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EVALUATION OF ANTIDIARRHEAL, ANTIMICROBIAL, AND ANTIOXIDANT ACTIVITIES OF 80% HYDRO-METHANOLIC ROOT AND LEAF EXTRACTS OF *CARISSA SPINARUM*

MSC THESIS

BY

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BISHOFTU, ETHIOPIA

Evaluation of Antidiarrheal, Antimicrobial, and Antioxidant Activities of 80% Hydro-Methanolic Root and Leaf Extracts of *Carissa Spinarum*

A Thesis Submitted to the Department of biomedical science in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Pharmacology.

MSc Thesis

By

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Bishoftu, Ethiopia

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College of Veterinary Medicine and Agriculture

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As members of the examining board of the final MVSc open defense, we certify that we have read and evaluated the thesis prepared by Berhanu Tafese entitled: **Evaluation of Antidiarrheal, Antimicrobial, and Antioxidant Activities of 80% Hydro-Methanolic Root and Leaf Extracts of *Carissa Spinarum*** and recommend that it be accepted as fulfilling the thesis requirement for the degree of Master of veterinary science in Veterinary Pharmacology.

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Internal Examiner	**Signature**	**Date**

Final approval and acceptance of the thesis is contingent upon the submission of its final copy to the CGS/FGC through the departmental graduate committee (DGC) of the candidate's major department. I hereby certify that I have read the revised version of this thesis prepared under my direction and recommend that it be accepted as fulfilling the thesis requirement.

Thesis advisor	**(Dr. Lishan Asefa)**	**Signature**	**Date**
Dean for Graduate Program		**Signature**	**Date**

DEDICATION

I dedicate this work to God, whose steadfast direction and blessings have been the foundation of my research journey, as well as to my best friend and valued staff member, Tolera Ayeno, for his unwavering support, commitment, and friendship, all of which have been driving forces behind this research. Your belief in me, both personally and professionally, has been a source of strength and inspiration. Your encouragement, hard work, and dedication to helping me have been critical to the success of my academic and research endeavors. Thank you for being my rock of strength and continuous companion. I am truly grateful for your presence in my life.

STATEMENT OF AUTHOR

I hereby affirm that this thesis is my original work and that all sources of materials utilized in its preparation have been properly acknowledged. This thesis is submitted as part of the requirements for the Master's degree in Veterinary Pharmacology at Addis Ababa University, College of Veterinary Medicine and Agriculture. It has been deposited in the university library to be made available to borrowers by the library's regulations. I certify that this thesis has not been submitted to any other institution to obtain any academic degree, diploma, or certificate.

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Date of submission: _____

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TABLES OF CONTENTS **PAGE**

LISTS OF ABBREVIATIONS

AMR	Antimicrobial Resistance
CS	Carissa spinarum
DPPH	2-2-Diphenyl-1-picrylhydrazyl
EM	Ethnomedical
EVM	Ethno-Veterinary Medicine
MDR	Multiple Drug Resistance
MHB	Mueller Hinton Broth
MIC	Minimum inhibitory concentration
PG	Prostaglandin
ROS	Reactive Oxygen Species
TTC	Tetrazolium Chloride

LISTS OF FIGURES

LISTS OF TABLES

LISTS OF ANNEXES

ABSTRACT

Carissa spinarum L is a medicinal plant with therapeutic and nutritional properties; however, scientific evidence of its antidiarrheal and antibacterial potential against resistant bacteria is lacking. Similarly, there is limited evidence available about its anti-oxidant properties, particularly on the root and leaf. Thus, the current study aimed to evaluate the antidiarrheal and antibacterial activities and antioxidant capacities of 80% methanolic root and leaf extracts of *Carissa spinarum*. Each root and leaf powder was extracted by maceration technique using 80% methanol as a solvent. The effects of the crude extract on entero-pooling, gastrointestinal motility tests, and castor oil-induced diarrhea were investigated at 100, 200, and 400 mg/kg doses. The antibacterial activity was conducted by the broth dilution method, and the antioxidant activity was carried out by the 2-2-Diphenyl-1-picrylhydrazyl method. The root and leaf extracts prolonged the onset of diarrhea and reduced the number of wet feces dropped and the total weight of feces significantly compared to the negative control at doses of 200 and 400 mg/kg ($p<0.001$). The results of the enteropooling test demonstrated that the extracts at 200 and 400 mg/kg ($p<0.001$) significantly decreased the weight and volume of the intestinal content. At the test dose of 400 mg/kg, both the leaf and root extracts significantly ($p<0.001$) decreased the weight and volume of intestine content in the enteropooling experiment compared to the negative control group, as well as the 100 and 200 mg/kg doses. The root and leaf extracts at all test doses significantly reduced intestinal motility compared to the negative control ($p<0.001$). In the antibacterial activity test, it was shown that both root and leaf extracts exhibited comparable activity against resistant and susceptible bacteria but showed superior efficacy against resistant strains. The antioxidant assay demonstrated that both plant extracts exhibited promising activity and were comparable to the standard with their inhibitory concentrations (IC_{50}) of (12.61±0.51), (13.6±0.28), and (5.86±0.35µg/ml) of root, leaf, and ascorbic acid, respectively. Thus, the methanolic root and leaf extracts of *Carissa spinarum* support the traditional claims of antidiarrheal, antibacterial, and antioxidant properties. However, further research is needed to elucidate the mechanisms behind antibacterial and antidiarrheal activity, and there is also a need to validate the antioxidant capacity through in vivo assays.

Keywords: *Antibacterial, Antidiarrheal, Antioxidant, Carissa spinarum, Extract*

1. INTRODUCTION

Diarrhea is a gastrointestinal disorder in which fecal mass and water content exceed usual (Khan *et al*., 2023). It is primarily divided into acute and chronic conditions depending on the duration of symptoms (Andargie *et al.,* 2022). Diarrhea can be caused by both infectious and non-infectious agents; however, infectious agents are the most common causes of diarrhea (Taylor *et al*., 2017; Torche et *al*., 2020). Diarrhea (Scours) is a common problem in calves under one month of age, especially those reared for dairy and beef (Torche *et al.,* 2020). It is the second most common cause of death for children under the age of five, next to respiratory diseases, with an estimated 4–8 million fatalities each year (Feyisa *et al*., 2020). Although diarrhea happens worldwide, it is more common in developing countries, especially in South Asia and sub-Saharan Africa (Demissie *et al*., 2021).

The increasing prevalence of AMR worldwide, particularly in bacteria that cause diarrhea, poses a serious risk to public health (Abolarinwa *et al*., 2022). Diarrheal bacteria are rapidly developing pan-drug resistance (PDR)**,** extensive drug resistance (XDR), and multiple drug resistance (MDR) (Ammar *et al*., 2021; Abolarinwa *et al*., 2022). AMR has emerged as a chronic public health problem worldwide, with 10 million deaths expected by 2050. The most important contributor to the current problem is the overuse and misuse of antimicrobials, notably the inappropriate use of antibiotics, which increases the worldwide burden of antimicrobial resistance, highlighting the need for traditional herbal remedies for disease treatment and prevention (Tang *et al*., 2023).

Nowadays, reactive species released into the environment as a result of industrialization processes are thought to be the source of many chronic ailments. Oxidative stress is recognized to be the cause of some of these illnesses, including cancer. It is a condition that occurs when the amount of reactive oxygen species (ROS) and other free radicals produced in the body exceeds the amount of natural antioxidant scavengers in the body (Salehi *et al*., 2019).

Ethiopia, with its unique geography and climate, has a rich history of using medicinal plants for traditional medicine since ancient times. With around 6,000 species, it has become an integral part of Ethiopian culture (Shure *et al*., 2022). Several varieties of medicinal plants are useful in Ethiopia for managing and treating diarrheal illnesses. A few of the Ethiopian medicinal plants that are frequently used to treat diarrhea are *Verbena officinalis L, Vernonia amygdalina Del, Carissa spinarum, Ensete ventricosum (Welw.) Cheesman, Calpura aurea, Justicia schimperiana (Hochst. Ex Nees) T.Anders, and Coffea arabica L* (Degu *et al*., 2022).

Carissa spinarum L. is categorized under the family Apocynaceae. This plant is locally known as Agam in Amharic and Hagamsa in Afaan Oromo in Ethiopia. In several African nations, *Carissa spinarumL*. is also known as the "Magic Shrub" since it is used as a source of treatment for a variety of illnesses and ailments (Berhanu *et al.*, 2020). Morphologically, it has forked branches, is woody, and can reach heights of up to 2–3 meters. Its appearance ranges from light brown to green, with the base being brown and the tip being deep brown (Rubaka *et al*., 2014). Several medicinal plants in Ethiopia are used directly in their raw form to treat a variety of ailments. The *Carissa spinarum* parts, which include roots, leaves, rootbarks, stems and stembarks, and ripe or unripe fruits, are widely used for the treatment of various diseases and exhibit anticancer, antimicrobial, antioxidant, anti-inflammatory, hepatoprotective, anti-arthritic, antipyretic, anthelmintic, vasorelaxant, and wound-healing properties (Sharma *et al.*, 2023).

Medicinal plants are essential sources of biologically active ingredients for traditional and modern medicine due to phytoconstituents like flavonoids, alkaloids, saponins, terpenoids, steroids, and tannins (Berhanu *et al*., 2020). The previous study on the various components of CS reported the presence of primary and secondary metabolites. Among the secondary bioactive metabolites (phytocompounds), the most common reported from *Carissa spinarum* include saponins, alkaloids, tannins, flavonoids, glycosides, phenols, cardiac glycosides, sterols, and terpenoids (Ayalew *et al*., 2022).

1.1. Statement of the Problem

In Ethiopia, the prevalence of diarrhea cases in both humans and animals remains high, despite the availability of synthetic medications for its treatment. Due to the adverse drug effects and reduced efficacy of current antidiarrheal agents, as well as the development and spread of AMR, it is imperative to explore alternative options that are safe, affordable, and cost-effective. One of the possible alternatives to antimicrobial agents is the use of natural plant-derived medicines. Medicinal plants have been used for the treatment of diarrhea for centuries in Ethiopia, and *Carissa spinarum* is one of the medicinal plants traditionally used by the Ethiopian community for the treatment of diarrhea, particularly their roots and leaves, based on ethnobotanical surveys. However, there is a lack of scientific study on the antidiarrheal activity of *Carissa spinarum* that validates the traditional claim. In addition, the effectiveness of *Carissa spinarum* against antibiotic-resistant bacteria, specifically in comparison to susceptible strains, as well as its therapeutic potential for oxidative stress-related disorders, is currently limited in the available literature. Therefore, there was a necessity to conduct a thorough investigation to bridge the knowledge gap regarding *Carissa spinarum's* efficacy against drug-resistant bacterial strains, its potential as an alternative treatment for diarrhea, and its antioxidant properties. Thus, this study aimed to explore the potential benefits of *Carissa spinarum L*, a medicinal plant traditionally used in Ethiopia, for treating diarrhea, combating bacterial infections, particularly multi-drug-resistant bacteria, and providing antioxidant benefits.

1.2. Objectives of the Study

General Objective

- To evaluate the in vivo antidiarrheal, in vitro anti-oxidant, and antibacterial activities of 80% hydro-methanolic root and leaf extracts of *Carissa spinarum.*

Specific objectives

- To evaluate and compare the antidiarrheal properties of root and leaf extracts of *Carissa spinarum.*
- To determine and compare the antibacterial activity of *Carissa spinarum* extracts against certain susceptible and resistant bacterial pathogens of known strains.
- To evaluate the potential antioxidant activity of the leaf and root extracts of *Carissa spinarum.*

2. LITERATURE REVIEWS

2.1. Background

Diarrhea (scours) is a condition in which feces are discharged from the bowels frequently and in a liquid form (Abolarinwa *et al*., 2022). It is the most commonly reported calf disease and the main reason behind neonatal deaths in calves younger than one month old, in both dairy and meat. It causes significant economic loss due to treatment expenses, stunted growth rates, and calf death (Torche *et al*., 2020). Diarrhea is a serious public health problem worldwide, causing substantial morbidity and mortality, particularly in underdeveloped nations (Naghavi *et al*., 2017). Diarrhea causes around 1.6 million fatalities worldwide each year, with underdeveloped countries and economically disadvantaged regions taking the majority of the burden (Abolarinwa *et al*., 2022; Wolde *et al.,* 2022). With a population of over 110 million, Ethiopia is the second-most populous country in Africa. Of them, almost 14% are children under the age of five (Wolde *et al*., 2022). The burden of diarrheal illness remains high despite the measures implemented, and the prevalence and contributing factors of diarrhea vary significantly across the nation's various regions. Ethiopia has a greater incidence of diarrheal diseases than other sub-Saharan African nations, which contributes to preventable fatalities (Shine *et al*., 2020). Diarrheal diseases also account for 9% of child mortality (Wolde *et al*., 2022).

Phytochemical studies have revealed *Carissa spinarum* is rich in numerous major secondary metabolites, suggesting the plant may have antidiarrheal effects. The pharmacological investigations conducted on Carissa spinarum in vitro and in vivo demonstrated its antipyretic, anticancer, wound-healing, anti-venom, anti-inflammatory, anthelmintic, anticonvulsant, antiarthritic, antibacterial, antioxidant, and hepatoprotective properties (Megersa and Tamrat, 2022; Sharma *et al*., 2023).

An ethnobotanical study in Ethiopia shows that *Carissa spinarum* is commonly used in Ethiopian society to treat diarrhea, with both the root and leaf parts of the plant being utilized for this purpose. The dry roots of Carissa spinarum are powdered, combined with salt, and consumed orally to treat diarrhea (Mekonnen *et al.,* 2022). Similarly, Carissa spinarum leaf is

powdered, combined with *Coffee arabica,* and traditionally drunk for the treatment of diarrhea (Megersa and Tamrat, 2022).

In recent years, a great deal of research has been done on the possible contribution of bioactive molecules derived from plants to the reduction of oxidative stress. Because of their structural appropriateness as antioxidants, phenolic phytochemicals have been recognized as having this role. ROS and other radicals are immediately neutralized by antioxidants as soon as they are formed (Salehi *et al.,* 2018). Medicinal plants, rich in antioxidant properties like phenols, flavonoids, and terpenoids, have been effectively used to treat ROS as a dietary supplement for scavenging radicals (Li *et al*., 2019).

2.2. Causes of Diarrhea

The causes of diarrhea can be classified as infectious and non-infectious agents; however, infectious agents are the most common causes of diarrhea (Torche *et al*., 2020). Bacteria, viruses, and protozoa are typically the most common causes of diarrhea in both people and animals. The most frequent cause of acute diarrhea is infectious pathogens (Akram *et al*., 2020). Among infectious agents, bacteria like Campylobacter, Vibrio cholera, Salmonella, Shigella, and Escherichia coli and viruses, particularly rotavirus, are regarded as typical causes of acute diarrhea (Thapar and Sanderson, 2004). Infectious diarrhea is also an intestinal infectious disease, including typhoid and paratyphoid fever, bacterial and amebic dysentery, and other infectious diarrhea (Liang *et al*., 2021).

Non-infectious diarrhea is commonly caused by drugs, toxins, food additives, medications, irritable bowel disorder, malabsorption, and inflammatory bowel syndrome (Ede, 2014). Antibiotics are prescribed for the treatment of various ailments, including diarrhea, but often the same agents can induce diarrhea (Akram *et al*., 2020; Khan *et al*., 2023). Diarrhea, regardless of its underlying cause, can result in electrolyte imbalances, dehydration, metabolic acidosis, and potentially even septicemia because of subsequent bacterial overgrowth in the small intestine (Taylor *et al*., 2017; Torche *et al*., 2020).

2.3. Medicinal Plants and Their Biological Activities

A plant is considered medicinal if any of its organs contain substances that can be used as medicine or to create potent pharmaceuticals. Traditional medicinal plants are increasingly used in primary healthcare worldwide, with scientists exploring new phytochemicals for antimicrobial treatment (Samie *et al*., 2010). Despite their rich secondary metabolites, few are being explored for novel antimicrobials. Medicinal plants are also used in the production of modern drugs, providing direct therapeutic agents, raw materials, and taxonomic markers. Rural areas rely on these plants due to a lack of facilities, clinics, and expensive modern medicines. Traditional medicines are locally available and cheaper than standard treatments, making them the only option for treating many diseases (Ramor and Ponnampulam, 2008; Berhanu *et al*., 2020).

2.4. Ethiopian Medicinal Plants for the Treatment of Diarrhea

Plants and plant extracts have long been used to treat gastrointestinal disorders, including diarrhea (Akram *et al*., 2020). Herbal treatments are thought to be useful in treating diarrhea. Modern medications are unlikely to quickly replace the traditional remedies utilized in rural African communities to treat diarrhea (Njume & Goduka, 2012). These days, there is strong support for incorporating herbal therapy into contemporary medical procedures. Moreover, the active ingredients in herbal medicines function as prototype leader compounds for the creation of novel medications (Fong, 2002). Some of the medicinal plants traditionally used frequently for diarrheal treatment and management in Ethiopia were shown in Table 1.

