ANTIBACTERIAL ACTIVITY OF *Hibiscus Sabdariffa* CALYX EXTRACT AGAINST SOME ENTERIC BACTERIAL ISOLATES

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A PROJECT SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN, BENIN CITY. IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF BACHELOR OF SCIENCE (B.SC.) DEGREE IN MICROBIOLOGY

SEPTEMBER, 2023

CERTIFICATION

This is to certify that this project was carried out by OSAGIE OSAGUMWENRO EMMANUEL of the Department of Microbiology under the supervision of Dr. (Mrs) R.O. OKOJIE.

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(Head of Department)

APPROVAL

I certify that this work has been accepted in partial fulfilment of the requirement for the award of Bachelor of Science (B.Sc) in the Department of Microbiology, University of Benin, Benin City.

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PROF. MR. D. **DATE**

(External Examiner)

DEDICATION

This project is heartily dedicated to God almighty for his guidance throughout my course of study

ACKNOWLEDGEMENTS

My greatest gratitude goes to God Almighty for His infinite and massive show of grace throughout the period of this work and the presentation.

I sincerely appreciate my supervisor Dr. (Mrs) R.O. Okojie for her patience, guiding and supervising me all through this work. The corrections she made to my work and several important adjustments to make sure it came out looking great. I remain entirely grateful for the way she pushed me to do better.

I am grateful to my parents Mr. Edwin Osagie and Mrs. Pat Osagie for their support and prayers throughout.

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ABSTRACT

The emergence of antibiotic-resistant pathogens has garnered considerable focus on the antibacterial attributes of natural compounds in recent years. This study aims to evaluate the antibacterial efficacy of Hibiscus sabdariffa calyx extract against several enteric bacteria, specifically Escherichia coli, Salmonella typhi, Staphylococcus aureus, and Enterococcus faecalis. A phytochemical analysis of the *Hibiscus sabdariffa* calyx extract was conducted to identify and quantify bioactive compounds that may contribute to its antibacterial properties. This analysis identified secondary metabolites, such as tannins, flavonoids, and alkaloids, recognised for their antimicrobial properties. These compounds likely contribute significantly to the antibacterial effects observed in the hibiscus sabdariffa calyx extract. Furthermore, the antibacterial efficacy was assessed utilising recognised microbiological methodologies, including minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), against a selection of enteric bacterial isolates. The results demonstrated a significant antibacterial effect linked to Hibiscus sabdariffa calyx extracts. Their inhibition of bacterial growth was dose-dependent, with ethanolic extracts demonstrating significantly greater efficacy than aqueous extracts.

CHAPTER ONE

INTRODUCTION

1.1 Background of Study

According to the World Health Organization (WHO) in 2008, traditional medicine encompasses the collective wisdom, abilities, and customs rooted in various cultural beliefs and experiences. It fulfils the objectives of preserving health, preventing, diagnosing, enhancing, or treating physical and mental disorders. Herbal medicine, a prevalent facet of traditional medicine, employs plants or plant components as its active constituents (WHO, 2008).

According to a WHO report from 2008, about 80% of people in Asia and Africa receive their primary medical care from traditional practitioners. In Nigeria, there will be 10,000 doctors for every 10,000 people in 2022.Just 24,000 licensed medical doctors are currently practicing in Nigeria, which is less than 10% of the required number to comply with a WHO recommendation (NMA, 2022).

This shows that some people rely primarily on medicinal plants, which are more readily available and less expensive for them, especially those who live in areas with low doctor to population ratios. Tropical and subtropical areas are home to the Malvaceae family medicinal plant Hibiscus sabdariffa. The Semi-Arid savanna region cultivates a large amount of it for both local consumption and export to the Middle East and Europe. In Nigeria, people refer to it as "zobo." It is also known as Florida cranberry, karkade, sorrel, and roselle (Morton, 1987). It is called Rarna, Sule (Hausa), Sakpa (Ga), and Eema (Ewe) in regional contexts (Dokosi, 1998).

The crimson, tangy calyx is cooked with sugar to make sorrel drink and is also used in crafting jellies, sauces, chutneys, and preserves (Gibbon and Pain, 1985). The seeds have an oil content of approximately 17%, which shares similar properties with cottonseed oil. Before there were

contemporary alternatives like maggi, these seeds were boiled, fermented, and dried to be used as a condiment in regional soup preparations called Yakuwa or Batso in Hausa. About 84.5% of the fruit is made up of water, 1.7% protein, 1.0% fats and oils, and 12% carbs. There is 4% citric acid in its calyx (Purseglove, 1969).

Hibiscus sabdariffa serves both as a dietary resource and a component of herbal medicine (Cisse *et al*., 2009). Its young shoots and leaves are consumed as vegetables, while the calyxes are used in creating beverages, jams, and as a source of natural coloring (Dokosi, 1998).

Hibiscus sabdariffa finds medicinal applications as a laxative, an anti-carcinogenic, an anti-hypertensive, and a medication for lowering cholesterol. It demonstrates significant antioxidant properties, reduces liver toxicity, and has fever-reducing capabilities. In certain regions of Africa, it serves as a remedy for various ailments, including abscesses, bilious conditions, cough, sores, wounds, dysuria, and scurvy (Morton, 1987). According to Fullerton *et al*. (2011), *Hibiscus sabdariffa* also shows promise as an antimicrobial agent. Hence, there is a pressing need for further research to evaluate the effectiveness of this medicinal plant.

1.2 Aim of the Study

To investigate the antibacterial activity of *Hibiscus sabdariffa* calyx extract against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi* and *Enterococcus faecalis.*

1.3 Objectives of the Study

The objective of this was, to:

1. evaluate phytochemicals in extract

2. determine the antibacterial activity of *Hibiscus sabdariffa* calyx extract against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi* and *Enterococcus faecalis.*
3. determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *Hibiscus sabdariffa* calyx extract against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi* and *Enterococcus faecalis.*

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview on Hibiscus sabdariffa

2.1.1 Botanical Description

Hibiscus sabdariffa known locally as asam kumbang, asam susur, and asam paya, belongs to the extensive Malvaceae family (Osman *et al*., 2011). It also goes by various names globally, such as roselle (in English), l'Oiselle (in French), Spanish (in Jamaica), karkade (in Arabic), and Krachiap daeng in Thailand (Maganha *et al*., 2010).

