of the formulated toothpaste containing *Acalypha wilkesiana* leaves extract.

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APPENDIX

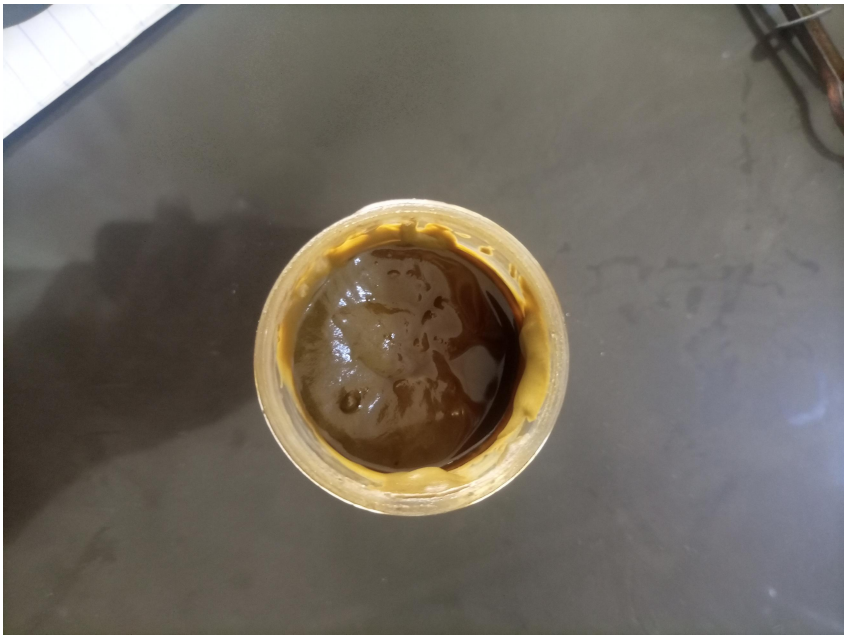

Figure 2: Formulated toothpaste containing *Acalypha wilkesiana* leaf extract

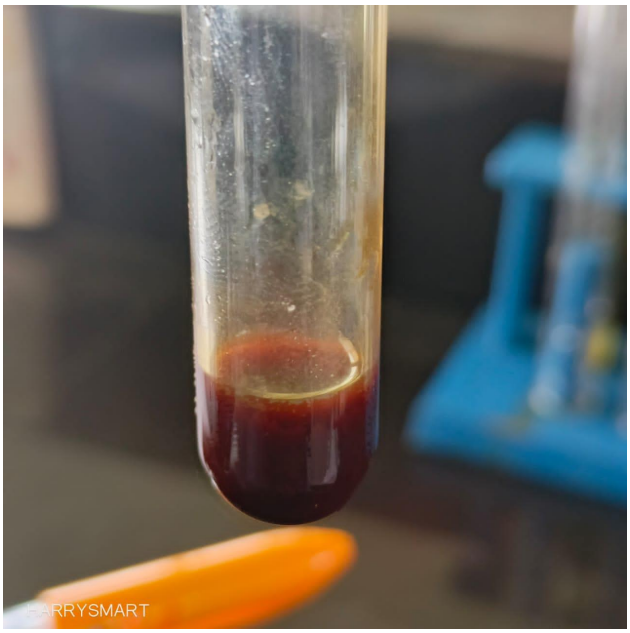

Figure 3: Reference sample with Wagner reagent

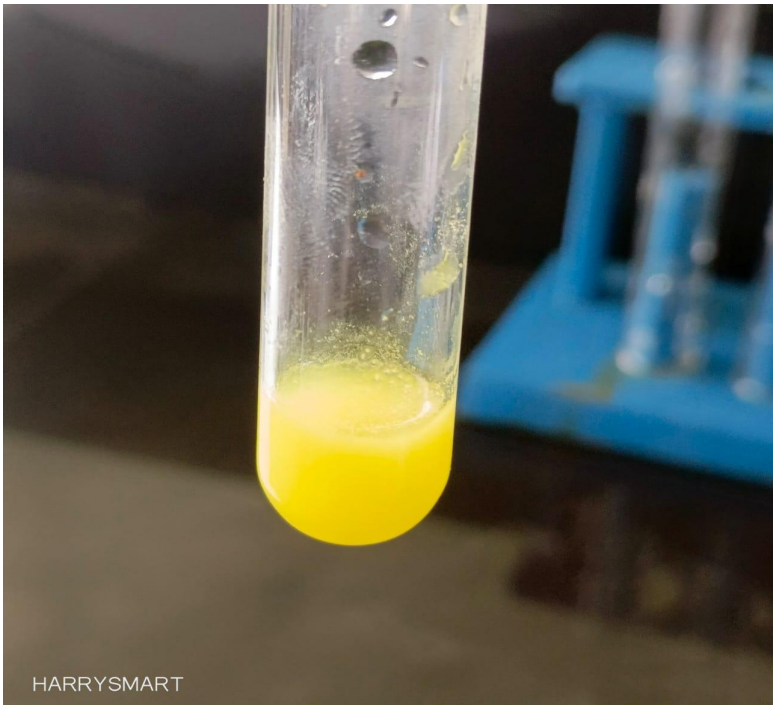

Figure 4: Reference sample with Hager reagent