Table 1: Some medicinal plants frequently used traditionally for diarrheal treatments

Plant Scientific Name	Family Name	Plant Parts	Methods Of Application	Disease	References
Coffea arabica L	Rubiacea	Seed	The roasted and powdered seed is consumed on an empty stomach for 2–3 days.	Diarrhea	Abera. (2014)
Lepidium sativum L	Brassicaceae	Seed	Seeds are ground into powder, mixed with honey and then taken for three day	Diarrhea	Behailu (2010)
Verbena officinalis L	Verbenaceae	Leaf root	Leaf and/or root juice drunk against diarrhea	Diarrhea	Araya *et al.* (2015)
Ensete ventricosum (Welw.) Cheesman	Musaceae	Root ,rhizome	Ensete-prepared meals are consumed with butter and meat. It's crushed, pounded, and then combined with water. Drink this mixture.	Diarrhea	Solomon (2011)
Carissa spinarum	Apocynaceae	root	The dry root is ground into a powder, mixed with salt, and consumed as a solution.	Diarrhea	Mekonnen *et al.(*2022)
Vernonia amygdalina Del	Asteraceae	leaf	Pounded, mixed, and dissolved with minimal water, then consumed for five days.	Diarrhea	Bekele and Reddy,(2015
Calpurnia aurea (Ait). Benth	Fabaceae	leaf	The leaf is mixed with water and administered orally.	Diarrhea	Enyew *et al*. (2014)
Zehneria scabra (Linn. f.) Sond.	Cucurbitaceae	leaf	The fresh leaf is squeezed and the juice is drunk	Diarrhea	Tadesse *et al* .(2014)
Cordia Africana Lam	Boraginaceae	leaf	The leaf is crushed and drunk it alone or by mixing it with boiled coffee	Diarrhea	Asrie *et al*.(2016)

2.5. Botany of *Carissa spinarum*

2.5.1 Description and distribution

Carissa spinarum (CS) is a species in the Apocynaceae family that grows up to 6 meters tall as a thorny, densely branching small tree or shrub, as shown in Figure 1. It features masses of fragrant evergreen leaves and, pink- or purple-tinged white flowers. The fruits start green, turn red, and ripen to a purplish-black color, ultimately becoming edible (Smyth and Sheridan, 2022). Of the species in the Apocynaceae family, CS is one of the most extensively dispersed. It lives in a variety of environments at elevations between 0 and 2450 meters, including thickets, savannahs, and wet or dry woods. However, it does not appear in extremely humid regions or equatorial rainforests (Leeuwenberg and Van Dilst, 2001; Smyth and Sheridan, 2022). Its wide ecological tolerance is shown in the global distribution of ethnobotanical and botanical samples. 74 nations and territories on three continents—Australia, Sub-Saharan Africa, the Arabian Peninsula, the Indian Ocean islands, and South, Southeast, and East countries—are home to CS (Smyth and Sheridan, 2022). *Carissa spinarum* is among the most well-known local plants in Ethiopia, where the raw form is used to treat various illnesses, including diarrhea, in various regions of the country (Tiruneh *et al*., 2022).

Figure 1: *Carissa spinarum* photo taken from the study area

2.5.2. Ethno medicinal uses of Carissa spinarum

Carissa spinarum has been used for a very long time. India, Ethiopia, and other African nations have long used the entire plant to treat a variety of illnesses, including gastrointestinal, respiratory, and venereal infections (Dhatwalia *et al*., 2021); fever, jaundice, hepatitis, cardiac conditions, diabetes, malaria, and pneumonia (Maobe *et al*., 2012); chronic joint pain (Wambugu *et al.*, 2011); sickle-cell anemia, hypertension, diarrhea, kidney complications, worms, cancer, gastric ulcers, and eye cataracts (Sharma *et al*., 2017). In addition, it has been used as an antivenin for snake bites and to treat microbiological illnesses such as polio, typhoid fever, syphilis, gonorrhea, herpes, and rabies (Kelemu and Wolde, 2018). According to Bhadane *et al*. (2018), it has also traditionally been used to treat male infertility and sexual issues such as asthenia and early ejaculation. Additionally, it is used as an ethnoveterinary medicine to treat anaplasmosis. Mosquito repellant is another application for CS leaves. According to reports, *C. spinarum* has been used to cure worm infestations in injured animals, reduce bleeding after birth, treat ulcers and muscle cramps, and others, as stated in Table 2 (Sharma *et al*., 2023).

Table 2: Ethnomedicinal uses of various parts of *Carissa spinarum*

Plant part	Ethinomedecinal uses	method of preparation and application	References
Root	Diarrhea	The dry root is ground into a powder, mixed with salt, and consumed as a solution.	Mekkonen *et al.*(2022)
Root	Diarrhea	One cup of freshly crushed and squeezed roots is consumed orally after being mixed with water.	Mengesha(2016)
Root	Diarrhea	Boiling the root part and taken it orally.	Tefera *et al*.(2019)
Leaf	Diarrhea	Powdered Carissa spinarum leaf is combined with Arabic coffee and consumed.	Megersa and Tamrat (2022)
Leaf	Diarrhea	After boiling the leaf with coffee arabica, the filtrate is consumed.	Usman *et al*.(2022)
Leaf	Diarrhea	Leaf was pulverised, combined with L. arabica coffee, and consumed.	Meragiaw *et al*.(2016)
Root	Wound	After being crushed, root bark is put to the incision for three days.	Jima and Megersa (2018)
Root	Evil eye	They add powdered dried root bark, fire it, and let them inhale it.	Amsalu *et al.* (2018)
Root	Intestinal Worm	Water is used to dissolve the roots, and then they are consumed.	Kefalew *et al*.(2015)
Root	Cold Disease	Crushing or chewing the root, boiling it, and then drinking one glass of water.	Fenetahu and Eshetu (2017)
Root	devil sickness	One cup of oral fresh roots is obtained by crushing and squeezing them with garlic and water.	Mengesha(2016)
Leaf	febrile illness	Squeezed, crushed, and liquidised leaves are consumed with coffee.	Araya *et al*.(2015)
Root	Gonorrhea	The fresh root of C. spinarum was minced and combined with cold water. To treat gonorrhea, one cup of tella (beer) is consumed over three days.	Smyth and Sheridan (2022)
Root	Brain tension	After crushing, fumigate	Chekole *et al*.(2015)
Fruit	Devils disease	Boil the fruit, and then combine it with the roots of P. schimperi, C. macrostachyus, and A. schimperiana. Drink the resulting suspension.	Mazengia *et al*.(2019)
Root	Snake Bite	After pounding the root, consume the extract.	Abebe (2021)
Root	Tooth Ache,Abdominal Pain	root is chewed	Abebe (2021)
Root	Dermatitis	crushing and mixing the *carissa spinarum* with water and drink	Fassil and Gashaw, (2019)
Root	Stomachache, Impotence	by crushing the root and leaf , then taken orally	Worku (2019)
Root	Malaria	Fresh root cut into pieces and mixed with cold water, then drunk after one day.	Megersa *et al*.(2023)
Leaf	Anthrax	Crushed leaves are applied onto the swelling	Birhan *et al*.(2023)
Leaf	Throat/oral cancer	Young twigs and fresh leaves are gathered, crushed to form a paste, and combined with honey.The mixture is then taken orally until it cures, and the fruits can be used to treat cancer.	Ayele (2018)

2.5.3. Ethnobotanical uses of Carissa spinarum in human medicine

The core features of the analysis in ethnomedicine (EM) of *Carissa spinarum* included plant parts used, preparation methods, administration route, and ailments managed by *C. spinarum.* The application of various plant components to the various disorders examined was then analyzed. The two primary regions where *C. spinarum* is commonly used in EM are South Asia (mostly India and Pakistan) and Eastern Africa (primarily Kenya and Ethiopia). Along with the Arabian Peninsula, Northern Africa, Australia, and South and East Asia, the remainder of Sub-Saharan Africa likewise uses *C. spinarum*, as stated in Figure 2 (Smyth & Sheridan, 2022).

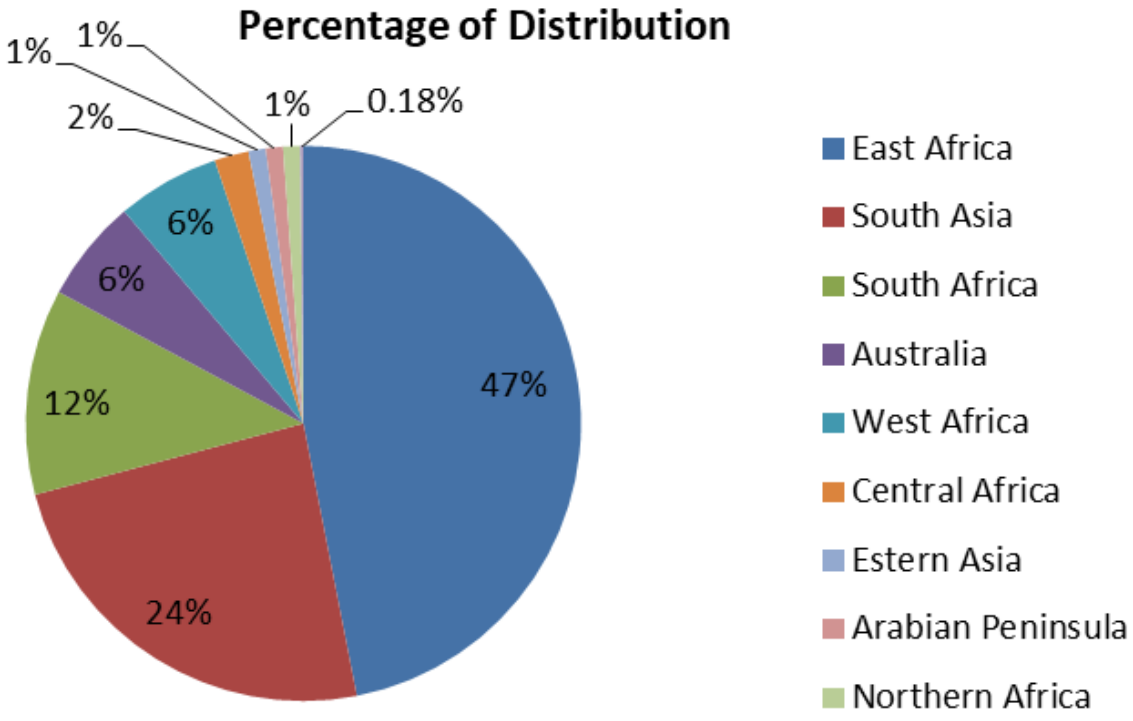

Figure 2: Geographical distribution of human EM use report (URs) for *Carissa spinarum*

2.5.4. Carissa spinarum plant parts used and their routes of administration

Most parts of the *Carissa spinarum* plant are employed in human medical formulations throughout the world. The root is the part of the *Carissa spinarum* that is most widely used by the majority of society across the globe. This indicates that the most often used plant part for *C. spinarum* is the root, with the leaf, fruit, stem, rootbark, and bark following in order of preference, as shown in Figure 3. Nine percent of reports (N/S) do not indicate which section of the plant is used (Smyth & Sheridan, 2022).

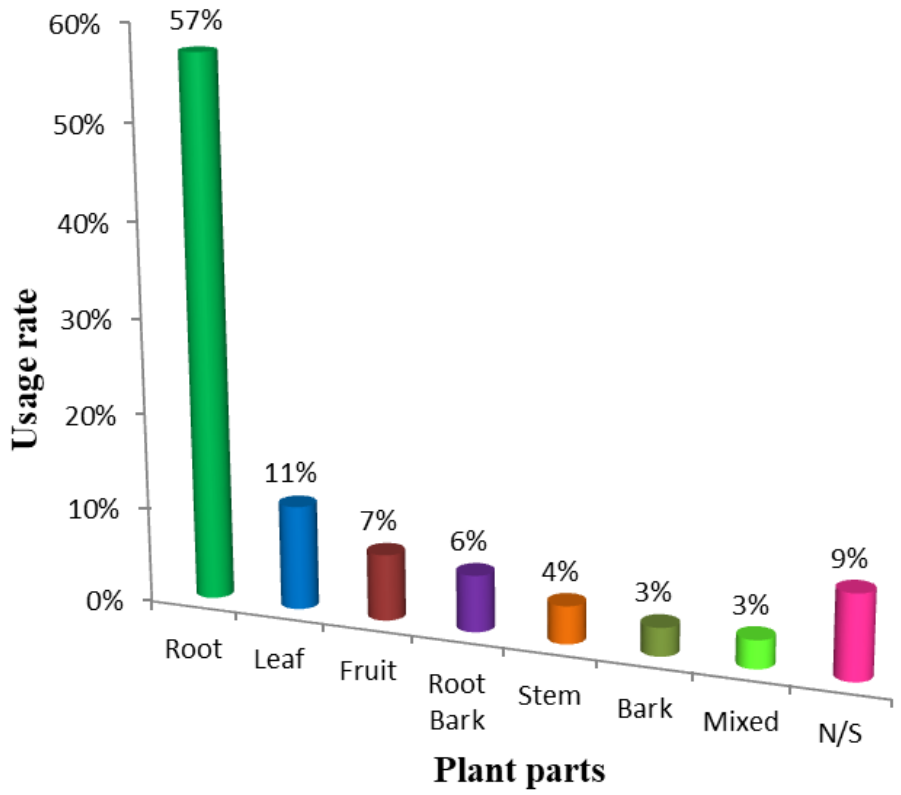

Figure 3: Parts of *Carissa spinarum* used for preparation of ethnomedicinal

2.4. Ethno-Veterinary Medicine (EVM) Uses of *Carissa Spinarum*

A study of traditional veterinary expertise in the Algerian steppe examined the common application of plant species used for human EM in animal healthcare (Miara *et al*., 2019). The authors proposed that zoo pharmacognosy—the practice of treating oneself by observation—may be the source of human use. This could influence the choice of treatment in the case of *C. spinarum*. The leaves of *C. spinarum* are known to be browsed by animals, and EVM uses them to cure helminths and other intestinal diseases in both humans and animals. When it comes to human use, the most commonly used part of the plant is the root, followed by the bark, leaf, fruit, and leaf (Smyth & Sheridan, 2022).

2.5. Phytochemical Component *Carissa Spinarum's*

Chemical components (phytochemical constituents) that cause a particular biochemical effect in humans and animals are what give ethnomedicinal plants their therapeutic efficacy. The leaves, fruits, stems, bark, seeds, and roots of medicinal plants, as well as their parts, are naturally rich in phytochemicals. The plant's defense system against harm and disease is largely dependent on these phytochemicals, which consist of both primary and secondary substances (Jaturapronchai, 2003). The leaves, roots, fruits, bark, and stem of CS have a wide variety of phytochemicals that provide the plant with a great deal of therapeutic benefit (Ansari *et al.,* 2018). With their complex structure, these phytochemicals are the active components of herbal medications that have combined antibacterial effects to increase their therapeutic effectiveness (Doshi *et al.,* 2017).

When the plant's root, leaf, stem, fruit, and bark are extracted using various solvents, important bioactive components are found, including acids, carinol, carindone, carissone, alanine, carinol, glucose, digitoxigenin, citric and lupeol, glycine, galactose, malic, glycolic, and malonic acids, sugars, oxalic acid, odoroside H, non-reducing sugars, phenyl alkaline, pectin, and vitamin C. Additional significant phytochemical components comprise carissol, which is an epimer of α-amyrin, anthraquinones, gallic tannins, emodins, lignins, anthocyanins, reducing agents, alkaloids, sugars, cardiac glycosides, and zavonoids (Mundaragi & Thangadurai, 2017). In addition, *Carissa spinarum* includes reducing sugar, tannins, tartaric

acid, sterols, glycosides, phenolic compounds, and triterpenoids (Afanyibo *et al*., 2019). However, prior studies have found that alkaloids, coumarins, fatty acids, flavonoids, leucoanthocyanins, saponins, and triterpenoids are absent from the ethanolic root extract of *Carissa spinarum* (Cs *et al*., 2013).

2. 6. Pharmacological and Antimicrobial Activities of Carissa Spinarum

Researchers were utilizing scientific pharmacological screening to confirm the usefulness of *Carissa spinarum* and other *Carissa spinarum* species, as suggested by their traditional applications. Several crude extracts and bioactive components that were separated from different plant parts have been tested for a range of biological activities, including wound healing, anthelmintic, hepatoprotective, anticonvulsant, antioxidant, antipyretic, antibacterial, anticancer, anti-inflammatory, and antidiabetic. Scientific evidence supporting the traditional use of this plant has been provided by observations of their therapeutic potential in a variety of animal models, both in vitro and in vivo (Dhatwalia *et al.,* 2021; Megersa and Tamrat, 2022; Sharma *et al*., 2023).

Numerous studies have documented the antibacterial properties of various components of *Carissa spinarum*. The antimicrobial activity of the CS root methanolic extract was evaluated against five selected bacteria and fungi. The results revealed the minimum inhibitory concentration values for *Bacillus subtilis*, *Escherichia coli*, Streptococcus sp., *Staphylococcus aureus*, and *Aspergillus niger* were 512 ± 43 µg/mL, 125 ± 10 µg/mL, 165 ± 20 µg/mL, 110 ± 28 µg/mL, and 256 ± 30 µg/mL, respectively. Rubaka *et al. (2014*) found high antibacterial activity against *E. coli* and *S. aureus* using methanolic and ethanolic root extracts of *Carissa spinarum*, with a diameter of relative inhibition widths of 54.66–57.24% and 66.97–70.13%, respectively. The summarized form of antimicrobial activities of *Carissa spinarum* is shown in Table 3.