As a part of the Malvaceae family, the Hibiscus genus has over 300 species that have been identified; many of these are grown for aesthetic purposes. Usually growing to a height of 5 to 7 feet, these plants have lobed leaves that are sometimes used as greens. Their thin stems and leaves have a reddish-green colour. The fleshy sepal, or calyx, that encloses the seed pod inside the flower, is the main edible part. Although the exact variety affects the size, the calyx usually has a diameter of between 1⁄2 and 1 1⁄2 inches. James (1994). Although the precise origin of H. sabdariffa is unknown, it is thought to be native to the area that stretches from Malaysia to India, where it is widely grown. It is most likely that Africa was exposed to it early on. This plant has spread widely over the years in both the tropical and subtropical regions of the two hemispheres. In several parts of Central America and the West Indies, it has also become native. It was originally brought to the West Indies mainly as an ornament.

H. sabdariffa is a relatively recent addition to Malaysia. Malaysia was first exposed to it in the early 1990s. In 1993, the Terengganu Department of Agriculture spearheaded the promotion of its commercial cultivation, a practice that has since spread to other states. As of right now, this

plant is only thought to be grown on a comparatively small 150 hectare plot of land (Osman et al., 2011).

Numerous components of *H. sabdariffa*, including its seeds, leaves, fruits, and roots, find application in various culinary preparations. However, it's the fleshy red calyxes that stand out as the most widely utilized part. These fleshy calyxes from *H. sabdariffa* have been incorporated into culinary traditions in numerous countries. They are used in various food products and recipes, including jellies, syrups, beverages, puddings, cakes, and wines, showcasing their versatility and popularity in different cuisines (Christian *et al*., 2006). The prominent red and enduring calyx of its flowers serves as a key component, known for its tangy flavor. It is a common ingredient in the preparation of both cold and hot beverages and is also utilized as a natural food coloring agent (Maganha *et al*., 2010).

2.2 Uses of Hibiscus Sabdariffa

2.2.1 Nutritional Use

The plant *H. sabdariffa* is mainly used as a vegetable and for preparation of beverage. The calyxes of *Hibiscus sabdariffa* are especially well-known for their role in beverage preparation, often serving as a tea or coffee substitute for those sensitive to stimulants (Irvine, 1961). They are also employed in making preserves, jelly, juice, and sauces (Stephens, 2012), as well as in crafting roselle wine, gelatin, pudding, and cakes. Furthermore, these calyxes can be utilized to add color to various food and drink items (Duke and Atchley, 1984).

The process of making Sooboro (Hausa) in Ghana involves boiling the calyxes in water, adding sugar to sweeten it, and sometimes adding ginger and other regional spices. Additionally, the leaves and calyxes are used in soups or as a cooked vegetable. Cereals and tubers are served with sauces prepared from the leaves and calyxes in Senegal. Furthermore, the leaves are used in

salads in the United States of America, and fruit salads contain the red, fleshy calyxes. These many applications highlight the plant's importance in both cuisine and culture.

2.2.2 Economic Uses

The idea that natural products are more effective than conventional medicines has led to a noticeable increase in interest in natural products over the past ten years in the global market. Particularly herbal products have helped many nations generate more income and job opportunities. For example, of the $10.4 billion dietary supplement market in the United States, botanical products make up $3.1 billion (NBJ, 1998).

Over the last ten years, there has been a significant increase in the global trade of Hibiscus sabdariffa calyxes, popularly known as roselle, with about 15,000 tonnes reaching the global market annually. Major importers such as Germany and the United States were prepared to pay between $1200 and $1700 US per tonne for Egyptian and Sudanese roselle in 1998. This demonstrates the enormous economic advantages that plants can have for the nations that grow them (McClintock and El Tahir, 2004).

2.2.3 Medicinal Uses

H. sabdariffa has found its place in numerous folk medicine practices. It is recognized as a traditional Thai remedy for kidney stones and urinary bladder stones (Hirunpanich *et al.*, 2006). Furthermore, *H. sabdariffa* is believed to possess diuretic properties and is used effectively in folk medicine to address inflammatory conditions (Dafallah and Al-Mustafa, 1996) and even as a potential treatment for cancer (Chewonarin *et al*., 1999). Studies have provided evidence of the positive impact of *H. sabdariffa* extract consumption on lowering blood pressure, observed in both human and rat studies (Faraji *et al.*, 1999; Onyenekwe *et al*., 1999). More recently, its antihypertensive properties have been validated in experimental hypertension research (Odigie *et*

al., 2003). Additionally, human studies have demonstrated the anti-inflammatory effects of *H. sabdariffa* consumption (Herrera *et al.*, 2004; Beltrán-Debón *et al*., 2010).

H. sabdariffa extract is reputed to possess antibacterial, anti-fungal, diuretic, uricosuric, and mild laxative properties (Farnworth and Bunyapraphatsara, 1992). Furthermore, components extracted from *H. sabdariffa* have exhibited characteristics such as anti-tumor effects, immune modulation, and anti-leukemic properties (Muller and Franz., 1992; Tseng *et al*., 2000). The oil extracted from *H. sabdariffa* seeds has shown in vitro inhibitory effects against Bacillus anthracis and *Staphylococcus albus* (Gangrade *et al*., 1979). Additionally, an ethanol extract from the dried leaves of the plant has demonstrated the ability to reduce aflatoxin formation (El-Shayeb and Mabrook, 1984) and inhibit some fungi, including *Aspergillus fumigatus*, *Rhizopus nigricans,* and *Trichophyton mentagrophytes* (Guerin and Reveillere, 1984).

2.3 Antioxidant Activity and Anticholesterol Effects of *Hibscus Sabdariffa*

The calyxes of H. *sabdariffa* have been extensively researched and consistently demonstrated to offer positive health benefits, primarily as a rich source of antioxidants. Notably, these calyxes contain antioxidants in higher levels than traditional sources like raspberries and blueberries (Juliani *et al*., 2009). Chemical constituents found in *H. sabdariffa,* such as anthocyanins and protocatechuic acid, have been identified as having potent antioxidant properties (Lee *et al*., 2002) and showing potential anti-tumor effects (Chang *et al*., 2005; Lin *et al.*, 2005).