Table 3: Antimicrobial properties of *Carissa spinarum* extracts against various microbes

Parts of Plant	Used Extract	Organism	Results	References
Roots	methanolic	*E.Coli*	Mic-125± 10µg/ml	Sanwal & Chaudhary (2011)
		B.subtilis	Mic-512± 43µg/ml	
		staphy.auereus	Mic-110± 28µg/ml	
		strept. spp	Mic-165± 20µg/ml	
		A.niger	Mic-256 ± 30µg/ml	
Root, Leaves,and Bark.	95%ethanol, methanol,and petroleum ether	*E.coli* DSM1103	ZOI-2.33±0.58-13.33±1.53mm	Rubaka *et al*(2014)
		S.auereus ATCC,25923	Mic-312 ± 5000µg/ml	
Leaf	n-hexane, ethyl acetate,and methanol	*E.coli.*ATCC- 25922	ZOI- 15mm at 0.5 mg/ml in ethyl acetate extract	Feyisa & Melaku (2016)
Fruit	nano emulsion	*S.auerues, salmonella typhi B.subtitils*	Mic- 30-50µg/ml	Doshi *et al.*(2017)
Root and Leaf	petroleum ether,hexane, ethyl acetate, and chloroform	MRSA, *E.coli,* proteus, P.fluerenses	ZOI - 20-30 mm	Joshi & Singh (2017)
Leaf	methanol and its solvent fractions	*S.auerues* and *S.pneumonia E.coli* & *k.pneumonia*	ZOI - 7-13 mm	Tiruneh *et al.* (2022)

Key notes: ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen; MIC, minimum inhibitory Concentration; ZOI, Zone of inhibition.

2.7. Antioxidant activity of *Carissa spinarum*

Free radicals are highly reactive molecules created during oxidation processes. These radicals then start a series of reactions that cause harm to cells. Free radicals are the source of many degenerative diseases that affect humans, such as cancer and damage to the central nervous system. Active ingredients included in natural products have been shown in studies to protect cells from the damaging effects of free radicals (Nigussie *et al*., 2023).

Oxidative stress is caused by an unbalanced free radical in the biological system. It is produced as a byproduct of oxygen metabolism. The development of many chronic diseases is attributed to the overproduction of unstable free radicals in the body (Pizzino *et al*., 2017). DPPH is a stable free radical with a nitrogen center that can take on an electron or hydrogen radical to transform into a stable diamagnetic molecule. Antioxidants give DPPH radicals an electron or hydrogen radical, which causes hydrazine to be produced in response to the radicals' reaction with reducing agents. Then, the solution changes from purple to yellow (Kumar *et al*., 2020). Some of the reported antioxidant properties of *Carissa spinarum* from different plant parts are shown in Table 4.

Table 4: The antioxidant activity reports of *Carissa spinarum* parts.

part/extract used	methods used	results	references
Stems by chloroform extract	DPPH	IC50—47.03	Raol et al.(2005)
fruit/ ripe fruit extract	DPPH	IC50—4. 69 mg/ml	Nazareth et al. (2021)
Fruit extract	DPPH	IC50–1013±2.00µM AEAC/100G dry wt	Nayak and Basak(2015)
	FRAP	IC50—2118±1.00µM AEAC/100G dry wt	
	Peroxidase(POX)	POX–0.001±0.0003 OD/min/gfwt	
	Catalase(CAT)	CAT—1.1119±0.004 U/ml	
	superoxide dismutase(SOD)	SOD—0.151±0.001 od/min/g tissue wt	
hydroalcolic and aqueous extract of root bark	DPPH	IC50—75.5±65.02 µg/ml (aqueous extract)	Afaanyibo *et al*(2019)
		IC50—96.10±1.11 µg/ml (hydro alcolic extract)	
methanolic root bark extract and its sub-fractions	DPPH	ethyl acetate showed a strong DPPH and FRAP activity as compared to that of other fractions	Liu *et al*.(2021)

Key note: DPPH, 2-2-Diphenyl-1-picrylhydrazyl; FRAP, Ferric reducing antioxidant power

2.8. Toxicity and Safety Profile

Numerous investigations have been carried out to assess the toxicity of various CS components. Hegde and Joshi (2010) stated that the ethanolic root extract of CS was safe to use in rats at a dose range of 0.5–1 g/kg body weight and that there was no rat mortality. Up to concentrations of 5000 mg/kg of plant extracts, the Carissa spinarum crude extracts did not result in any detectable alterations in behavior or physical characteristics, nor fatalities.

The chloroform and hydromethanolic extracts did not considerably change the hematological or physical parameters seen in groups treated relative to the control groups in subacute toxicity experiments ($p > 0.05$) (Gebrehiwot, 2019). In addition to studying the cytotoxicity of hydroalcoholic CS root extract on Artemia salina, Dossou-Yovo *et al*. (2021a**)** also investigated the oral toxicity of the extract on Wistar rats, both acute and subacute (28 days). The CS root hydroalcoholic extract's lethal concentration (LC50) was determined to be 0.9 mg/mL. No toxicity indications or fatalities were observed after a 28-day dosage of 500–1000 mg/kg of CS root hydroalcoholic extract. Regarding subacute toxicity, no harmful or lethal results were seen during the investigation. Similar results were previously reported about the oral toxicity of 500–1000 mg/kg body weight of ethanolic CS root extract in Wistar rats (Dossou-Yovo *et al.*, 2021b).

2.9. Antibiotic Resistance

Antibiotics are thought to be the most important medical advancement of the 20th century and are the "magic bullets" for combating bacteria (Salam *et al*., 2023). Antibiotic resistance is one of the most serious and critical public health challenges of the twenty-first century. When compared to drug-susceptible microorganisms, infections caused by multidrug-resistant (MDR) pathogens are associated with higher mortality rates (WHO, 2013; Keita *et al*., 2022).

A high selection pressure resulting from the overuse and abuse of antibiotics over time has led to the development of acquired antimicrobial resistance (AMR) in microbes to numerous medications (Salam *et al*., 2023). The 2022 Global Antimicrobial Resistance and Use Surveillance System (GLASS) report reveals alarming rates of resistance in common bacterial infections. The WHO revealed a list of pathogens in response to the rise in antibiotic

resistance. This list includes the pathogens known by the acronym ESKAPE, which stands for *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and Enterobacter species. These pathogens were assigned the highest "priority status" because of their significant threat to humans (Mancuso *et al*., 2021).

Because of the major threat posed by the growth and spread of drug-resistant bacteria around the world, there is an urgent need for new drugs with different modes of action capable of mitigating the prevalence of antimicrobial resistance. Secondary plant metabolites are one of the most underexplored natural sources of antibacterial agents. It is estimated that less than 1% of tropical plant species worldwide have been tested for medicinal uses and studied phytochemically (Keita *et al*., 2022). Similarly, numerous scientific studies reported over the last three decades indicate that medicinal plants could be a promising alternative to ineffective antibiotics in fighting infectious infections. In recent years, alkaloids, phenolic compounds, terpenoids, and saponins have shown significant antibacterial activity, notably through membrane disruption mechanisms, anti-quorum sensing, and interference with intermediate metabolism, protein binding, and anti-biofilm activity (Abdallah *et al*., 2023).

3. MATERIALS AND METHODS

3.1. Description of Plant Collection Area

The roots and leaves of *Carissa spinarum* were gathered from the Welmera district for experimental purposes. Welmera is a district in the Oromia region and located at 29 kilometers to the west of Addis Ababa city on the main road to Ambo. Holeta and Kolobo are the two towns located in the Welmera district. It is bordered by the west Shewa zone on the west, Addis Ababa city on the east, Sululta on the northeast, and Sebeta Hawash on the south. The district's altitude ranges from 2060 meter above sea level to 3380 meter above sea level. The Welmera district lies between latitudes 8 050' and 9 0 15 N and longitudes 38025' and 390 45' E. There are two agroecological zones in Welmera woreda: highland and midland. The midlands make up about 39% of the total, with the highlands making up 61%. The average annual temperature ranges from 0 to 27 °C (degree centigrade), while the average annual rainfall is between 834 mm and 1300 mm (Gudina & Alemu, 2023).

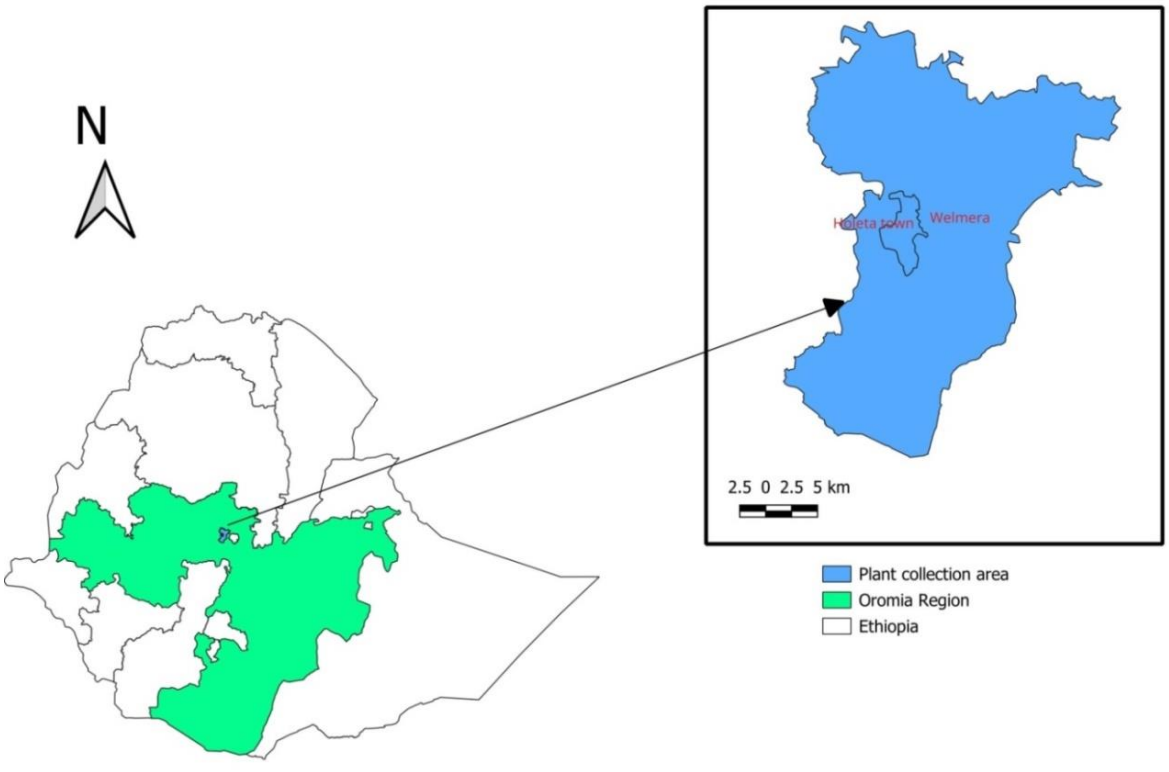

Figure 4: Description of plant collection area

3.2. Experimental Study Area

The experimental study, which focused on evaluating the in vivo antidiarrheal, in vitro antibacterial, and antioxidant activities of the 80% methanolic root and leaf extracts of *Carissa spinarum*, was conducted at the esteemed Armauer Hansen Research Institute (AHRI). This renowned research institute provided the ideal setting for carrying out the study and ensuring correct and reliable results.

3.3. Study Design

The study employed an experimental research design, conducted from January to May 2024, to evaluate the in vivo antidiarrheal (using mice model), in vitro antibacterial, and antioxidant activity of the 80% methanolic root and leaf extracts of *Carissa spinarum*. Swiss albino mice were used as the experimental subjects for their antidiarrheal activity, and the extracts were tested at various concentrations in all experimental studies. The selected bacteria included both MDR and susceptible strains obtained from Addis Ababa University, the College of Veterinary Medicine and Agriculture, Veterinary Microbiology, and the Ethiopian Public Health Institute (EPHI), respectively. This study design allowed for a comprehensive assessment of the effectiveness of the *Carissa spinarum* extracts in addressing antidiarrheal, inhibiting bacterial growth or killing, and exhibiting antioxidant properties.

3.4. Chemicals, Equipment, and Materials

The chemicals used during this experimental study include methanol (loba chemie pvt. Ltd.), atropine sulfate (sigma-aldorich), activated charcoal, loperamide hydrochloride (Medochemie Ltd.), ciprofloxacin references (200 mg), Muller-Hinton agar and broth, distilled water, and castor oil (Humanwell Pharmaceutical Ethiopia PLC), DPPH (sigma-aldorich), and ascorbic acid (sigma-aldorich). A pestle and mortar, a transparent plastic ruler, plastic paper, measuring cylinders, a funnel, a beaker, a surgical scalpel blade, forceps, an oral gavage, and an Erlenmeyer flask. Electrical grinder (karl kolb), orbital shaker (DS-500), drying oven (Genlab oven), what man number 1 filter paper, suction filter (Rocker 400), refrigerator, ordinary ruler, petri dishes, volumetric flask, water bath, and incubator, UV-spectrophotometer (uv-1800 shimadzu), uv-visible spectrophotometer (Evolution 60S), Sensitive Balance (Mettler AE

160), Vacuum Pump (V-300), Heating Bath (B-300 Base), Rota-Vapor (R-300) were the materials used.

3.5. Collection of Plant Materials and Preparation

Fresh and healthy roots and leaves of *Carissa spinarum* were collected. The collected plant sample was also identified and authenticated by a botanist (Melaku Wondafrash) from the Department of Biology at Addis Ababa University, and a voucher specimen (BT001) was given and deposited at the national herbarium for future reference. Then, the plant root and leaf material were carefully washed with tap water to get rid of any remaining dust. The plant material was dried in the shade for two weeks at room temperature as soon as it was collected. Once the target plant's roots and leaves were shaded and dried properly, they were then electrically ground to a uniform powder size. Ultimately, it was weighed, after which the powdered sample was kept in a sealed container for this study's extraction purposes.

3.6. Extraction of Plant Material

The extraction was carried out by macerating seven hundred grams (700 g) of powdered *Carissa spinarum* root and leaves separately in an 80% methanol solvent using a 1:10 solute-solvent ratio. Using a mini-orbital shaker at 150 rpm and occasionally stirring helped facilitate the extraction process for 72 hours. Then, after three days, first using gauze and followed by a suction filter along with Whatman number 1 filter paper, the macerates were filtrated. For thorough extraction, the marc was re-macerated with a new solvent. The methanol solvent was removed from the filtrate with the aid of Rota vapor at 40 °C, and the filtrate was kept inside the water bath at 40 °C to remove the remaining water for drying purposes. Then, the plant extract's yield percentage was determined after concentrating the crude extracts. Finally, it was transferred to a tightly closed glass container and refrigerated to keep the dried crude extract at 4 °C until the commencement of the actual experiment (Kefe *et al*., 2016; Zewdie *et al*., 2020). Each plant extract's yield was expressed as a yield percentage:

$$\text{Yield percentage (\%)} = \frac{\text{weight of dry crude extract}}{\text{weight of dried powder}} * 100$$

3.7. Experimental Animals

A total of 150 Swiss albino mice, of either sex or weighing between 30 and 35 g at 8 to 10 weeks of age, were used in the experiment, including for the pilot study (one group containing six mice was used for the pilot study). All of the mice appeared healthy. The animals for experimental purposes were obtained from the Ethiopian Public Health Institute (EPHI). Each animal had access to pellet food and clean water at all times while being kept in plastic cages at room temperature in an air-conditioned environment with a 12-hour light/dark cycle. Before starting the main experiment, they were given seven days to acclimate to the laboratory environment.

Acute Oral Toxicity

An acute oral toxicity study of the CS plant has been conducted previously by Hegde *et al*. (2010). The plant crude extract was found to be safe up to a level of 2000–5000 mg/kg with no reported death or signs and symptoms of toxicity when conducted on the model rats. This indicated that the plant has a wide safety margin, and it is the foundation for the current experimental study of the in vivo antidiarrheal activity of CS.

3.8. Grouping and Dosing of Animals

Experimental animals, categorized into eight groups each with six mice, were formed by the random division of the mice. Group I was chosen as the test group and given distilled water (10 mL/kg), while groups II, III, and IV were chosen as the test groups and given the lowest dose (100 mg/kg), middle dose (200 mg/kg), and highest dose (400 mg/kg) of CS root extract, and groups V, VI, and VII were given leaf extract of similar dose to root extract. As a positive control, group VIII was given the standard medication loperamide (2 mg/kg) for tests involving castor oil-induced diarrhea and anti-enteropooling and atropine (3 mg/kg for tests involving anti-motility). Negative and positive control groups were common for both root and leaf extract-taking groups since they were administered the same test substance. Each therapy was administered only once (Andargie *et al.,* 2022).

In the present investigation, before starting the main experiment, a pilot study was conducted with six mice of either sex to experiment properly and efficiently. The dose used in the experimental mice was determined based on the oral acute toxicity study. As a solvent, distilled water was used for orally administered plant extract preparation since the plant extracts were easily dissolved and mixed well in distilled water.

3.9. Antidiarrheal Activity Determinations

3.9.1. Castor oil-induced diarrhea in mice

For this study, the approach outlined by Feyisa *et al*. (2020) was employed with minor modifications. The mice were randomly divided into eight groups of each of the six animals, fasted for 18 hours, and given free access to water. The vehicle-treated group was given distilled water (10 ml/kg) as a negative control, whereas group 8 was also given the standard dose of loperamide (2 mg/kg) orally as a positive control. The root extract was given orally to the animals in groups II, III, and IV at doses of 100 mg/kg, 200 mg/kg, and 400 mg/kg, respectively. Similarly, the leaf extract was given to the animals in groups 5, 6, and 7, with the same dose of root extract (100, 200, and 400 mg/kg) given to the animals, respectively. One hour after treatment, 0.5 ml of castor oil was given orally to each mouse to induce diarrhea. Then, the animals were kept in a separate cage with transparent, non-wetting plastic paper lined at the bottom. Every hour for four hours, the non-wetting plastic paper was changed. The beginning of diarrhea, the frequency of wet feces, and the overall quantity and weight of feces produced were all noted during the observational period. Finally, using the formulas mentioned below, the percentage of fecal output (% FOP) and diarrheal inhibition (% inhibition of defecation) were determined.

$$\% \text{ of fecal output} = \frac{\text{Mean faecal weight of each treatment groups}}{\text{Mean faecal weight of negative control group}} * 100$$

$$\% \text{ inhibition of defecation} = \frac{\text{Mo} - \text{M}}{\text{Mo}} * 100$$

Where Mo is the mean number of wet feces of the negative control and M stands for the mean number of wet feces of the test sample or standard drug.

3.9.2. Castor oil-induced enteropooling in mice

The method employed by Andargie *et al*. (2022) was used to determine the amount of fluid accumulated in the intestine. One hour prior to the oral administration of castor oil (0.5 ml/mouse), the animals were fasted for 18 hours and grouped and treated as explained in the grouping and dosing section. Then, the mice were killed by cervical dislocation after one hour of receiving castor oil. Each mouse's abdomen was opened, and the entire length of the intestine from the pylorus to the caecum was tied off, carefully dissected, and removed. The volume of the intestinal contents was measured after the small intestines were weighed and milked into a graduated tube. After reweighing the empty intestines, the difference between the two weights was computed. Finally, using the following formulas, the percentage reduction in intestinal output and weight of intestinal contents were calculated.

$$\% \text{ Inhibition by using MVSIC} = \frac{\text{MVICC} - \text{MVICT}}{\text{MVICC}} * 100$$

Where, MVICC is the mean volume of the small intestinal content of the negative control group and MVICT is the mean volume of the intestinal content of the test group.

$$\% \text{ inhibition by using MWSIC} = \frac{\text{C} - \text{T/D}}{\text{C}} * 100$$

Where C is the mean weight of the small intestinal content of the control and T is the mean weight of the intestine content of the test or drug group.

3.9.3. Gastro-intestinal motility test

The experimental mice were grouped and given the appropriate treatment, as stated above, in grouping and dosing sections after fasting for 18 hours with free access to water. After treatment, each mouse received 0.5 ml of castor oil. Then, all mice were administered 1 ml of 5% activated charcoal suspension after one hour of castor oil administration. The animals were killed by cervical dislocation after 30 minutes of receiving activated charcoal, and the full length of the intestine from the pylorus to the cecum was removed and laid out lengthwise on white plastic paper. The overall length of the intestine and the distance covered by the activated charcoal were then measured by ordinary ruler. The following formula was used to determine the peristaltic index and percentage of inhibition.

$$\text{Peristaltic index (PI)} = \frac{\text{Mean distance travelled by charchoal meal}}{\text{Mean length of smll intestine}} * 100$$

$$\% \text{ Inhibition} = \frac{Dc - Dt}{Dc} * 100$$

Where Dc is the mean distance traveled by the negative control and Dt stands for the mean distance traveled by the test group (Ayele *et al*., 2023).

3.9.4. In-vivo antidiarrheal index

The in vivo antidiarrheal index for the positive control and test substance-treated groups was calculated by taking the major parameters like delay in defecation time, gut meal travel reduction, and purging frequency using the formula employed by Terefe *et al*. (2023).

$$\text{ADI } \textit{in vivo} = \sqrt[3]{\text{Dfeq} * \text{Gmeq} * \text{Pfeq}}$$

The above-mentioned parameters were calculated using the formula:

$$\text{Dfeq} = \frac{\text{Average onset of diarrhea in minutes(test} - \text{control)group}}{\text{Average of diarrhea in minute of the control group}} *_{100}$$

$$\text{Gmeq} = \frac{\text{Distance travelled by charcoal marker in the (control} - \text{test)group}}{\text{Distance travelled by the charcoa marker in the control group}} *_{100}$$

$$\text{Pfeq} = \frac{\text{Average number of wet feces of (control} - \text{test)group}}{\text{Average number of wet feces of control group}} *_{100}$$

Where Dfreq is the delay in defecation time or diarrhea onset obtained from the castor oil-induced diarrheal test, Gmeq is the gut meal travel reduction (as % of control) obtained from the charcoal meal test (% inhibition), and Pfreq is the purging frequency or reduction in the number of wet stools (as % of control) obtained from the castor oil-induced diarrheal model (% inhibition of defecation).

3.10. Antibacterial Activity Test

3.10.1. Test microorganisms

Four standard strains and clinical isolates of known MDR bacteria were used to test the antibacterial activities of the target medicinal plant. For this study, both the American Type Culture Collection (ATCC) and clinical isolates of bacteria that were known to be MDR were utilized. The standard strains of bacteria such as *Staphylococcus aureus* (ATCC-25923), *Escherichia coli* (ATCC-25922), *Klebsiella pneumonia* (ATCC-700603), and *Salmonella typhimurium* (13311) and multi-drug-resistant bacteria of known strains like methicillin-resistant *Staphylococcus aureus* (ATCC-300), ESBL-*Escherichia coli* (BAA-2471), carbapenem-resistant *Klebsiella pneumonia* (BAA-1705), and *Salmonella typhimurum* (29630) were obtained from the Ethiopian Public Health Institute and Addis Ababa University, College of Veterinary Medicine, Veterinary Microbiology Laboratory, respectively. The selections of bacteria were based on availability, diarrheal-causing ability, their zoonotic nature, and being multidrug-resistant.

3.10.2. Media preparation and culture of organisms

By following the manufacturer's preparation protocol, standard media used for bacterial growth and multiplication were prepared, boiled, sterilized, and poured into sterile Petri dishes. Then, the test organisms containing the sample were dispensed, spread on agar, and incubated for 24 hours at 37°C, following a biosafety cabinet (BioAir) setup. These freshly cultured organisms were used for MIC determination.

3.10.3. Determinations of minimum inhibitor concentrations

An antibacterial activity test was carried out to determine the MIC of 80% hydro-methanolic root and leaf extract of *Carissa spinarum* by using the broth dilution method on 96-well microtiter plates. The determination of the minimum inhibitory concentration of plant extract against bacterial strains was conducted according to the protocol developed by the Clinical and Laboratory Standards Institute (Sahu *et al*., 2018). To prepare stock solutions with a concentration of 32 mg/ml, plant extracts were made by dissolving the extract in distilled

water. In each well of the 96-well microplate, 100 µL of Muller-Hinton broth was added. Subsequently, one hundred microliters of the extract were loaded into the first row, and multichannel micropipettes were used to make two-fold serial dilutions from the first row down to the end. Thus, MHB was used to prepare serial dilutions in 96-well microplates, starting from a maximum concentration of 16 mg/mL to a minimum of 0.0078 mg/mL, with a stock solution serving as the source.

For standardization of the inoculum, a few colonies of bacteria (3-5) have been transferred from the sub-cultured plate into tubes holding sterile MHB using a disposable sterile inoculating loop to prepare a bacterial suspension. Then, the absorbance of the suspension was measured using a UV-visible spectrophotometer with a 1 cm light path until an absorbance reading of 0.08–0.1 at 625 nm was achieved. The standardized inoculum was diluted in MHB to prepare suspensions of test organisms containing a concentration of roughly 5×10^5 CFU/mL. A bacterial suspension containing roughly 5×10^5 colony-forming units/ml was prepared using a fresh culture. 100 µl of suspension containing test bacteria (5×10^5 CFU/mL) were loaded into the entire well in the microtiter plate within 15 minutes of standardization, except for the sterility control (last column). The plate was subsequently incubated at 37 oC for 18 to 24 hours.

Following the incubation period, each well was loaded with 40 µl of a 0.4 mg/mL solution of 2, 3, 5-Triphenyltetrazolium chloride (TTC; Tetrazolium chloride) as a measure of microbial growth. Then, the plates were incubated for 30 minutes at 37 °C. With the use of magnifying glasses, the absence or presence of the pink color after the incubation period of 30 minutes was used for determining the MIC values. The lowest extract concentration (MIC) that did not show any visible pink color, a sign of no microbial growth and development, was recorded for each extract. The tests were carried out in triplicate to determine the MIC values. For each strain in the study, there was a growth control and a sterility control. The bacterial sensitivity was assessed by doing parallel tests with ciprofloxacin as a standard, starting initially at a 10 µg/mL concentration in sterile water (with a range of 10 µg/mL to 0.0049 µg/ml).

3.11. Anti-Oxidant Capacity Assay

The plant extracts' antioxidant capacity was assessed using 2-2-diphenyl-1-picrylhydrazyl. The free radical scavenging activity of antioxidants was determined by the DPPH method employed by Dessalegn (2020), with a few modifications. First, 10 mg of plant extract was dissolved in 10 ml of distill water to prepare 1000 µg/ml of stock solution, and then serial dilution with methanol was performed to prepare the intended concentrations of the solutions (1000, 500, 250, 125, 62.5, and 31.25 µg/ml). Similarly, the same setting was adjusted for the standard ascorbic acid as well. Next, a DPPH solution was made by dissolving 6 mg of the crystalline solid DPPH in 100 mL of analytical-grade methanol in a 200-ml holding conical flask and thoroughly mixing it with gentle shaking.

Each test tube was loaded with 2 ml of DPPH solutions containing varying concentrations of the extracts and ascorbic acid solutions. Subsequently, the mixes were vortexed and allowed to stand in a dark area for half an hour. The DPPH solution was used as the control and methanol as the blank. The mixtures were carefully transferred from the test tube into the cuvete after 30 minutes of incubation, and the cuvete was then placed inside the UV-visible spectrophotometer. The resultant mixture's absorbance was measured at 517 nm. The concentration of extracts (in µg/ml) that scavenges 50% of the DPPH radicals was established as the IC_{50} value. Plant extracts and standards (ascorbic acids) were tested for their antioxidant capacity in triplicate. The capacity to scavenge the DPPH free radical was calculated using the formula:

%age of DPPH radical scavenging activity $= \frac{Ac-As}{Ac} * 100$

Where, Ac= Absorbance of the control and As= Absorbance of the sample/ standard.

3.12. Data Analysis

The statistical analysis of the data results was performed using the statistical package for social science software (SPSS) version 27, and the findings were presented as the mean standard error of the mean (mean±SEM). The statistical significance of antidiarrheal data was assessed by a one-way ANOVA followed by a post hoc Tukey test for multiple comparisons of the mean. Anti-bacterial and anti-oxidant activity data results were expressed as the mean ± standard deviation of the mean (mean±SD). The inhibitory concentration (IC_{50}) values of the antioxidant capacity assay result data were calculated by GraphPad Prism version 10. The results were considered statistically significant at a P value of less than 0.05.

3.13. Ethical Clearance

An application letter for ethical clearance has been submitted to the animal ethics review committee of Addis Ababa University, College of Veterinary Medicine and Agriculture. An ethical approval letter was obtained from the committee with the reference number (VM/ERC/04/38/16/2024). In addition, the animal care and welfare guidelines were properly followed when handling and using all experimental animals (Yuan, 2011).

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4. RESULTS

4.1. The Percentage Yield of Extracted Plant Material

At the end of plant extraction, out of 700 g of each root and leaf powder, the percentage yields were found to be 82 g (11.71%) and 132 g (18.86%), respectively.

4.2. Minimum Inhibitory Concentration (MIC)

The medicinal plant extract may show different antibacterial activities against susceptible (ATCC) and MDR bacterial strains. According to the current findings of this experimental investigation on the antimicrobial properties of *Carissa spinarum*, 80% methanolic root and leaf extract demonstrated similar activities against susceptible and multi-drug-resistant *Staphylococcus aureus* bacteria with MIC values of 4 mg/mL. In contrast to other bacterial strains, carbapenem-resistant *Klebsiella pneumonia* was found to be the most susceptible strain, with MIC values of 1 mg/mL, as shown in Table 5 and Annex 2. Similarly, the root extract of *Carissa spinarum* showed greater activity against carbapenem-resistant *Klebsiella pneumonia* than corresponding susceptible *Klebsiella pneumonia* strain (ATCC), with MIC values of 1 mg/mL and 16 mg/mL, respectively. On the other hand, both susceptible and resistant strains of *Klebsiella pneumonia* showed the same sensitivity for the standard drug ciprofloxacin, with MIC values of 0.625 µg/mL.

The plant extracts used in this investigation have antibacterial activity (MIC) values ranging from the lowest (1 mg/mL) to the highest (16 mg/mL). Similarly, the range of MIC values of standard antibiotics (ciprofloxacin) ranges from the lowest (0.02 µg/ml) on susceptible salmonella and *E. coli* to the highest (>10 µg/ml) on MRSA. Comparatively, the leaf extract had more activity than the root extract against *E. coli* bacteria with 4 mg/mL and 16 mg/mL, respectively. Similarly, the leaf extract (MIC, 8 mg/ml) exhibited greater antibacterial activity than the root extract (MIC, 16 mg/ml) against both susceptible strains of *Klebsiella pneumonia* and *Salmonella typhimurium*. The resistant bacterial strains like MRSA, *Salmonella typhimurium*, and *Klebsiella pneumonia* were inhibited at the same dose with a MIC of 4 mg/mL for both extracts. Thus, the result of the experimental study on antibacterial activity

indicated that *Carissa spinarum* root and leaf extract had greater activity against resistant bacterial strains compared to susceptible strains, except for *Staphylococcus aureus*.

An AST was also performed alongside the antibacterial activity of the medicinal plant *Carissa spinarum*, which was expressed as the minimum inhibitory concentration measured in terms of µg/ml. The AST revealed that the standard antibiotic (ciprofloxacin) exhibited less inhibitory activity against resistant bacterial strains in comparison to American-type culture collection (ATCC), and greater resistance developed with MRSA (MIC > 10 µg/ml) and extended-spectrum beta-lactamases (*E. coli)* (MIC = 10 µg/ml), as shown in Table 5 and Annex 2. Moreover, the standard drug ciprofloxacin showed significant antibacterial activity against the susceptible bacteria in comparison to the resistant ones, in contrast to the plant extracts, which showed better activity against resistant bacteria relative to the susceptible ones. The minimum inhibitory concentration of ciprofloxacin against carbapenem-resistant *Klebsiella pneumonia* was statistically significant ($p<0.001$) compared to ESBL- *E. coli* and *MSRA*, and also with *Salmonella typhimurium*-resistant ($p<0.01$).

Table 5: Comparison of the minimum inhibitory concentrations of multi-drug-resistant and susceptible bacterial strains

Dose of the extract in (mg/ml)	Minimum inhibitory concentration (MIC) results in mg/ml (mean ±SD)							
	Susceptible Bacterial Strains(ATCC)				Multi-Drug Resistant Bacterial Stains			
	Staph aureus	*E.coli*	*S.typhm*	*k.pneum*	MRSA	ESBL. *E.coli*	R.S. typhm	Carbapen K.pneum
MERE	4±0.0	16±0.0	16±0.0	16±0.0	4±0.0	4±0.0	4±0.0	1±0.0
MELE	4±0.0	4±0.0	8±0.0	8±0.0	4±0.0	4±0.0	4±0.0	2.67±1.16
Cipro (µg/ml)	0.3125±0.0	0.02±0.0	0.02±0.0	0.625±0.0	>10±0.0	10±0.0	1.042±0.36	0.625±0.0

Key notes: Experimental results were expressed as Mean ±Standard deviation of the mean (mean ±SD).

MERE, Methanol Root Extract; MELE, Methanol Leaf Extract; MELE (100, 200, and 400 mg/ml) represents group V, VI, and VII. Cipro, Ciprofloxacin; ATCC, American Type Culture Collections; *staph aueres*, *staphylococcus aureus* (ATCC-25923); *E.coli*, *Escherichia coli* (ATCC-25922); S.typhm, *salmonella typhyimurium*(ATCC-13311); k.pneum, *klepsiella pneumonia* (ATCC-700603); MRSA, methicillin resistant *staphylococcus auereus* (ATCC-300); ESBL.*E.coli*, Extended Spectrum Betalactames *Escherichia coli* (BAA-2471); R.S.typhm, Resistant *salmonella typhymurium* (29630); and carbapen.k.pneum, carbapenem resistant *klepsiella pneumonia.*

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4.3. Anti-Oxidant Assay

The findings of the in vitro anti-oxidant assay by the DPPH method revealed that both root and leaf extracts have shown very promising free radical scavenging activity that was almost similar to the standard (ascorbic acid). At the highest concentration (1000 µg/mL), the radical scavenging percentage of root and leaf were found to be 96.67±0.51 and 97.15±0.70%, respectively, which were comparable to the standard ascorbic acid 98.17±0.12% at the same concentration as shown in Table 6. There was a comparable value between the plant parts and the positive control. Similarly, the results of the findings showed that the inhibitory concentration 50 (IC_{50}) of the root was almost similar to that of leaf extract, but the IC_{50} of both methanolic extracts was found to be lower than the ascorbic acid standard. 12.61±0.51, 13.6±0.28, and 5.86±0.35 were the recorded IC_{50}s of root, leaf, and ascorbic acid, respectively. The lower the value of IC_{50}, the stronger the substance is at scavenging free radicals. The percentages of free radical scavenging at the lowest concentrations of root, leaf, and ascorbic acid were found to be 69.9±5.90, 67.93±4.25, and 82.51±5.97%, respectively.