Previous studies have indicated that extracts derived from dried *H. sabdariffa* calyxes can protect rat hepatocytes from cytotoxicity and genotoxicity induced by tert-butyl hydroperoxide through various mechanisms (Tseng *et al*., 1997; Liu *et al.,* 2002). Protocatechuic acid from *H. sabdariffa* calyxes demonstrated the ability to inhibit rat hepatic damage induced by

lipopolysaccharide. Additionally, Hibiscus protocatechuic acid has been shown to counteract the carcinogenic effects of various chemicals in different rat tissues, including diethylnitrosamine in the liver (Lin *et al.*, 2003). This protective effect is believed to be linked to the scavenging of free radicals by antioxidant compounds present in H. *sabdariffa* calyxes (Taneka *et al*., 1993). Additionally, it has been discovered that administering dried calyx extracts from H. sabdariffa significantly lowers levels of LDL, triglycerides, and serum cholesterol. In vivo, the calyxes of H. sabdariffa show hypolipidemic and antioxidant effects against LDL oxidation. But more research is needed to fully understand the specific mechanisms underlying these effects (Hirunpanich et al., 2006).

2.4 Phytochemical Constituents

The main anthocyanin in the calyx of H. sabdariffa is delphinidin-3-glucoxyloside, or hibiscin. The calyx of H. sabdariffa is known to be rich in a variety of chemical constituents. H. sabdariffa calyx also contains other flavonoid constituents, such as anthocyanins such as cyanidin-3-rutinoside, delphinidin, cyanidin-3-monoglucoside, cyanidin-3-sambubioside, and cyanidin-3,5-diglucoside. Additionally present are flavonol glycosides like sabdaritrin, hibiscetin-3-monoglucoside, gossypetin-3-glucoside, gossypetin-7-glucoside, and gossypetin-8-glucoside. Alkaloids, ascorbic acid, β-carotene, anisaldehyde, arachidic acid, citric acid, malic acid, tartaric acid, glycinebetaine, quercetin, protocatechuic acid, pectin, polysaccharides, mucopolysaccharides, stearic acid, and wax are also present in H. sabdariffa calyx (Hirunpanich et al., 2005).

About 65% (dry weight) of the mucilage produced by the calyx is hydrolysed to produce galactose, galacturonic acid, and rhamnose. The pharmacological effects of plant extracts are

thought to be caused by these molecules, which have demonstrated bioactivity in a variety of biological models. In addition, the calyx and flower petals of roselle contain various antioxidant constituents, including anthocyanins, quercetin, ascorbic acid, β-sitosteroid glycoside, and protocatechuic acid (Tseng *et al.*, 1997). Furthermore, *H. sabdariffa* calyx is found to contain polyphenolic acids (1.7% dry weight), flavonoids (1.43% dry weight), and anthocyanins (2.5% dry weight) (Tsuda *et al.*, 2000). These chemical components contribute to the plant's potential health benefits and medicinal properties.

2.6 Enteric Bacteria

Bacteria classified as enteric primarily infect and cause diseases in the human intestines or gastrointestinal tract (GI tract). These pathogens are usually spread by contaminated food, water, or direct contact between people. They can cause diarrhoea, gastroenteritis, and in rare circumstances, more serious gastrointestinal disorders. *Salmonella* sp. and *Escherichia coli* are common human pathogens that enter the body.

2.6.1 *Escherichia coli*

Escherichia coli belongs to the family Enterobacteriaceae and genus *Escherichia*. The majority of the sources of the bacteria's spread are contaminated food and water, and members are widely dispersed throughout the environment. Well-known for causing simple cystitis, *Escherichia coli* can also cause extraintestinal infections like bacteremia, pneumonia, and spontaneous bacterial peritonitis, as well as abdominal infections. A sub group called *Enterohaemorrhagic Escherichia coli* (EHEC) can cause food borne illness as the *E. coli* O157:H7 strain which causes severe and potentially fatal illness known as hemorrhagic colitis which is characterized by bloody diarrhea and severe abdominal pain (Mueller and Tainter, 2023). Since the presence of *Escherichia coli* in

food typically indicates both direct and indirect faecal contamination, it is frequently used as a faecal indicator. Escherichia coli infections can result in severe dehydration or septic shock, which calls for the replacement of lost fluids with crystalloids. Escherichia coli-caused traveler's diarrhoea can be effectively treated with antibiotics such as azithromycin, fluoroquinoles, and rifaximin (Collier, 2023).

2.6.2 *Staphylococcus aureus*

A gram-positive bacterium that is resistant to radiation, heat, and drying is *Staphylococcus aureus*. Both pathogenic and comparatively non-pathogenic strains exist. When the bacteria contaminate food, they cause illness. According to Prescott et al. (2005), they produce a number of extracellular substances, including heat-stable enterotoxins, which make food dangerous even though it looks normal. They also produce some enzymes that are linked to staphylococcal invasiveness. The food can be thoroughly cooked to kill the bacteria without destroying the toxin once the bacteria have produced it. A large number of their toxins are plasmid-borne genes. The quantity of tainted food consumed and an individual's susceptibility to the toxin can both affect how severe the symptoms are. Staphylococcal food poisoning can cause a number of symptoms, such as diarrhoea, vomiting, cramping in the abdomen, nausea, and vomiting. Antibiotics such as vancomycin, daptomycin, oxacillin, nafcillin, cefazolin, and linezolid are frequently prescribed by physicians to treat staph infections. Since many staph bacterial strains have become resistant to other antibiotics, vancomycin may be required in cases of severe infections. Intravenous administration of vancomycin and other antibiotics is used to treat resistant staph infections.

2.6.3 *Salmonella typhi* (genus *Salmonella*)

Enteritis salmonella Typhoid fever is caused by the gram-negative bacterium serotype typhi, which has plagued developing countries for many years. Pierre Louis first used the term "typhoid

fever" in 1829 after discovering lesions in the lymph nodes of patients who had passed away from "gastric fever." The word was coined to characterise the delirium that patients with the illness would experience, and it was derived from the Greek word "typhus," which meant "smoky." The organism responsible for typhoid fever was not identified until 1880, despite being first reported in the early 1800s. German pathologist Karl Eberth discovered S. enterica in 1880. Georg Gaffky cultured it for the first time in 1884. Almroth Wright created a vaccine for the illness a few years later. Typhoid fever remains a major global public health concern despite tremendous research and medical advancements (Barnett and Lancet, 2016).

Salmonella enterica serotype typhi is usually contracted by ingestion of food or water that is contaminated with the excrements of people that carry the organism and must survive the gastric pH barrier in the stomach prior to adherence in the small intestine. An infectious dose of *Salmonella enterica* serotype typhi in healthy individuals ranges between 1000 and 1 million organisms but can be related to the host's defense mechanisms (Parry *et al.,* 2002).