Table 6: The free radical scavenging activity of hydro-methanolic root and leaf extracts of *Carissa spinarum* and ascorbic acid by the DPPH methods

Concentration	**DPPH% free radical scavenging activity**		
in µg /mL	Root Extract	Leaf Extract	Ascorbic Acid
1000	96.67±0.51	97.15±0.70	98.17±0.12
500	96.54±0.34	96.09±0.65	97.04±0.14
250	93.38±0.24	95.18±0.83	96.37±0.07
125	89.78±0.37	91.13±1.15	95.31±0.10
62.5	82.1±5.92	83.08±4.97	91.47±3.53
31.25	69.9±5.90	67.93±4.25	82.51±5.97
IC_{50}	12. 61±0.51	13. 6±0.28	5.86±0.35

The results were expressed as the mean ± standard deviation of the mean (mean±SD).

The result demonstrated that the anti-oxidant potential of the plant was concentration-dependent. When the concentration of plant extract increases, the percent of free-radical

scavenging activity also increases; this indicates that a higher concentration has greater antioxidant capacity, as reported by several studies.

4.4. Antidiarrheal Effect of the 80% Hydromethanolic Root and Leaf Extracts of CS in Mice Model

At doses of 200 and 400 mg/kg, both root and leaf extracts of medicinal plants significantly prolonged the onset of diarrhea and reduced the number of wet and total weight of feces in comparison to the negative control. At the highest doses of root and leaf extract ($p<0.001$), the experimental results demonstrated a significant delay in the onset of diarrhea and a reduction in the number of wet feces drops and the total weight of feces as compared to the lowest doses (100 mg/ml) and negative control. Similarly, a significant reduction in the frequency and weight of feces was observed in addition to a prolonged onset of diarrhea for both leaf and root extract at 200 mg/kg doses. However, the root and leaf extracts were statistically not significant in delaying the onset of diarrhea when compared to the negative control at 100 mg/kg doses. The dose-dependent percentage reduction in diarrheal inhibition was found to be 25.43, 35.81, and 68.67 for root extract and 14.95, 49.24, and 67.14 for leaf extract, which indicated that leaf and root extracts show comparable activity against diarrheal inhibition, particularly at the highest concentrations. Similarly, there was a dose-dependent decrease in the test substance's percentage of fecal output. The reference drug showed significant ($p<0.001$) activities against all parameters in comparison with all test doses except for 400 mg/kg, as shown in Table 7.

Table 7: Effect of the methanolic root and leaf extracts of *Carissa spinarum* on castor oil induced diarrhea in mice

Dose in mg/kg	onset time of diarrhea(min)	frequency of wet feces	total weight of feces (gm)	% inhibition of diarrhea	% of fecal output
DW 10	41.50±2.884	11.17±1.078	2.678±0.049	-	-.
MERE 100	56.00±3.777	8.33±0.715[1a]	2.112±0. 088[1c]	25.43	78.86
MERE 200	68.33±4.924[1b]	7.17±0.307[1b]	1.818±0. 071[1c]	35.81	67.89
MERE 400	82.33±3.283[1c2b]	3.50±0.428[1c2c3b]	1.038±0. 076[1c2c3c]	68. 67	38.76
Loperamide 2	86.17±6.300[1c2b]	2.67±0.333[1c2c3c]	.713±0. 077[1c2c3c4a]	76.1	26. 62
MELE 100	50.50±3.212	9.50±0. 764	2.045±0. 072[1c]	14.95	76.36
MELE 200	63.83±5.747[1a]	5.67±0. 558[1c2b]	1.717±0.100[1c2a]	49.24	64.12
MELE 400	78.00±4.733[1c2b]	3.67±0. 333[1c2c]	.920±0. 081[1c2c3c]	67.14	34.35

Keynotes**:** The values of the results were expressed as the mean ± standard error of the mean (SEM) (n = 6). Data were analyzed by one-way ANOVA followed by a post-hoc Tukey test: 1, compared to DW10; 2-4, compared to MERE/MELE extracts; and 5, compared to loperamide 2. [a]p<0.05, [b]p<0.01, [c]p<0.001. MELE (100, 200, and 400 mg/kg) represents group V, VI, and VII. DW10, distill water (10 ml/kg); MERE, methanol root extract of *Carissa spinarum*; MELE, methanol leaf extract of *Carissa spinarum.*

4. 5. Enteropooling Effects of 80% Hydro-Methanolic Root and Leaf Extracts of CS in Mice

At the test dose of 400 mg/kg ($p<0.001$), the CS root and leaf extracts significantly decreased the weight as well as the volume of intestine contents in the enteropooling experiment in comparison to the negative control group, 100, and 200 mg/kg. When compared to the test doses of 100 mg/kg, the extract at 200 mg/kg significantly decreased the weight of the small intestinal contents. At 100 mg/kg doses of both extracts, there was no statistically significant decrease in weight or volume of small intestine content in comparison to the negative control. The largest percentage inhibition of weight of intestinal contents (56.84%) was demonstrated by the root extract at 400 mg/kg doses. This was higher than that of the leaf extract (47.58%) and comparable to the reference drug (66.86%). A 13.46, 31.47, and 67.3% decrease in the intestinal contents' volume was observed at the doses of 100, 200, and 400 mg/kg, respectively. The standard drug demonstrated an 82.27% percentage decrease in the intestinal contents' volume, as shown in Table 8.

Table 8: Enteropooling Effects of 80% hydro-methanolic root and leaf extracts of *Carissa spinarum* on Castor Oil-induced Mice

Group (dose in mg/kg)	**weight of intestinal content**	**%inhibition of MWSIC**	**volume of intestine contents**	**%Inhibition by MVSIC**
DW10	1.177±0.060	-	1.055±0. 054	-
MERE 100	1.027±0. 038	12.74	.913±0. 038	13.46
MERE 200	.933±0. 045^{2a}	20.73	.723±0. 067^{1c}	31.47
MERE 400	.508±0.057^{1c2c3c}	56.84	.345±0. 039^{1c2c3c}	67.3
Loperamide 2	.390±0.045^{1c2c3c}	66.86	.187±0.016^{1c2c3c}	82.27
MELE 100	1.110±0.059	5. 69	.892±0. 064	15.45
MELE 200	.925±0.05^{1a}	21.41	.732±0. 078^{1b}	30. 62
MELE 400	.617±0.046^{1c2c3b}	47.58	.433±0.027^{1c2c3b}	58.96

Key Notes**:** The values of the results were expressed as the mean ± standard error of the mean (SEM) (n = 6). Data was analyzed by one-way ANOVA followed by a post- hoc Tukey test: 1, compared to DW10; 2-4, compared to MELE extracts; 5, compared to loperamide 2. $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.00$. MELE (100, 200, and 400 mg/ml) represents groups V, VI, and VII. MERE, methanol root extract; MELE, methanol leaf extract; DW10, distill water (10 ml/kg); MWSIC, mean weight of small intestine content; MVSIC, mean volume of the small intestine content.

4. 6. Intestinal Motility Effects of 80% Hydromethanolic Root and Leaf Extracts of CS in Mice Model.

In comparison to the negative control, the root and leaf extracts significantly decreased intestinal motility at all test doses ($p<0.001$) except at 100 mg/kg of leaf extract, for which ($p<0.05$). At 400 mg/kg, the peristaltic index of the root extract produced a better peristaltic index (38.84%) than that of the leaf extract (41.86%) at comparable doses, despite both peristaltic index values being below that of the conventional drug (30.17%). At 100, 200, and 400 mg/kg, the root and leaf extract percentage inhibition of intestinal transit charcoal meal was found to be 38.88, 52.69, and 59.08% for the root, and 15.61, 23.78, and 55.24% for the leaf, respectively. This suggested that, at comparable concentrations, root extract was relatively more effective than leaf extract in inhibiting intestinal motility. The maximal intestinal motility inhibition (59.08%) was recorded at 400 mg/kg doses of root extract, which was comparable to the conventional medication inhibition (67.52%) of intestinal motility as shown in Table 9.

Table 9: Effects of root and leaf 80% hydromethanolic extracts of *Carissa spinarum* on the gastro-intestinal motility of mice

Group (dose in mg/kg)	length of Small intestine(cm)	Distance moved by charcoal meal(cm)	% Peristaltic index (PI)	% Inhibition
DW10	70.67±1.333	65.17±2.272	92.22	-
MERE 100	68.50±1.544	39.83±1.579^{1c}	58.15	38.88
MERE 200	68.67±2.186	30.83±3.458^{1c}	44.9	52. 69
MERE 400	68.67±0.882	26.67±1.801^{1c2b}	38.84	59.08
Atropine 3	70.17±0.910	21.17±2.120^{1c2c}	30.17	67.52
MELE 100	71.00±0.577	55.00±2.324^{1a}	77.46	15. 61
MELE 200	72.00±.0.577	49.67±1.856^{1c}	68.99	23.78
MELE 400	69.67±0..882	29.17±1.424^{1c2c3c}	41.86	55.24

Key notes: The values of the results were expressed as the mean ± standard error of the mean (SEM) (n = 6). Data was analyzed by one-way ANOVA followed by a post hoc Tukey test: 1, compared to DW10; 2-4, compared to MERE/MELE extracts; 5, compared to Atropine 3. $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$. MELE (100, 200, and 400 mg/ml) represents groups V, VI, and VII. MERE, methanol root extract; MELE, methanol leaf extract; DW10, distill water (10 ml/kg).

4.7. In-Vivo Anti-Diarrheal Index

The in vivo antidiarrheal indices (ADI) of the root and leaf extract were found to be 32.57, 49.59, 73.63, and 17.17, 39.79, and 68.84%, respectively, at test doses of the lowest (100), medium (200), and highest (400) mg/kg, as stated in Figure 7. At 400 mg/kg doses, the maximal in vivo antidiarrheal index of the root and leaf extract was achieved with 73.63 and 68.84%, respectively. Hence, root extract at 400 mg/kg (73.63%) showed better antidiarrheal activity compared to leaf extract (68.84%) at a similar dose, and the root extract exhibited comparable antidiarrheal activity to the standard drug with an ADI of 82.08% as illustrated in Table 10.

Table 10: In vivo antidiarrheal indices of 80% hydromethanolic root and leaf extracts of *Carissa spinarum*

Group (Dose in mg/kg)	Delay in Defecation (Dfeq) %	Gut meal travel distance (Gmeq) %	purging frequency of wet feces (Pfeq)%	In vivo ADI%
DW 10	-	-	-	-
MERE 100	34.94	38.88	25.43	32.57
MERE 200	64. 65	52. 69	35.81	49.59
MERE 400	98.39	59.08	68.67	73. 63
loperamide 2	107. 64	67.52	76.1	82.08
MELE 100	21. 69	15. 61	14.95	17.17
MELE 200	53.81	23.78	49.24	39.79
MELE 400	87.95	55.24	67.14	68.84

Key note: DW10, Distil Water (10 ml/kg); MERE, Methanol Root Extract; MELE, Methanol Leaf Extract

5. DISCUSSION

Diarrhea is one of the gastrointestinal disorders, and its treatment is worsened by increasing antibiotic resistance, which makes it a global healthcare concern (Megersa *et al*., 2023). The potential use of natural plant-derived extracts for medicinal purposes has grown dramatically because of the emergence and spread of resistant pathogenic microbes and the adverse effects of synthetic medications used for the treatment of diarrhea (Lone *et al*., 2024). Thus, the current study aimed to explore the antidiarrheal and antibacterial activity and antioxidant properties of hydro-methanolic root and leaf extracts of *Carissa spinarum L.*

The findings of the current study demonstrated that both root and leaf extracts had remarkable antidiarrheal activity, and their percentage of diarrheal inhibition was comparable with root (68.67%) and leaf (67.14%), as shown in Table 7. At a dose of 200 and 400 mg/kg ($p< 0.001$) for both extracts, the plant extract significantly decreased the frequency and total weight of wet feces, as well as delayed the commencement of diarrhea in comparison to the negative control. This finding was in line with other studies elsewhere that revealed the hydro-methanolic crude extract of *Ruta chalepensis* (Degu *et al.,* 2020) and the hydro-methanolic crude extract of *Indigofera spicata* (Komal & Rana, 2013) at 200 and 400 mg/kg doses showed statistically significant inhibition of the frequency of defecation and the onset of diarrhea.

On the other hand, at 100 mg/kg doses of root and leaf extracts, there was a statistically insignificant delay in the onset of diarrhea. Teferi *et al*. (2019) and Megersa *et al*. (2023) found a statistically significant delay in the onset of diarrhea at doses of 100 mg/kg, which was inconsistent with the current findings. It was also observed that an increase in the percentage of diarrheal drops was directly proportional to an increase in the dose of the plant extract. At the highest dose of the extract, the activity of the medicinal plant was comparable with that of the reference drug. This could be due to an increase in the concentration and composition of phytochemicals in the plant extracts as the dose increases.

The significant activity in diarrhea caused by castor oil was probably caused by intestinal lipase enzyme activity, which stopped ricinoleic acid from being liberated and discharged. In turn, this led to an increase in the intestinal absorption of Na+, other electrolytes, and water and indirectly restored the normal absorptive capacity of the Na+/K+ ATPase. The Na+/K+-ATPase activity has been demonstrated to be enhanced by a variety of herbal extracts; this is accompanied by an apparent increase in intestinal absorption and a decrease in secretory capacity (Ahmed *et al*., 2022). Thus, the root and leaf extracts of *Carissa spinarum* may contain phytochemicals that can either directly or indirectly stimulate the intestinal mucosa's Na+/K+-ATPase.

Moreover, localized intestinal mucosa irritation and inflammation brought on by ricinoleic acid result in the release of PG and an increase in the net secretion of water and electrolytes into the small intestine. Research suggests that prostaglandin production inhibitors delay the onset of diarrhea caused by castor oil (Ugwuja *et al*., 2022). Beck and Namdeo (2015) reported that *Carissa spinarum* leaf extracts have potent anti-inflammatory activities similar to analgin. This potent anti-inflammatory activity of *Carissa spinarum* is crucial in reducing the occurrence of diarrhea. These activities can diminish the inflammatory processes and motility triggered by castor oil in the GIT. Consequently, the reduction in inflammation can limit the production of prostaglandin caused by castor oil, thereby promoting normal absorptive functions and alleviating the onset of diarrhea. Thus, the root and leaf extracts of *Carissa spinarum* may contain phytochemicals that interfere with the production of prostaglandin.

According to the results of the castor oil-induced enteropooling model, the 80% hydro-methanolic root and leaf extracts of *Carissa spinarum* considerably reduced the weight and volume of intestinal contents at the middle and highest concentrations in comparison to the vehicle. This finding was in agreement with the result reported by Alemu *et al*. (2022), who demonstrated that the methanol seed extract of *coffee Arabica linn* significantly reduced the weight and volume of intestinal contents at doses of 200 and 400 mg/kg. The presence of flavonoids in the root and leaf extracts inhibits the expression of COX-2 and the synthesis of prostaglandin E2, two important inflammatory process regulators (Hämäläinen *et al.,* 2011). Thus, the presence of flavonoids and others in the plant extracts could be the cause of the

decreased weight and volume of intestinal content, as well as the enhanced intestinal absorption of water and electrolytes.

The apparent decrease in enteropooling caused by castor oil in the plant extracts may also be due to the presence of phytochemicals like tannins and others that alleviate castor oil-mediated effects and improve the absorption of electrolytes and water. Tannins in the methanol extract tend to reduce intestinal secretion and peristalsis movement due to their capacity to precipitate the proteins of the enterocytes and the layers that are created on the enterocytes' mucosal surface when proteins precipitate, preventing the growth of microorganisms (Degu *et al*., 2016). The increase in weight and volume of intestinal content could be due to the activation of the nitric oxide pathway by the effect of ricinoleic acid (Mascolo *et al*., 1994). According to a prior study, the production of nitric oxide is inhibited by the presence of phytochemical compounds like flavonoids, terpenoids, alkaloids, and steroids (Sisay *et al*., 2017).

Moreover, with an increasing dose of the extract, there was a notable increase in the percentage of inhibition that decreased the weight and volume of intestinal contents. This study finding was consistent with a study reported by Tessema *et al*. (2022), who demonstrated that methanol extracts tend to reduce weight and volume of intestinal content in dose-dependent ways. This indicates that as the dose of the plant extracts increases, the amount of phytochemicals (secondary metabolites) having anti-secretory properties also increases correspondingly. The outcomes of this model also showed that the highest extract dose affected intestinal fluid buildup in a way that was comparable to and closer to loperamide's inhibitory effect. The findings imply that the advantages of plant extracts might be attributed to improved fluid retention through enhanced electrolyte absorption and/or decreased intestinal hypermotility, a mechanism similar to that of loperamide.

The gastrointestinal propulsion test with charcoal meal was used to assess the effects of extracts on castor oil-induced gastrointestinal transit in mice. The percentage reduction in gastrointestinal charcoal meal transit was found to be 59.08% at a dose of 400 mg/kg of the root extract, which is closer to the percentage reduction recorded by a standard drug (atropine) (67.52%), while the percentage reduction recorded by leaf (55.24%) at a similar dose was slightly lower than the roots. A reduction in intestinal motility lengthens the time that

intestinal contents remain in the intestine, which could cause the absorption of water and electrolytes from the small intestine to take much longer. The effects of the extract in models of diarrhea and enteropooling caused by castor oil may thus be responsible for this.

The present study's findings were in line with previous research on plants used for the treatment of diarrhea. For instance, Tessema *et al.* (2022) and Megersa *et al*. (2023) reported that methanol extract and solvent fractions of the leaves of *Withania somnifera* and *Maesa lanceolata Forssk* (Myrsinaceae) demonstrated a significant reduction in intestinal motility at all test doses. However, this finding was in contrast with the finding of Teferi *et al.* (2019), which showed the methanol leaf extracts of *Osyris quadripartite* failed to significantly reduce intestinal motility at all test doses. This difference might be attributed to variations in phytochemicals responsible for anti-motility activity.