Patients usually show up 7–14 days after the initial Salmonella enterica serotype typhi inoculation without any symptoms. Patients will show up with a feverish, influenza-like illness after the first period of no symptoms. Throughout the course of the illness, abdominal symptoms are always present and can include pain, nausea, vomiting, constipation, or diarrhoea. The patient may experience sporadic disorientation and apathy as the illness worsens. In contrast to malaria, patients will not experience rigours and fevers will vary little to not at all during the day (Parry et al., 2002; Crump et al., 2015).

Those who reside in endemic areas or who have visited an endemic area and are exhibiting symptoms of a febrile illness should be suspected of having typhoid fever. While a single laboratory value is not pathognomonic for typhoid fever, basic laboratory data can help the

clinician make the diagnosis. Depending on the patient's age, a complete blood count may reveal leukocytosis or leukopenia with a left shift. Furthermore, relative anaemia might exist. Abnormal liver function tests could be seen if a complete metabolic profile is acquired (Parry et al., 2002; Crump et al., 2015).

Whenever possible, the clinician should obtain stool and blood cultures in order to confirm the diagnosis. 40% to 80% of people have positive blood cultures, and 30% to 40% have positive stool cultures (Edelman et al., 1986; Parry et al., 2002; Crump et al., 2015). But when systemic symptoms appear, stool cultures lose their sensitivity (Edelman et al., 1986). A bone marrow aspirate is the most sensitive diagnostic test available to medical professionals. Despite being intrusive, Salmonella enterica serotype typhi infections result in more than 90% of positive cultures, which can continue to be positive for days even after antimicrobial therapy is started (Parry et al., 2002: Crump et al., 2015).

Agglutinating antibodies against the lipopolysaccharide O and flagellar H antigens are measured by the Widal test. Although a four-fold increase in antibody titers taken ten days apart is ideal for a positive test, many clinicians base treatment decisions on a single acute-phase sample. False-negative and positive results are possible with this method.

Chloramphenicol was the first antibiotic used to treat infections brought on by the Salmonella enterica serotype typhi. Salmonella enterica serotype typhi resistant strains were found in the community in just two years (Parry et al., 2002). Nowadays, the cornerstone of treatment is either ofloxacin or ciprofloxacin (Parry et al., 2002; Bhuta 2006; Crump et al., 2015). Quinolone therapy is acceptable for use in children with severe infections or in the absence of other therapies, despite the risks involved. When quinolone resistance is detected, ceftriaxone, an extended-spectrum cephalosporin, may be utilised. Azithromycin is an additional treatment

option for people infected with a strain of Salmonella enterica serotype typhi that is resistant to quinolones. When standard therapies are ineffective, patients have been treated with a combination of fluoroquinolones, cephalosporins, and macrolides (Parry et al., 2002; Crump et al., 2015).

2.6.4 *Enterococcus faecalis*

Formerly classified as part of the group D *Streptococcus* system – is a Gram-positive, commensal bacterium inhabiting the gastrointestinal tracts of humans (Ryan *et al*.,2004; de Almeida *et al.*,2018) Like other species in the genus *Enterococcus*, *E. faecalis* is found in healthy humans and can be used as a probiotic.

The infection known as *Enterococcus faecalis* arises when the enterococci bacteria, which are found in the gut and bowel, multiply excessively or spread to other areas of the body. It may result in symptoms in every part of the body.

A hardy species of bacteria called *Enterococcus faecalis* is becoming more and more resistant to antibiotics. The bacteria can join together to create "microbial communities," where it clings to other microorganisms to create biofilms. According to Hashem et al. (2021) these biofilms are particularly resistant to antibiotic therapy.

Being that it is an opportunistic pathogen, *Enterococcus faecalis* is harmless to most people with healthy immune systems, but it does pose a threat to the elderly as well as those who are immunocompromised (have weakened immune systems) (Del Turco *et al*.,2020).

It is typical to find *E. faecalis* in plants, animals, water, and soil. Consuming unclean food can make you more susceptible to infection. Symptoms may include; fever and chillls, painful or burning urination, Nausea and vomiting, lightheaded or confusion, Headache, pain or pressure in the lower abdomen (Agudelo *et al*., 2014).

Your doctor will order bacterial culture and antibiotic sensitivity testing if they think you have an E. faecalis infection. A urine sample or blood drawn into special bottles and sent to the lab will depend on the infection site.

Techniques will be employed in the lab to observe whether bacteria grow from the samples. To determine which antibiotics will or won't be effective in treating the infection, those samples will be tested.

An antibiotic called ampicillin is frequently used to treat infections caused by *E. faecalis* (Agudelo et al., 2014). Ampicillin kills *E. faecalis* by preventing the bacteria from forming their outer cell wall.

However, because *E. faecalis* infections may be resistant to a number of antibiotics, including aminoglycosides, vancomycin, and daptomycin, they are typically very difficult to treat (Kristich et al., 2014).

To boost their effectiveness, combination antibiotics are used for severe infections like endocarditis. Penicillin and gentamicin together may be one such example of this (Kristich et al., 2014). Nevertheless, different antibiotics that might be more useful in treating E. faecalis infections are being studied by researchers.

CHAPTER THREE

MATERIALS AND METHOD

3.1 Test Bacterial Isolates

Salmonella typhi, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Escherichia coli* clinical isolates were obtained from the University of Benin Teaching Hospital and brought to the microbiology laboratory for antimicrobial analysis and confirmatory testing.

3.2 Sample Collection and Extraction

The *Hibiscus sabdariffa* calyxes were collected from Ovia North East, located in Benin City, Edo State, Nigeria. After being air dried and ground into a powder using an electric blender, the calyxes were kept in an airtight container pending additional examination. Aqueous extraction involves soaking 500g of the sample in water, filtering it using Whatman No. 1 filter paper, and then allowing it to evaporate to extract the oil (Abdul-Hammed et al., 2021).

3.3 Determination of Phytochemicals

3.3.1 Total flavonoid:

The methodology for determining total flavonoid content (TFC) will slightly deviate from Pandey et al.'s 2020 findings. Four millilitres (ml) of distilled water, three millilitres of a 5% $NaNO_2$ (Sodium Nitrite) solution, and five millilitres of the sample will be mixed together. 0.3 ml of 10% $AlCl_3$ (aluminium chloride) will be added after five minutes, and 2 ml of 1M NaOH (sodium hydroxide) will be added after six minutes. The absorbance was measured at 510 nm against a blank solution after 30 minutes. TFC, which is expressed as mg of quercetin equivalents (QE/g) of extracts, will be computed using a quercetin calibration curve.

3.3.2 Total phenols:

The Folin Ciocalteu method will be used to determine the total phenol content (Slinkard and Slingleton, 1997). A known aliquot of the samples was created up to 1.5 ml using distilled water. Subsequently, 10 ml of sodium carbonate and 0.5 ml of Folin-Ciocalteu reagent were added, and the mixture was then allowed to sit at 37 °C for an hour. At 750 nm, the absorbance values were measured. Using the gallic acid calibration curve, the total phenolic content will be expressed as mg gallic acid equivalent (GAE)/g of extracts.

3.3.3 Tannins:

Using the Folin Denis reagent, the tannin content will be ascertained (AOAC 2005). In a 100 ml volumetric flask with 75 ml of water, a known aliquot of the sample was added. Afterwards, 10 Na_2CO_3 solution and 5 ml of Folin Denis reagent were added. Following 30 minutes, the colour at 760 nm was assessed in comparison to an experimental blank with an absorbency of 0. Tannic acid percentage was calculated using a standard curve and milligrammes of tannic acid.

3.4 Preparation and Sterilization of Culture Media

Every culture medium were prepared in compliance with the manufacturer's guidelines. Sterilization will be at 121ºC at 15psi for 15 min unless otherwise stated by manufacturer.

3.4.1 Nutrient agar

In a conical flask corked with cotton wool and foil paper, twenty-eight grammes (28 g) of nutrient agar will be dissolved in one thousand millilitres (1000 ml) of distilled water and left to dissolve. The medium will be sterilised for 15 minutes at 121°C in an autoclave. Following sterilisation, cooling was permitted for the flask.

3.4.2 Mueller Hinton agar

Mueller Hinton agar weighed thirty-eight grammes (38g) and was fully dissolved in 1000 millilitres of distilled water by boiling. At 15 psi (121°C), the autoclave was sterilised for 15 minutes. After cooling to 60°C, it was poured into sterile Petri dishes.

3.5 Confirmatory Test for Isolates

Morphological analysis

3.5.1 Gram staining:

The purpose of this test is to verify the type of bacteria's cells that will be utilised. Gram-positive and Gram-negative bacteria were distinguished using staining techniques. Gramme positive organisms are those that maintain the primary stain after being decolorised, whereas Gramme negative organisms do not. The cell composition is the reason for the stain's non-retention. The Gram stain procedure is as follows:

The bacteria isolate was spread out on a grease-free slide and heated using a flame. The primary stain, crystal violet, was applied to the smear for one minute, and then it was rinsed with distilled water. After being submerged in Lugol's iodine solution for 30 seconds, the slides were cleaned with distilled water. After 10 seconds of decolorisation with 95% alcohol, the area was quickly cleaned with distilled water. The smear was then rinsed off after one minute of counterstaining with saffranin. After letting the slides air dry, they were examined under a microscope with a ×100 magnification oil immersion objective lens.

3.6 Biochemical Identification

Biochemical test will be carried out so as to help in the identification of the bacteria isolates as phenotypic (cultural) characteristics is not sufficient. The various biochemical test carried out are shown below;

3.6.1 Oxidase Test

This is primarily employed to distinguish Pseudomonas from other rods that are Gram-negative. To determine which species of bacteria would produce the enzyme cytochtome oxidase, an oxidase test was performed. As a control, the Gram-positive bacteria Staphylococcus aureus and the Gram-negative bacteria Escherichia coli were used. A sterile wire loop with a piece of filter paper on it The oxidase reagent (1% aqueous tetramethyl-3-phenyl nediamine dichloride) was added in two to three drops upon preparation. Within ten seconds, purple colouration indicates a positive oxidase test.

3.6.2 Urease Test

This is employed in the testing of organisms capable of producing the urease enzyme, which catalyses the conversion of urea into ammonia. Proteus mirabilis and other non-urease positive organisms are typically distinguished from one another using this test. Aseptic test tubes were filled with a sterilised medium, which was then inoculated with the isolated test bacteria and incubated for 24 hours at 37°C. Urease was identified by its reddish-pink colour shift from yellow to red.

3.6.3 Indole Production Test

The purpose of this test is to identify the isolate that can separate indole from the tryptophan in the peptone water. The test is typically used to distinguish between enterobacteriaceae-related and other Gram-negative bacteria. One litre of distilled water was used to dissolve five grammes

of peptone broth that was available for sale. After that, the medium was autoclaved for 15 minutes at 121 degrees Celsius to sterilise it. Each of the bacterial isolates was inoculated into the peptone broth after 4 millilitres of the medium were poured into a sterile test tube. Following a 24-hour incubation period at 37 °C, a few drops of KOVAC reagent were added to the inoculated media. The 150 ml of concentrated hydrochloric acid, 10 g of dimethylamino benzaldehyde, and 150 ml of amyl alcohol make up the KOVAC reagents. The red colouration that appears right away at the top of the test tube indicates a positive test.

3.6.4 Citrate Utilization Test

This test determines which isolates can use citrate as their only carbon source for metabolism. Simon's citrate agar is the test medium used in this investigation. A litre of distilled water was used to dissolve 22 grammes of commercially available Simon's citrate agar, which was then autoclaved for 15 minutes at 121 degrees Celsius to sterilise it. The test organism was inoculated by stabling the medium on the test tubes with sterile straight inoculation wire that contained culture. The medium was then dispensed into the test tubes. The tubes were incubated for roughly twenty-four hours at 37 °C. A shift in colour from green to bright blue indicates a positive outcome.

3.6.5 Catalase Test

This test looks for the catalase enzyme and determines whether it is present or not. The breakdown of hydrogen peroxide to produce free oxygen gas and water is catalysed by the enzyme catalase. The bacterial isolates that were spread out on a slide were given a few drops of recently made 3% hydrogen peroxide. A gas bubble's formation suggested that the catalase enzyme was active.

$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

3.6.6 Sugar Fermentation and Production of Gases Using Triple Sugar Iron Agar (TSI)

The manufacturer's instructions will be followed in the preparation of TSI. The prepared media was put in a test tube and left slanted to solidify. Using a sterile loop, the test bacterium was added to the medium's slant and butt, and it was then incubated for eighteen to twenty-four hours. The production of acid or alkaline in the tube's slant or butt region was used to interpret the results, and the presence of cracks or air bubbles in this area indicated the production of gas. Moreover, the medium's blackening indicated the production of hydrogen sulphide. In accordance with standard microbiological protocol, a prepared laboratory chat was utilised to interpret the results in addition to additional biochemical tests performed on the isolates to verify or identify them.