Several studies have shown that diarrhea is associated with an increase in peristalsis in the GI system (Zhao *et al*., 2019). Reducing GI peristalsis is one of the ways antidiarrheal medications function. For example, the standard drug used in this research, atropine, decreased the propulsive activity in the charcoal meal because of its anticholinergic action, which prolonged the intestinal transit time (Obiorah *et al*., 2022). This delays the absorption of gastrointestinal contents by extending the amount of time that nutrients stay in the intestine. Additionally, enhancing the absorption of water and electrolytes may lessen the watery consistency of diarrheal feces (Ahmad *et al*., 2021). Therefore, the anti-motility and antispasmodic properties of the extracts, which also contribute to the plant's antidiarrheal effects, may be the reason for the reduction in intestinal transit brought on by castor oil.

The highest concentrations of the root extract and atropine showed comparable percentages of motility inhibition, suggesting that the plant extract may have an anti-motility effect comparable to that of atropine. On the other hand, the leaf extract at the highest doses revealed a slightly lower percentage of motility inhibition compared to the standard drug atropine, even if the plant extracts had significant anti-motility compared to the negative control. This difference between root and leaf extracts might be due to variations in the concentration and composition of phytochemical constituents in the plant extracts. This indicated that the proportion of tannin, flavonoids, and terpenoids might be higher in root extract relative to leaf

extract since flavonoids are known to reduce intestinal motility due to their ability to relax intestinal smooth muscles (Maamori, 2011). Similarly, according to several reports, tannin reduces intestinal peristalsis by preventing intracellular inflow of Ca2+ (Yacob *et al*., 2016; Adela *et al*., 2022). On the other side, it has been shown that terpenoids block the release of prostaglandin (PGs), which in turn limits intestinal motility and secretion (Salgado *et al*., 2005).

The effectiveness of plant extracts in treating diarrhea is determined by their anti-diarrheal index values. The plant extract's effectiveness in treating diarrhea increases with antidiarrheal index (ADI) values (Zayede *et al*., 2020). At higher doses, it showed the highest ADI values with root (73.63) and leaf (68.84) extracts in treating diarrhea compared to the other two lower concentrations. Similar findings were reported by Terefe *et al.* (2023) and Degu *et al.* (2020), who demonstrated that antidiarrheal activity had a dose-dependent nature and that the maximum antidiarrheal activity that was comparable to the standard drug was achieved at the highest doses (400 mg/kg). Large amounts of phytochemical components (phenols, tannins, alkaloids, flavonoids, and terpenoids) could be the cause of the increased effect (greater antidiarrheal activity) and may also be responsible for the antidiarrheal activity.

The increasing ADI values with increasing concentrations indicate that the parameters have a dose-dependent nature in their antidiarrheal activity. Based on these findings, the root extract demonstrated better antidiarrheal activities than the leaf extract, which signifies the root was almost endowed with antidiarrheal activity and closer to the antidiarrheal activity of the standard drug loperamide. This difference might be due to the presence of a carissin compound, which is found in the root but not in the leaf and has potential antimicrobial properties. These differences could also be due to variations in the chemical composition and concentrations of bioactive secondary metabolites since roots most often serve as storage organs for various secondary metabolites, which can contribute to a higher concentration of bioactive compounds in the roots having antidiarrheal properties compared to the leaves.

Based on the current findings, the *Carissa spinarum* antidiarrheal mechanism of action was linked to delayed diarrheal onset, decreased intraluminal fluid accumulation, enhanced water absorption, and suppression of water secretion. These results were in line with the objective of medication therapy for diarrhea, which was to decrease the amount of water excreted in the feces by either enhancing fluid absorption, decreasing fluid secretion, or doing both (Wibiwo *et al.*, 2021; Alkandahri *et al*., 2023).

Finding novel antimicrobial medications with plant origins has become extremely popular due to the worldwide rise in multi-drug- resistant pathogenic microorganisms in humans and animals and also the undesirable side effects of some antibiotics (Mir *et al*., 2021). Finding secondary metabolites from medicinal plants with antibacterial properties is therefore currently a hot topic since the discovery of new drugs derived from medicinal plants offers a promising means of resolving socioeconomic issues and mitigating the long-term impact caused by bacteria resistant to drugs, such as *K. pneumoniae*, *E. coli*, and MRSA (Dholaria and Desai, 2018). Antibacterial activity tests were performed to verify the medicinal plant's therapeutic potential in inhibiting the growth and multiplication of multi-drug-resistant bacteria at minimum concentrations in comparison to susceptible ones.

The result of the current study revealed that root and leaf extracts have shown considerable activity against both gram-positive and gram-negative bacteria, with different degrees of antibacterial activity. This could be due to the variety of phytochemical constituents, their mechanisms of action, and the synergistic effects of several bioactive secondary metabolites present in the plant extracts that contributed to the broad-spectrum antimicrobial activity of the root and leaf of *Carissa spinarum.* This finding was supported by Ayalew *et al*. (2022). Similarly, this study demonstrated that hydro-methanolic root and leaf extracts exhibited better activity against selected multi-drug-resistant bacterial strains compared to susceptible bacteria, except for *Staphylococcus aureus*. In the case of MRSA and *Staphylococcus aureus*, no observable difference in terms of activity against both types of bacteria was seen. Their intrinsic characteristics, which have to do with how permeable their cell surfaces are to the extracts, may be the reason for the comparable susceptibilities of the tested *staphylococcus aureus* since both susceptible and MRSA showed similar sensitivity to both plant extracts. This could also be due to the broad-spectrum properties, mode of action, and common

sensitivity parts of bacteria targeted by plant extracts shared by both MRSA and the corresponding susceptible *staphylococcus aureus.*

According to the current study findings, the MIC values of leaf and root were recorded as (4 mg/ml) and (16 mg/ml), respectively, against the test organism *E. coli.* There was a similar finding reported by Sanwal and Chaudhary (2011), who demonstrated MIC values of 0.125 mg/ml against *E. coli* from the methanolic root extract of *Carissa spinarum* that support the current finding. In addition, Joshi and Singh (2017) reported that methanolic and ethanolic root extracts exhibited antibacterial activity with MIC values of 0.312 mg/mL against *E. coli.* Similarly, *the* petroleum ether extract of *Carissa spinarum* leaf showed antibacterial activity against *Staph aureus* with a MIC of 0.312 mg/mL. This finding was in contrast with the current finding of leaf extract with a MIC of 4 mg/mL against *S. aureus*. This difference might be attributed to variations in the solvent of extraction and geographical location.

The methanolic leaf extract of *Carissa spinarum* has shown promising activity against carbapenem-resistant *Klebsiella pneumonia* bacteria with considerable antibacterial activity with a MIC of 2.67 mg/mL from the present study results. There was a similar report by Rath and Padhy (2015), who demonstrated that ethyl acetate, ethanol, and methanol leaf extracts of *P. foetida* demonstrated antibacterial activity with MICs of 3.125, 1.56, and 3.125 mg/ml, respectively, which was comparable with the current findings. Similarly, root extract also showed the maximum inhibitory activity against carbapenem-resistant *Klebsiella pneumonia,* with the lowest MIC of 1 mg/mL. This significant antibacterial activity of methanolic root and leaf extracts against this and other resistant bacteria could be due to the phytochemicals found in the root and leaf extracts of *Carissa spinarum,* like flavonoids and other major bioactive secondary metabolites. Flavonoids have been shown to have antibacterial activity through several methods, such as suppression of bacterial nucleic acid synthesis, disruption of cell membrane function, and disruption of metabolic pathways. Flavonoids also impair bacterial cell walls and lysosomal permeability due to their interactions with bacterial DNA (Manihuruk *et al*., 2017).

The root, as well as the leaf extract, exhibited almost comparable activities against multi-drug-resistant bacteria; however, the leaf extract had slightly better activities against susceptible bacteria relative to the root extract, except against *Staphylococcus aureus* and carbapenem-resistant *Klebsiella pneumonia*, but their differences were insignificant. This could be due to differences in the complex structure and nature of the bioactive compounds found in *Carissa spinarum*, since previous studies reported that *Carissa spinarum* is endowed with several secondary and primary bioactive metabolites (Sharma *et al*., 2023). The presence of those numerous phytochemicals in this medicinal plant could potentially enable them to target the multiple pathways or mechanisms of resistance in bacteria, thereby hindering the development of resistance. Furthermore, several compounds in *Carissa spinarum* might have synergistic activities that could potentially enhance their antibacterial activity.

Ciprofloxacin is a broad-spectrum antibiotic that acts by preventing DNA gyrase from doing its job (Serizawa *et al*., 2010). As a result, a double-stranded DNA break is formed, which slows down DNA replication (Shariati *et al*., 2022). The standard drug showed less sensitivity to the resistant bacteria compared to the susceptible ones, particularly MRSA and ESBL. *E. coli*. This indicated that MRSA bacteria were found to be the most resistant bacteria against the standard ciprofloxacin from the selected and tested organisms in comparison to other test organisms with a MIC greater than the initial concentration (greater than 10 µg/ml). This showed that MRSA developed resistance to ciprofloxacin, one of the most important antibiotics in health care.

In the present study, the antibacterial sensitivity test of ciprofloxacin against *E. coli* (MIC, 0.02µg/ml) and *Staph aureus* (MIC, 0.3125µg/ml) demonstrated almost similar antibiotic sensitivity values to the MIC of reference ciprofloxacin, which ranges from 0.004–0.016 and 0.12-0.5µg/ml, respectively, with standards established by Clinical and Laboratory Standards Institute (CLSI) guidelines.

The present finding has been supported by the findings of Campoli-Richards *et al*. (1988), Chin *et al.* (1984), and LeBel (1988), who found and documented the antibacterial activity of ciprofloxacin against the resistant bacteria MRSA with MIC ranges of 0.12–1 mg/L, 0.1–0.8 mg/L, and 1 mg/ml, respectively. Similarly, Chin and Neu (1984) and Eliopoulos *et al.* (1984) showed that ciprofloxacin has antibacterial activity against *E. coli* and *Klebsiella pneumonia* (ATCC), with MIC values of 0.01–2 μg/ml and 0.06–0.125 μg/ml, respectively. This was nearly identical to the current result, which suggested that ciprofloxacin has antibacterial activity against *Klebsiella pneumonia* and *E. coli,* with minimum inhibitory concentrations (MICs) of 0.625 mg/mL and 0.02 mg/mL, respectively.

Furthermore, the in vitro plant extract test results also demonstrated that the plant extracts had significant antibacterial properties against susceptible and resistant strains of *Salmonella typhimurium* and *Klebsiella pneumonia*. The antibacterial properties of *Carissa spinarum* against resistant *Salmonella typhimurium* and *Klebsiella pneumonia*, however, have not been supported by any earlier research or publications. Nevertheless, Ayalew *et al.* (2022) reported the solvent leaf extracts of *Carissa spinarum*, which had zones of inhibition of 11.0 ± 0.23 mm, 10.0 ± 0.72 mm, and 7.0 ± 0.41 mm for methanol, acetyl acetate, and chloroform extracts, respectively, at 100 mg/ml doses. This supported the current finding on the antibacterial activity of *Carissa spinarum* against *Klebsiella pneumonia* (ATCC).

Even if there were no prior reports of *Carissa spinarum* on ESBL-*E.coli*, the antibacterial activities of *Carissa spinarum* against ESBL-*E.coli* (MIC, 4 mg/ml) and MRSA (MIC, 4 mg/ml) of the current finding were almost similar to those of Manilal *et al*. (2023), who demonstrated the ethyl acetate extract of *C. asiatica* exhibited antibacterial activity of (MIC, 5 mg/ml) and ethyl acetate of *S. marianum* (MIC, 5 mg/ml) against ESBL-*E.coli* and MRSA, respectively. However, compared to the ethyl acetate extract of *S. marianum* (MIC, 10 mg/mL) against ESBL-*E. Coli*, the methanol extract of *Carissa spinarum* (MIC, 4 mg/mL) showed superior antibacterial activity. The variation in phytochemical content and composition between the plants may be the cause of this difference. The antibacterial activity of methanol leaf extract of *Carissa spinarum* against *Salmonella typhimurium* (ATCC) (MIC, 8 mg/ml) was found to be comparable to the result obtained by Marasini *et al.* (2015), who

reported an antibacterial activity (MIC, 6.25 mg/ml) shown by *Artemisia vulgaris*, *Drymaria cordata*, and *Rauwolfia serpentine*.

Antibiotic-resistant bacteria develop different mechanisms against the existing antibiotics. The formation of an efflux pump and biofilm are among the pillar mechanisms of resistance for bacteria against antibiotics. The capacity of efflux pumps to eliminate a broad variety of structurally varied substances and the formation of biofilm are key factors in the development of bacterial resistance to different antibiotics (Gaurav *et al.*, 2023). Hence, the reason why the plant extracts have shown better activity against resistant bacteria could be attributed to the presence of phytochemicals that might block an efflux pump and biofilm formation used by resistant bacteria as a resistance mechanism. Similarly, the several bioactive compounds in the plant extract may inhibit the production of enzymes that confer resistance to bacteria, and the synergetic effect of those multiple phytochemicals could also contribute to their promising broad-spectrum antibacterial activities against multidrug-resistant bacteria. Moreover, the presence of various secondary metabolites in the plant could potentially target the multiple pathways of the bacteria. Those bioactive plant metabolites responsible for this antibacterial activity include flavonoids, tannins, coumarins, triterpenes, alkaloids, sterols, terpenoids, and others.

In general, the plant extracts studied exhibited significant antibacterial activity, particularly against drug-resistant bacteria. This enhanced activity may be attributed to the presence of phytochemicals that target bacterial resistance mechanisms, the diverse bioactive compounds present in the extracts, including flavonoids, tannins, coumarins, and alkaloids, contributing to their broad-spectrum antibacterial effects, and the synergistic effects of phytochemicals in the plant extracts. The extracts showed comparable antibacterial efficacy between root and leaf extracts. Overall, the bioactive substances in the plant extracts play a crucial role in their antimicrobial activity in general and against multidrug-resistant bacteria in particular.

DPPH assays have been frequently used in the investigation of natural compounds' antioxidant capacity. The DPPH antioxidant test showed the ability of test samples to scavenge free radicals (Okello *et al*., 2021). In the present experimental study, the antioxidant activity of medicinal plants was assessed by the DPPH method. According to the in-vitro antioxidant assay of medicinal plants, the findings demonstrated that root and leaf had remarkable and comparable inhibitory concentrations of IC_{50} 12.61±0.51 and 13.6±0.28 µg/mL, respectively, and the standard ascorbic acid 5.86±0.35 µg/mL. The current leaf and ascorbic acid results are almost comparable with the findings reported by Mahdi-Pour *et al*. (2012), who revealed that a methanolic extract of the leaf of *Lantana camara* showed inhibitor concentrations (IC_{50}) of 16.02±0.94 and 6.21±0.04 µg/mL for leaf and ascorbic acid, respectively. However, the methanolic root extract of *Lantana camara* demonstrated lower free radical scavenging capacity of 31.52±0.74 µg/mL compared to the methanolic root extracts of *Carissa spinarum* in the current finding. This difference could be attributed to the concentration and type of phytochemical variations found in the two plant parts. This suggests that the root of *Carrisa spinarum* was endowed more with phenolic compounds like flavonoids, tannins, and others relative to the root of *Lantana camara*, which are responsible for free radical scavenging activities.

The IC_{50s} of methanolic root extract 12.61±0.51 obtained in this experimental study were slightly lower than the findings of Sisay *et al*. (2022), who found an IC_{50} of 21.28 that was obtained from the methanol-soluble fraction of smoke oil from the root of *Carissa spinarum.* However, the 98.17±0.12% DPPH inhibition observed at 1000 µg/mL in this finding was higher than the finding of Sisay *et al*. (2022), who found 92.60% at 100 µg/mL of concentration. These differences might be due to variations in concentrations and types of secondary metabolites in the plant extract, in addition to differences in methods, techniques, and solvents of extraction. Similarly, the present findings were in contrast to the finding reported by Thida *et al.* (2019), who reported a radical scavenging capacity with an IC_{50} of 33.85 ± 2.1µg/mL from the root bark extract of *Carissa spinarum.* This difference might be due to the lower amount of phenolic and other bioactive compounds responsible for antioxidant properties found in the root bark compared to the root and leaf parts.

The current study findings of root and leaf extract exhibited better antioxidant capacity IC_{50} of 12.61±0.51 and 13.6±0.28 µg /mL, respectively, in contrast to IC_{50} of 75.65±5.02 µg /mL from the aqueous extract and IC_{50} of 96. 10±1.11 µg /mL from hydroalcoholic root bark reported by Afanyibo *et al*. (2019). This significant difference might be due to variations in the composition and concentration of phytochemicals found in different plant parts. It may also be due to variations in the solvents used for extraction and geographical location. Moreover, the IC_{50} of 47.03 µg/ml from chloroform extracts of stems with a similar method (DPPH method) found by Rao *et al*. (2005) had lower antioxidant capacity in contrast to current findings. This considerable difference could be attributed to the variation in the solvent used for extraction and the parts of the plant used.