3.7 Antimicrobial Sensitivity of Extract

3.7.1 Inoculation of Plates

The flood-inoculation technique, a modified version of Acar and Goldstein's method, will be used to accomplish this. Freshly prepared bacterial suspension with turbidity equal to 0.5 McFarland was used to make 2 ml of the suspension, which was then gently spread over the medium's surface by rocking the plate gently. After the excess fluid was removed, the plate was dried for 30 minutes at 37ºC in an incubator.

3.7.2 Agar Well Method

This will be carried out using the modified method of (Bauer *et al.,* 2012). Muellar Hinton Agar was prepared and after sterilization, cooled and poured into the petri dishes and allow to solidify. Upon solidification of the agar, sterile corn-bore of 6mm was used to make well into the agar plates, about 4mm deep. The bacteriocin in different concentrations was transferred aseptically

into the hole and labeled accordingly, and the plate was flooded with bacteria isolates. The petri dishes were incubated at 37°c up to 24 hours after which the radius of the zones of growth of the isolates were measured using graduated ruler in millimeter (mm).

3.7.3 Determination of Minimum Inhibitory Concentration (MIC) and MBC Using Broth Dilution Method

The oil extract's Minimum Inhibitory Concentration (MIC) against bacteria was ascertained using the broth dilution method (Nagalakshmi et al., 2019). Two grammes of the powdered extracts were dissolved in ten millilitres of sterile distilled water to create an aqueous extract solution containing 200 mg/ml of Hibiscus sabdariffa calyxes. In a similar manner, 10ml of 5% dimethyl sulfoxide (DMSO) was used to dissolve 2g of ethanolic extract. The extracts were diluted in test tubes with sterile nutritional broth to achieve different concentrations of 25 µg/ml, 50 µg/ml, 75 µg/ml, and 100 µg/ml. A loopful of the bacterial culture was inoculated into test tubes holding different concentrations of oil extract in Nutrient broth using a standard wire loop (Hi-media). After a 24-hour incubation period at 37 °C, the tubes were checked for growth or turbidity.

3.8 Data Analysis

The SPSS software, version 21.0, was used to analyse the data. The mean of three replicates is used for all data. For every parameter, the mean, range, and standard deviation were found. Duncan's Multiple Range test (SPSS, 2010) was used to separate the means.

CHAPTER FOUR

RESULTS

4.1 Phytochemical Screening of *Hibiscus Sabdariffa* Calyx Extract

Phytochemical screening was carried out on the plant extract to determine it's constituents, the result is showed the presence of Terpenoid, flavonoid, Tanin, phenol and Alkaloid , and also the absence of saponin.

TABLE 1: The result of the phytochemical screening of the *Hibiscus sabdariffa* calyx is represented in the table below:

PHYTOCHEMICALS	**INFERENCE**
Terpenoids	+
Flavonoid	+
Tanin	+
Saponin	-
Phenol	+
Alkaloid	+

Key

+ = Present

- = Absent

4.2 Results for Antibacterial Activity, Minimum Inhibitory Concentration and Minimum Bactericidal Concentration.

The antibacterial effect of *Hibiscus sabdariffa* calyx extract against *Escherichia coli, Staphylococcus aureus, Salmonella typhi* and *Enterococcus faecalis* are presented on Table 2 and 3.

According to the findings, the aqueous extract of Hibiscus sabdariffa calyx showed the strongest antibacterial activity against *Enterococcus faecalis*, with an inhibition zone of 20 mm (100 mg/ml). The alcohol-based extract from the calyx of Hibiscus sabdariffa showed the most potent antibacterial activity against Salmonella typhi, with inhibition zones measuring 20 mm (100 mg/ml), and the least potent antibacterial activity against *Salmmonella typhi*, measuring 7 mm (25 mg/ml).

Tables 4 and 5 display the *Hibiscus sabdariffa* calyx extract's Minimum Inhibitory Concentration (MIC) against *Salmonella typhi*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Escherichia coli*.

According to the findings, the minimum inhibitory concentration (MIC) of *Hibiscus sabdariffa* calyx aqueous extract against *Salmonella typhi*, *Enterococcus faecelis*, *Staphylococcus aureus*, and *Escherichia coli* in aqueous form was 50 mg/ml for *Enterococcus faecalis*, 25 mg/ml for *Staphylococcus aureus*, 100 mg/ml for *Salmonella typhi*, and 100 mg/ml for *Salmonella typhi*. The minimum inhibitory concentration (MIC) of *Hibiscus sabdariffa* calyx alcohol extract in alcohol form was 50 mg/ml for *Escherichia coli*, 25 mg/ml for *Staphylococcus aureus*, 25 mg/ml for *Salmonella typhi*, and 25 mg/ml for *Enterococcus faecalis*.

The Minimum Bactericidal Concentration (MBC) of *Hibiscus sabdariffa* calyx extract against *Escherichia coli, Staphylococcus aureus, Salmonella typhi* and *Enterococcus feacelis* are represented in Table 9 and 10.

From the results, the MBC of the aqueous extract against *Escherichia coli* was 100mg/ml, *Staphylococcus aureus* was 50mg/ml*, Salmonella typhi* was void and *Enterococcus faecalis* was 100mg/ml. The MBC of the alcohol extract against *Escherichia coli* was 50mg/ml*, Staphylococcus aureus* was 50mg/ml*, Salmonella typhi* was 100mg/ml and *Enterococcus feacelis* was 100mg/ml.