As demonstrated by the current study's findings, *Carissa spinarum* root and leaf extracts have potent antioxidant capacities and nearly similar percentages of radical scavenging. Both plant parts may contain similar secondary bioactive compounds, especially polyphenols, as indicated by the nearly comparable inhibitory concentrations of the extracts. Numerous studies have shown a strong correlation between polyphenols and the antioxidant activity of medicinal plants, which suggests that the plant extract's remarkable ability to scavenge free radicals may be due to the plant's high concentration of polyphenols, including tannin, flavonoids, and other phenols. Plants with higher levels of phenols and flavonoids in their tissues have more antioxidant activity, making them better. The higher the concentration of flavonoids and phenols in plant tissues, the stronger the plant's antioxidant activity and its ability to protect body tissues from oxidative stress. Thus, the comparable activity of plant extract to ascorbic acid in terms of both percentage of free radical scavenging and inhibitory concentration makes the medicinal plant the candidate plant for the formulation of natural products with better antioxidant activities.

6. CONCLUSION AND RECOMMENDATION

Based on the results obtained in this study, *Carissa spinarum* has shown promising properties in terms of antidiarrheal effects, antimicrobial activities against multidrug-resistant bacteria, and significant antioxidant effects, particularly in its leaf and root components. The root extract exhibited superior antidiarrheal properties compared to the leaf extract, and both extracts demonstrated effectiveness against resistant bacteria. Both extracts demonstrated potent antioxidant activity that was comparable to standard ascorbic acid. These findings highlight the potential of *Carissa spinarum* as a natural remedy for diarrhea and infections caused by resistant bacteria and as a potential source of antioxidants. However, the specific mechanism underlying the antidiarrheal and antimicrobial activity, especially against resistant bacteria, remains unknown. Additionally, the promising antioxidant activity of *Carissa spinarum* has not been validated through in vivo assays. Based on these conclusions, the following recommendations have been proposed:

- Further research should be conducted to gain a deeper understanding of the specific mechanism behind the antidiarrheal and antibacterial effects, particularly against multidrug-resistant bacteria.
- Discovering the potential of *Carissa spinarum* as a natural remedy for combating infections caused by multidrug-resistant bacteria is of great importance.
- Additional studies should focus on conducting in vivo antioxidant assays to validate the promising antioxidant properties of *Carissa spinarum.*

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8. ANNEXES

Annex 1: Plant Material Preparation and Extraction Procedure

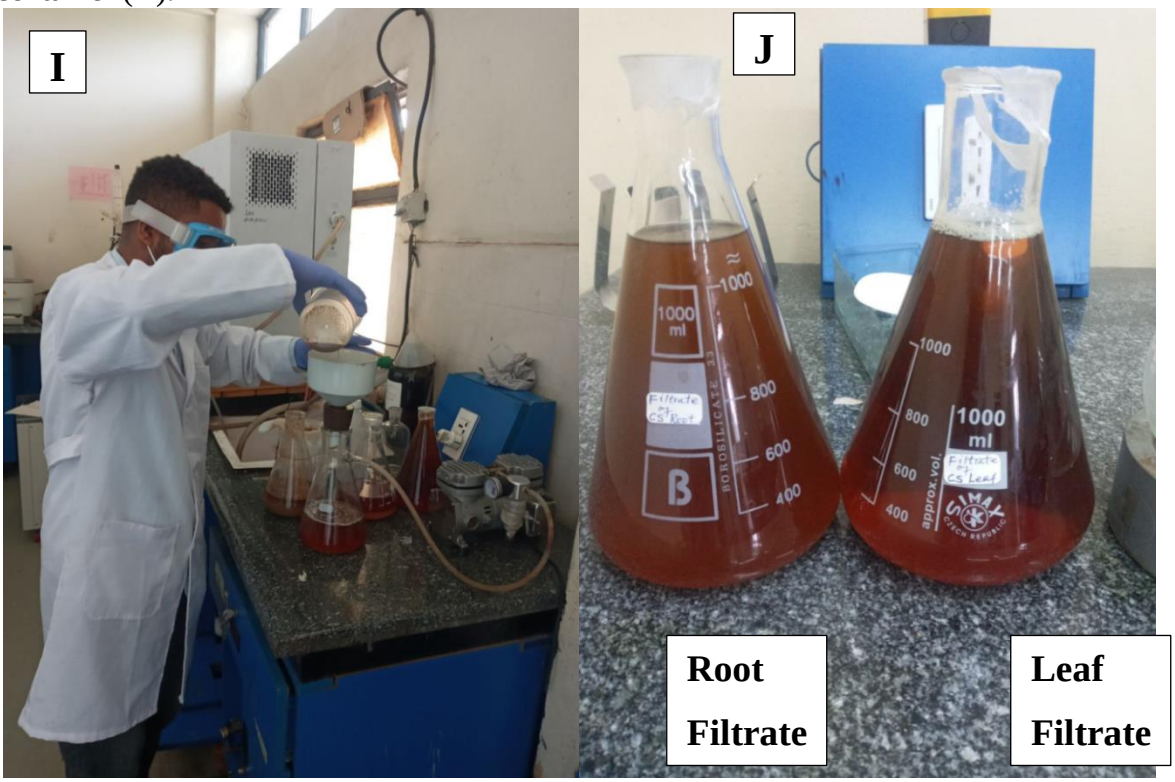
First, the collected root and leaf of *Carissa spinarum* were properly washed with tap water to remove debris after being identified and authenticated by a botanist, as shown in figures A and B.

Second, the plant materials were powdered by an electrical grinder after the plant parts dried in the shaded area for two weeks, as depicted in figures C and D, respectively, for root and leaf.

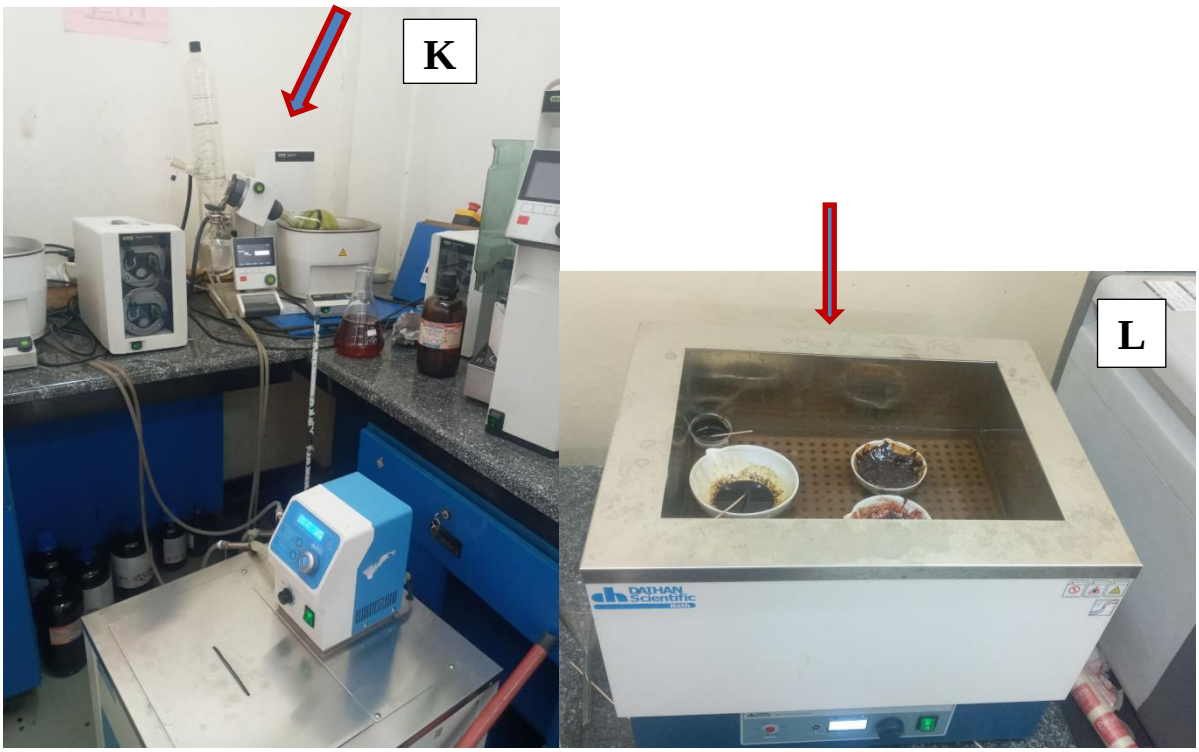
Then, the root and leaf powder weighing 700 g were macerated in the methanol solvent separately in an 80% methanol solvent using a 1:10 solute-solvent ratio, as illustrated in figures E and F. The extraction of this plant material was facilitated by a mini orbital shaker for three days, or 72 hours.

Then, after three days, the macerates were filtered first by gauze, followed by a suction filter with Whatman number one filter paper for efficient extraction of plant materials, as shown in figures G, H, I, and J, respectively, in sequential order.

Filtration was done by using gauze (G) and then, placing filter paper in a suction filter container (H).



The methanol solvent was removed from the filtrate with the aid of Rota vapor at 40 °C, and the filtrate was kept inside the water bath at 40 °C to remove the remaining water and to concentrate, as shown in K and L, respectively.



Finally, the extracted plant materials were kept inside the refrigerator at 4 °C until the beginning of the main experiment.

Annex 2: Antibacterial activity determination of *Carissa spinarum* procedure

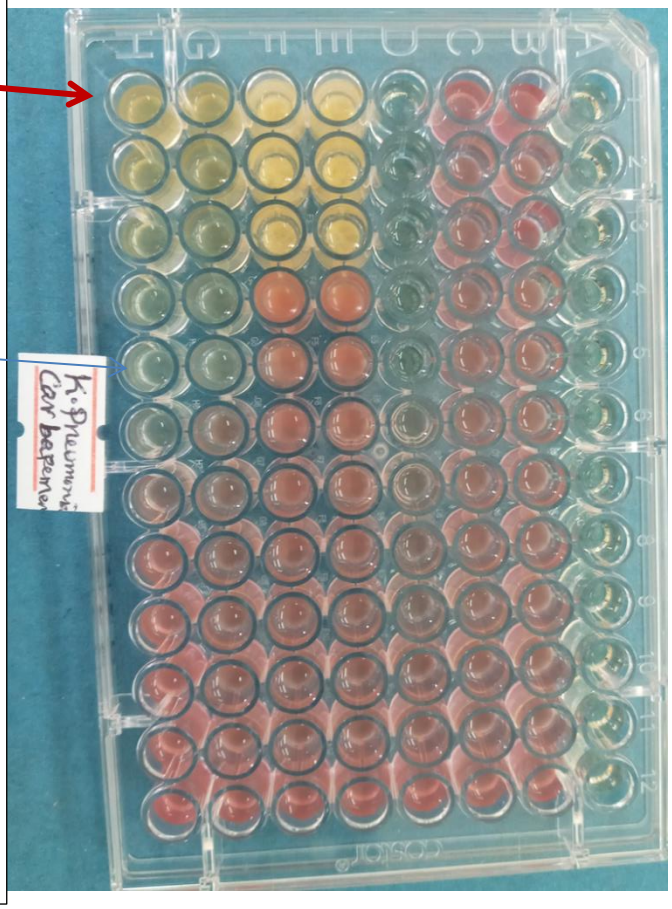
- ✓ The minimum inhibitory concentration (MIC) was determined by using the broth dilution method on 96-well microtiter plates.
- ✓ Standard media used for bacterial growth and multiplication were prepared, boiled, sterilized, and poured into sterile Petri dishes.
- ✓ Then, the test organisms containing the sample were dispensed, spread on agar, and incubated for 24 hours at 37 °C.
- ✓ In order to prepare stock solutions with a concentration of 32 mg/ml, plant extracts were made by dissolving the extract in distilled water.

- ✓ In each individual well of the 96-well microplate, 100 µL of Muller-Hinton broth was added.
- ✓ Then, 100 µL of the extract were loaded into the first row and, in a similar fashion, down to the end.
- ✓ Thus, Mueller-Hinton broth was used to prepare serial dilutions in 96-well microplates, starting from a maximum concentration of 16 mg/mL to a minimum of 0.0078 mg/mL.
- ✓ For standardization of the inoculum, a few colonies of bacteria (3-5) have been transferred from the sub-cultured plate into tubes holding sterile MHB using a disposable sterile inoculating loop to prepare a bacterial suspension.
- ✓ Then, the absorbance of the suspension was measured using a UV-visible spectrophotometer with a 1 cm light path until an absorbance reading of 0.08–0.1 at 625 nm was achieved.
- ✓ The standardized inoculum was diluted in MHB to prepare suspensions of test organisms containing a concentration of roughly 5×10^5 CFU/mL.
- ✓ A bacterial suspension containing roughly 5×10^5 colony-forming units/ml was prepared using a fresh culture.
- ✓ 100 µl of suspension containing test bacteria (5×10^5 CFU/mL) were loaded into the entire well in the microtiter plate within 15 minutes of standardization, with the exception of the sterility control (last column).
- ✓ The plate was subsequently incubated at 37 oC for 18 to 24 hours.
- ✓ Following the incubation period, each well was loaded with 40 µl of a 0.4 mg/mL solution of 2, 3, 5-Triphenyltetrazolium chloride (TTC; Tetrazolium chloride) as a measure of microbial growth. Then, the plates were incubated for 30 minutes at 37 °C.
- ✓ With the use of magnifying glasses, the absence or presence of the pink color after the incubation period of 30 minutes was used for determining the MIC values.
- ✓ The lowest extract concentration (MIC) that did not show any visible pink color, a sign of no microbial growth and development, was recorded for each extract.
- ✓ The tests were carried out in triplicate to determine the MIC values.
- ✓ For each strain in the study, there was a growth control and a sterility control.

- ✓ The bacterial sensitivity was assessed by doing parallel tests with ciprofloxacin as a standard, starting initially at a 10 µg/mL concentration in sterile water (with a range of 10 µg/mL to 0.0078 µg/mL).

The results of the minimum inhibitor concentration of root and leaf extracts of Carissa spinarum against multi-drug-resistant bacteria

Extract Conc.
16mg/ml
8mg/ml
4mg/ml
2mg/ml
1mg/ml
0.5mg/ml
0.25mg/ml
0.125mg/ml
0.0625 mg/ml
0.03125mg/ml
0.0156 mg/ml
0.0078mg/ml



Cipro conc.
10 µg/ml
5 µg/ml
2.5 µg/mL
1.25 µg/ml
0.625 µg/ml
0.3125µg/ml
0.0156 µg/ml
0.078 µg/ml
0.039 µg/ml
0.0195 µg/ml
0.0097 µg/ml
0.0049 µg/ml

From the picture, H&G represent (root extract), E&F (leaf extract), C&D (-Ve and +Ve control, respectively), B (growth control), and A (sterility control). This pattern works for all the rest. This pattern works for all the rest. The picture shows the superior activity of the root extract of *Carissa spinarum* against carbapenem-resistant *Klebsiella pneumonia,* with the lowest MIC values of 1 mg/mL.