TABLE 2: Antibacterial activity of different concentrations of aqueous *Hibiscus sabdariffa* calyx extract against *Escherichia coli, Staphylococcus aureus, Salmonella typhi* and *Enterococcus faecalis.*

ORGANISMS	**Zones of inhibition of different concentrations of aqueous *Hibiscus sabdariffa* calyx extract (mm)**				
	100mg/ml	**50mg/ml**	**25mg/ml**	**12.5mg/ml**	**LEV (500mg)**
Escherichia coli	15 (I)	6 (R)	5 (R)	5 (R)	30 (S)
Staphylococcus aureus	10 (R)	5 (R)	5 (R)	-	24 (S)
Enterococcus faecalis	20 (S)	15 (I)	9 (R)	5 (R)	17 (S)
Salmonella typhi	13 (R)	10 (R)	5 (R)	-	27 (S)

KEY

LEV: Levofloxacin

R: Resistant (0-13mm)

I: Intermediate (14-16mm)

S: Sensitive (17mm and above)

TABLE 3: Antibacterial activity of different concentrations of alcoholic extract of *Hibiscus sabdariffa* calyx extract on *Escherichia coli, Staphylococcus aureus, Salmonella typhi* and *Enterococcus faecalis.*

ORGANISMS	**Zones of inhibition of different concentrations of alcohol *Hibiscus sabdariffa* calyx extract (mm)**				
	100mg/ml	**50mg/ml**	**25mg/ml**	**12.5mg/ml**	**LEV (500mg)**
Escherichia coli	15 (I)	15 (R)	10 (R)	8 (R)	30mm (S)
Staphylococcus aureus	15 (I)	10 (R)	8 (R)	8 (R)	24mm (S)
Enterococcus faecalis	15 (I)	10 (R)	10 (R)	8 (R)	17mm (S)
Salmonella typhi	20 (S)	18 (R)	10 (R)	7 (R)	27mm (S)

KEY

LEV: Levofloxacin

R: Resistant (0-13mm)

I: Intermediate (14-16mm)

S: Sensitive (17mm and above)

TABLE 4: The Minimum Inhibitory Concentration (MIC) of aqueous *Hibiscus sabdariffa* calyx extract against *Escherichia coli, Staphylococcus aureus, Salmonella typhi* and *Enterococcus faecalis.*

ORGANISMS	**MIC (mg/ml)**
Escherichia coli	100
Staphylococcus aureus	25
Enterococcus faecalis.	50
Salmonella typhi	100

TABLE 5: The Minimum Inhibitory Concentration (MIC) of alcoholic *Hibiscus sabdariffa* calyx extract against *Escherichia coli, Staphylococcus aureus, Salmonella typhi* and *Enterococcus faecalis.*

ORGANISMS	**MIC (mg/ml)**
Escherichia coli	50
Staphylococcus aureus	25
Enterococcus faecalis.	25
Salmonella typhi	25

TABLE 6: The Minimum Bactericidal Concentration (MBC) of aqueous *Hibiscus sabdariffa* calyx extract against *Escherichia coli, Staphylococcus aureus, Salmonella typhi* and *Enterococcus faecalis.*

ORGANISMS	**MBC (mg/ml)**
Escherichia coli	100
Staphylococcus aureus	50
Enterococcus faecalis.	100
Salmonella typhi	-

TABLE 7: The Minimum Bactericidal Concentration (MBC) of alcohol *Hibiscus sabdariffa* calyx extract against *Escherichia coli, Staphylococcus aureus, Salmonella typhi* and *Enterococcus faecalis.*

ORGANISMS	**MBC (mg/ml)**
Escherichia coli	50
Staphylococcus aureus	50
Enterococcus faecalis.	100
Salmonella typhi	100

CHAPTER FIVE
DISCUSSION AND CONCLUSION

5.0 Discussion

The extract derived from the calyx of *Hibiscus sabdariffa* exhibits antimicrobial properties. It was effective against *Escherichia coli* and *Enterococcus faecalis* at all tested concentrations, with the most substantial inhibitory zones measuring 15mm and 15mm in both alcohol and aqueous solutions at 100mg/ml. In alcohol and aqueous solutions, the inhibitory zones were 15mm and 20mm at 100mg/ml for *Escherichia coli* and *Enterococcus faecalis*, respectively. *Staphylococcus aureus* and *Salmonella typhi* also displayed susceptibility to the calyx extract at most concentrations, except for one. They exhibited greater susceptibility to the calyx extract in ethanol form and resistance at a concentration of 12.5mg/ml in the aqueous extract.

The results in Tables 7 and 8 indicated that the minimum extract concentration required to inhibit the growth of *Escherichia coli* was 100mg/ml in both aqueous and alcohol extracts, while for *Staphylococcus aureus*, it was 25mg/ml in both aqueous and alcohol extracts. *Enterococcus faecalis* required 50mg/ml and 25mg/ml in aqueous and alcohol extracts, respectively, as the minimum inhibitory concentration. *Salmonella typhi* needed 100mg/ml in aqueous extract and 25mg/ml in alcohol extract as the minimum inhibitory concentration.

Conversely, Tables 9 and 10 revealed that 100mg/ml of aqueous extract and 50mg/ml of alcohol extract were the minimum concentrations required to eliminate *Escherichia coli*. For *Staphylococcus aureus*, 50mg/ml of the extract in both aqueous and alcohol forms was sufficient for eradication. *Enterococcus faecalis* required 100mg/ml of the extract in both aqueous and alcohol forms, and *Salmonella typhi* could be eliminated with 100mg/ml of the extract in alcohol form. All test isolates exhibited sensitivity to the control drug, Levofloxacin (500mg).

The medicinal use of plants, particularly as antimicrobial agents, has been extensively documented by numerous scientists (Cowan, 1999; Sharaf *et al*., 1966; González-Lamothe *et al*., 2009). Research on the antimicrobial properties of Hibiscus sabdariffa has revealed varying degrees of inhibition in microbial growth against both Gram-positive and Gram-negative bacteria. This highlights the wide-ranging effectiveness of the plant extract. Additionally, studies have demonstrated its efficacy against specific strains such as *E. coli* 0157:H7 (Fullerton *et al*., 2011), as well as pathogenic bacteria like *Pseudomonas aeruginosa* and *Escherichia coli* (Khalaphallah and Wagdi, 2014), along with *Klebsiella pneumoniae* (Alshami and Alharbi, 2014).

5.1 CONCLUSION

In summary, our study confirms the antibacterial properties of hibiscus sabdariffa calyx extract against enteric bacteria, aligning with prior research showcasing its antimicrobial potential. These results offer valuable insights into the potential utilization of H. sabdariffa calyx extract as a natural antibacterial remedy against enteric bacteria, presenting an encouraging path for additional research and the exploration of alternative therapeutic possibilities.