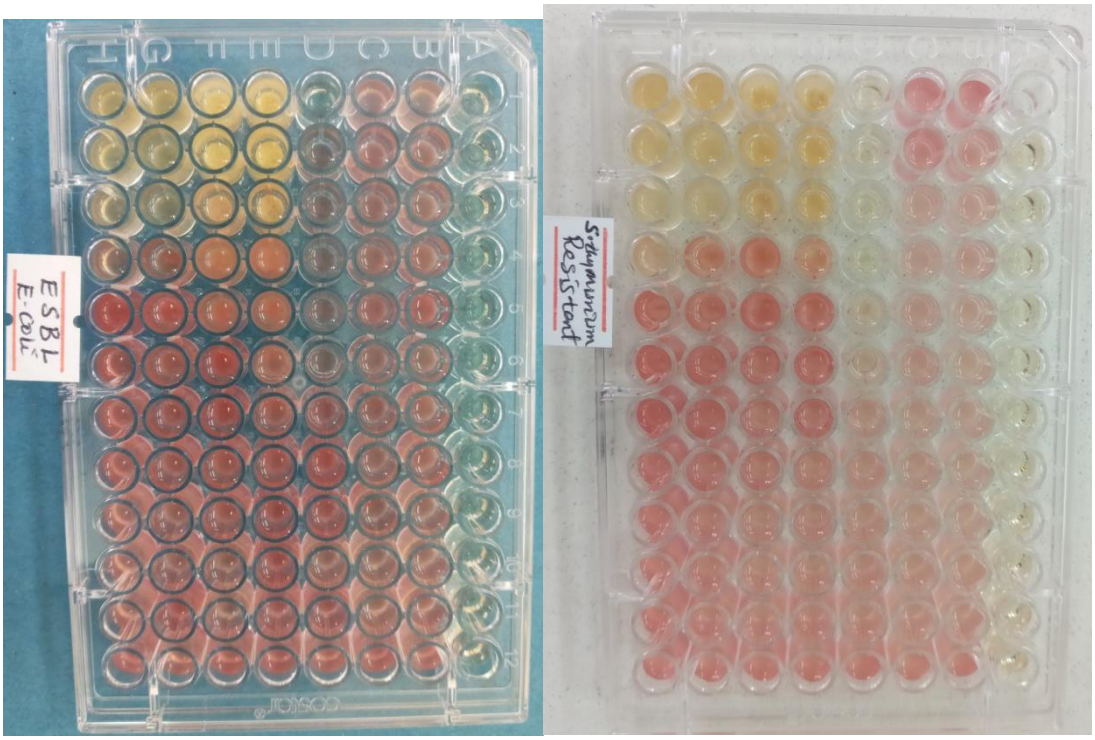
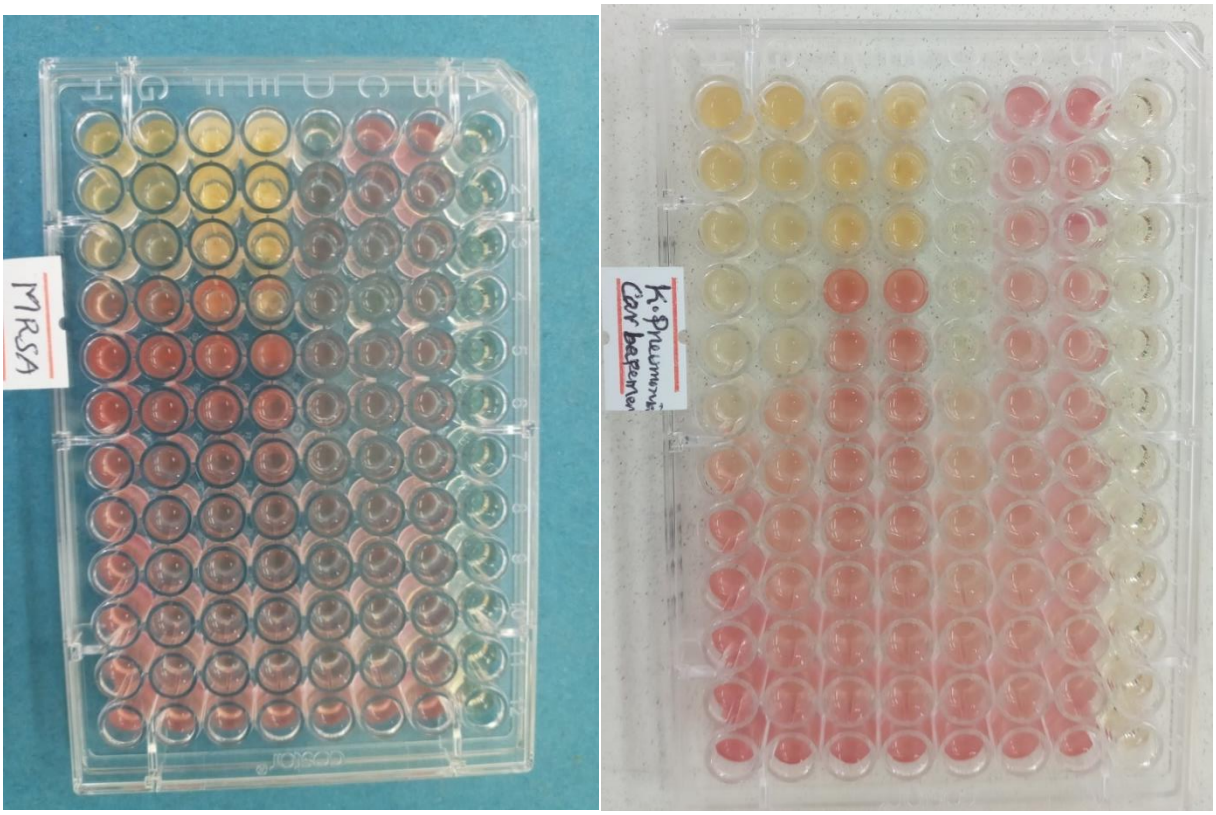
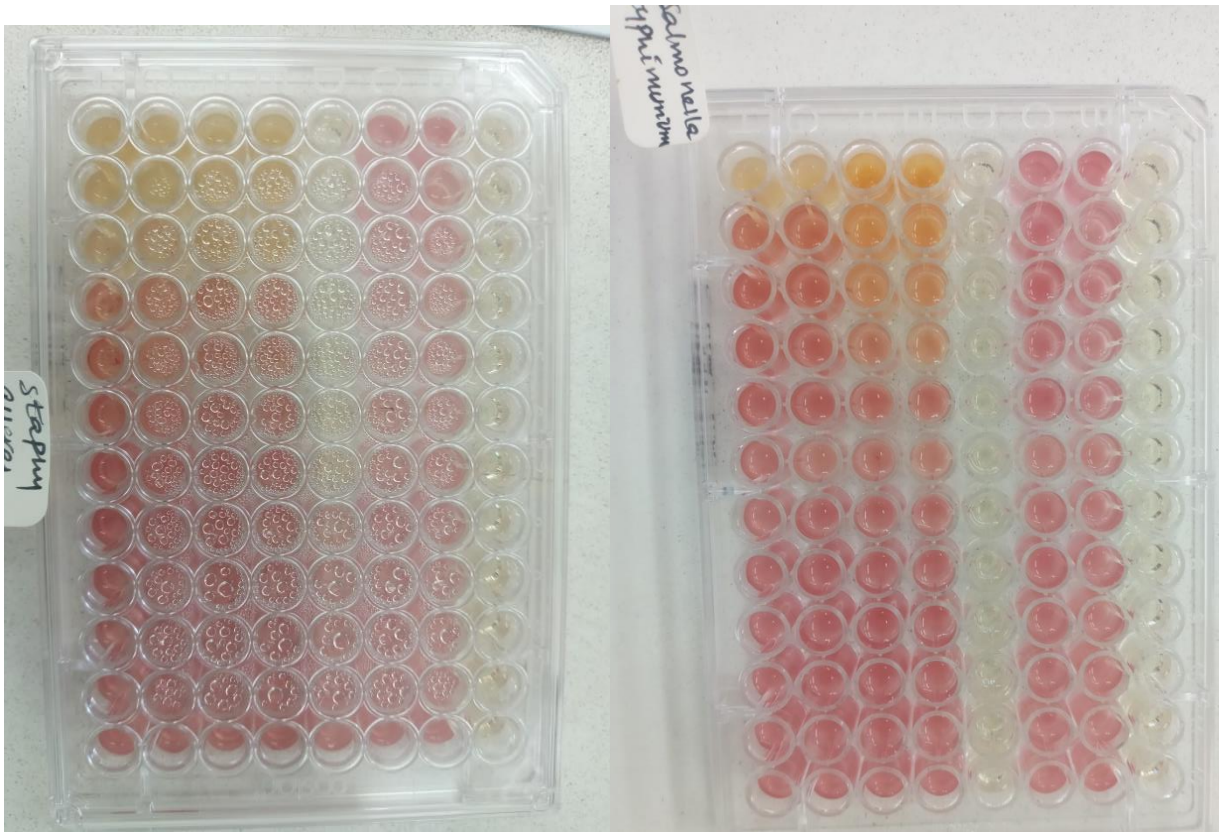


Figure showing ESBL-*E.coli* on the left side and *Salmonella typhimurium* resistant on the right side with their minimum inhibitory concentration.

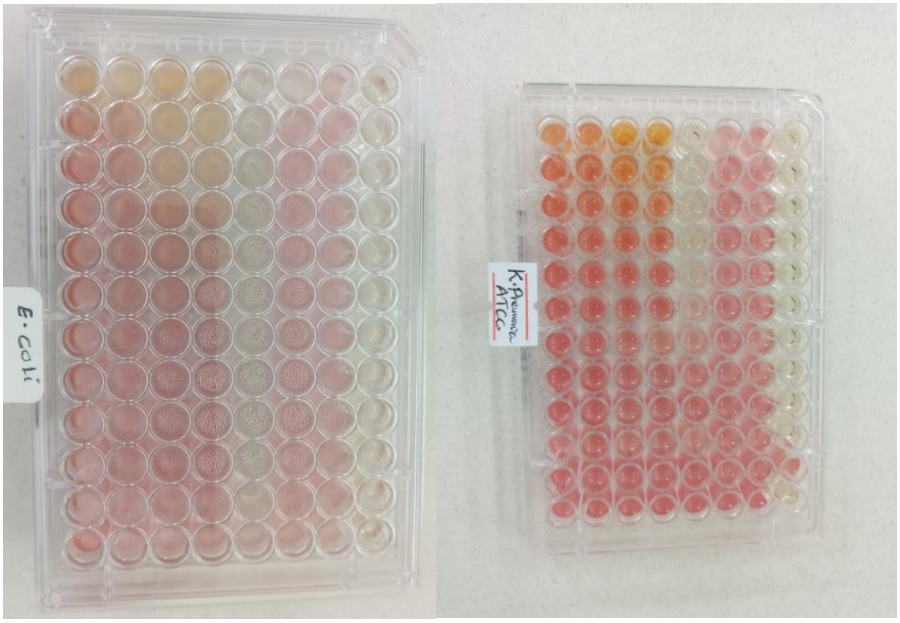


Picture demonstrating MRSA (left side) and carbapenem resistant *klebsiella pneumonia*(right)

The results of the minimum inhibitory concentrations of root and leaf extract against susceptible bacteria strains.



The picture displays the MIC of *Staphylococcus aureus* (left side) and S*almonella typhimurium (*Right side) with their respective MICs.



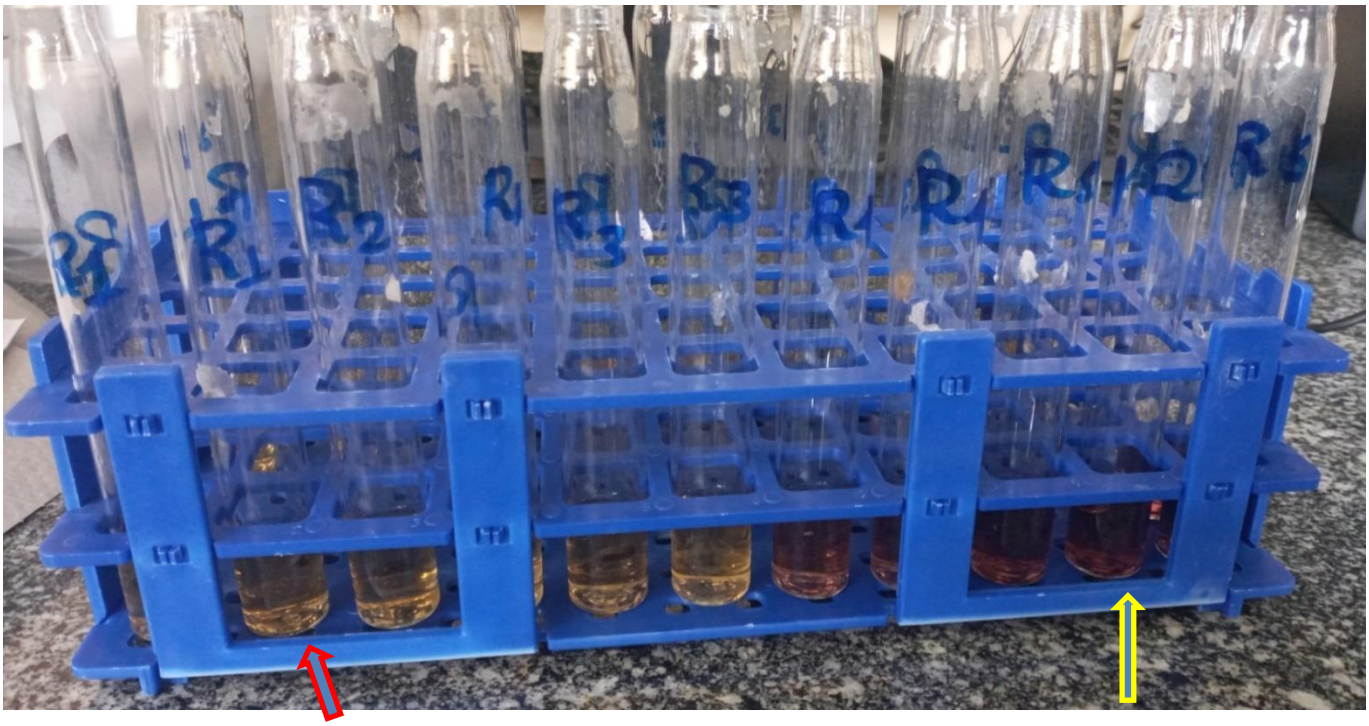
The picture on the left depicts *E. coli* and, on the right, *Klepsiella pneumonia* (ATCC) with their respective minimum inhibitory concentrations.

Annex 3: Antioxidant capacity assay procedures

- The free radical-scavenging activity of antioxidants was determined by the DPPH method.
- First, 10 mg of plant extract was dissolved in 10 ml of distill water in order to prepare 1000 µg/ml of stock solution, and then serial dilution with methanol was performed to prepare the intended concentrations of the solutions (1000, 500, 250, 125, 62.5, and 31.25 µg/ml).
- Next, a DPPH solution was made by dissolving 6 mg of the crystalline solid dpph in 100 mL of analytical-grade methanol in a 200-ml holding conical flask and thoroughly mixing it with gentle shaking.
- Each test tube was loaded with 2 ml of DPPH solutions containing varying concentrations of the extracts and ascorbic acid solutions.
- Subsequently, the mixes were vortexed and allowed to stand in a dark area for half an hour. The DPPH solution was used as the control and methanol as the blank.
- The mixtures were carefully transferred from the test tube into the cuvete after 30 minutes of incubation, and the cuvete was then put inside the UV-visible spectrophotometer.
- The resultant mixture's absorbance was measured at 517 nm.
- The concentration of extracts (in µg/ml) that scavenges 50% of the DPPH radicals was established as the IC50 value.
- Plant extracts and standards (ascorbic acids) were tested for their antioxidant capacity in triplicate.

The figure displays the presence of the root extract in the test tube before the addition of the DPPH solution.

The picture shows the addition of DPPH solution to the root extract and vortexing in a slightly dark area on the left side, and the picture on the right side demonstrates the mixture of extracts with the DPPH solution before incubation.



Concentrations of Plant extracts	1000 µg /ml	500	250	125	62.5	3 1.25 µg /ml

The picture shows free radical scavenging of root extract after incubation for 30 minutes, which reveals changes in color from purple to yellow following the scavenging of the radicals in dose-dependent ways.

The figure demonstrates transferring the mixture of plant extract and DPPH solution from the test tube into a cuvette and placing it inside the UV-visible spectrophotometer after 30 minutes of incubation to appreciate the absorbance values (left side figure). The right-side figure shows the result of absorbance values measured by a UV-spectrophotometer.

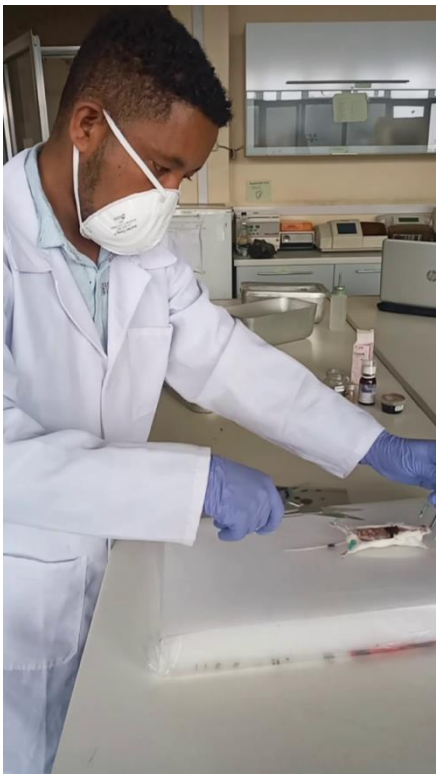
Annex 4: Antidiarrheal activity determination using castor oil-induced diarrhea procedures

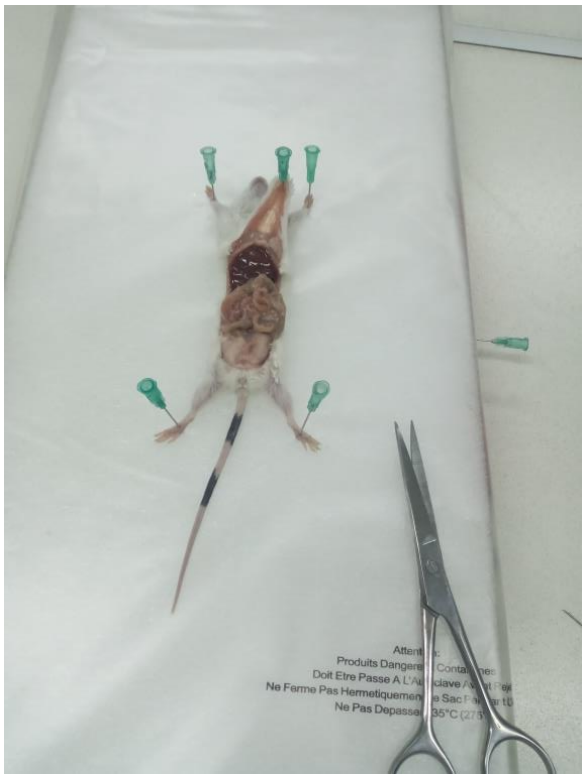
Marking for identification weighing mice after marking

Administration of the test substance by using oral gavage (left) and Diarrhea induced by castor oil in a cage lined at the bottom by plastic transparent paper (right)

Killing mice by cervical dislocation

Abdominal dissection of mice for evisceration of intestinal content





Preparation of mice for removing small intestines from the pylorus to the caecum for both castor oil enteropooling and gastro-intestinal motility models.

Annex 5: In vivo antidiarrheal efficacy evaluation recording format

Castor oil - induced diarrheal evaluation recording format.

Plant Part	concentration of the methanolic extract	Target Animal	Onset of Diarrhea (Min)	Frequency of Wet Feces	Total Weight of Feces (gm)

Castor oil-induced enteropooling evaluation recording format

Plant Part	Concentration of methanolic extract	Target Animal	weight of intestine before removing content	weight of intestine after evacuation	volume of intestinal content

Castor oil-induced gastro-intestinal motility evaluation recording format

Plant Part	Concentration of methanolic extract	Target Animal	Total length of small intestine	The distance of the small intestine covered by activated charcoal