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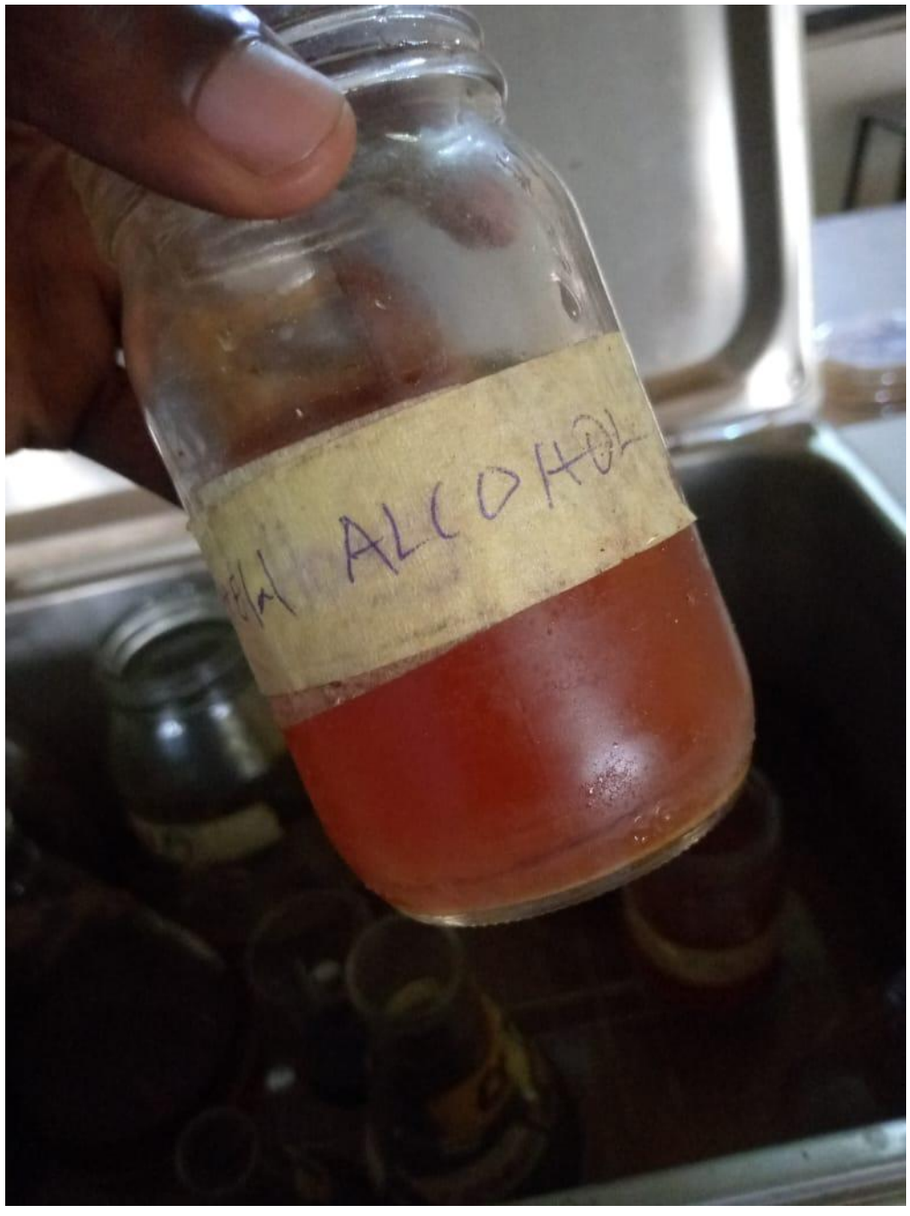
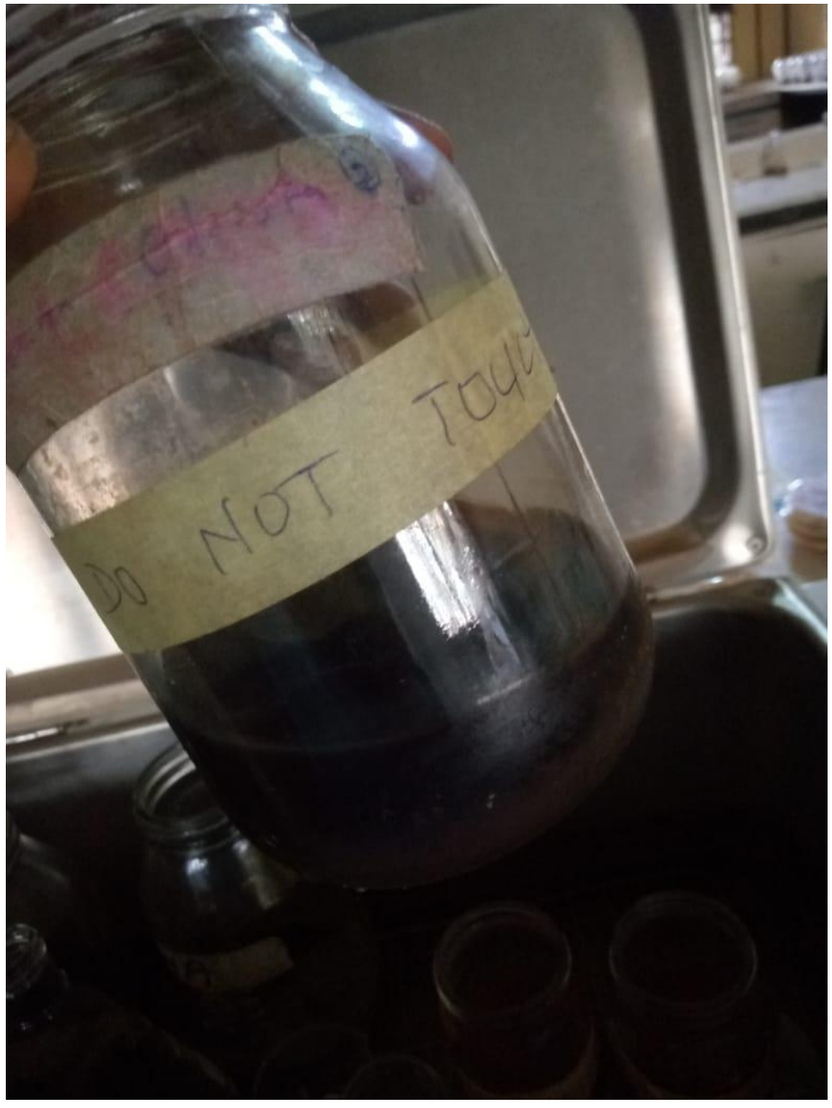
APPENDIX

Hibiscus Sabdariffa plant

Hibiscus Sabdariffa Calyx



Hibiscus Sabdariffa Alcoholic solution



Hibiscus Sabdariffa aqueous solution

Hibiscus Sabdariffa alcoholic extract in Dimethyl sulfoxide (DMSO)

Hibiscus Sabdariffa extract in water bath