PHYTOCHEMICAL INVESTIGATION AND ANTIMICROBIAL ACTIVITY STUDY OF *Artemisia annua L.* LEAVES (*ASTERACEAE*)

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A thesis written in the Department of Pharmaceutical Chemistry and submitted to the College of Postgraduate Studies in partial fulfilment of the requirements for the award of **Master of Science** Degree of the University of Benin, Benin City, Nigeria

June, 2025

CERTIFICATION

We certify that this work was carried out by Miss Bukola Esther **OLOWOEYO** in the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

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(Head of Department)

CERTIFICATION OF THESIS

We the undersigned attest and declare that the thesis of Miss Bukola Esther **OLOWOEYO**

Titled:

Phytochemical investigation and antimicrobial activity study of *Artemisia annua l.* Leaves (A*steraceae*) has successfully passed the anti-plagiarism test and does not violate any copyright regulations.

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Dr. Osayemwenre Erharuyi **Date**

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Dr. Vincent Imieje **Date**

(Head of Department)

DEDICATION

This project is dedicated to the Almighty God, whose wisdom, strength, and guidance have brought me this far. His grace has been my foundation and source of unwavering support throughout this journey.

I also dedicate this work to my beloved parents and family, whose endless love, encouragement, and sacrifices have shaped my academic and personal growth. Their belief in my potential has been a driving force behind my achievements.

Finally, this work is dedicated to all researchers and scientists striving to discover natural therapeutic agents, and contribute to global health and the fight against antimicrobial resistance.

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LIST OF ABBREVIATIONS

CIA	Collagen-induced arthritis
DCM	Dichloromethane
G	Growth
GAE	Gallic acid equivalents
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
HSV	Herpes Simplex Virus
LD	lethal dose
LPS	lipopolysaccharide
MBC	Minimum bactericidal concentration
MDR	M*ultidrug-resistant*
MIC	Minimum inhibitory concentration
mRNA	Messenger ribonucleic acid
NG	No growth
QE	Quercetin equivalents
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
TFC	Total flavonoid content
TPC	Total phenolic content
TNF	Tumor necrosis factor

ABSTRACT

Artemisia annua (Asteraceae), commonly known as sweet wormwood, has been widely used in traditional medicine for treating fevers, malaria, and various infections. While it's active component, artemisinin, is well known for its antimalarial properties, recent studies have suggested that other bioactive compounds in *A. annua* may also exhibit significant antimicrobial activity. However, the antimicrobial potential of *A. annua* leaf extract, particularly against multidrug-resistant bacteria, remains underexplored. This study was therefore designed to evaluate the antimicrobial potential of *A. annua* leaves and identify constituents with potential antimicrobial activity.

The research involved the collection and authentication of *A. annua* leaves, extraction and fractionation using organic solvents of varying polarity (n-hexane, dichloromethane, ethyl acetate, and methanol), acute toxicity screening and antimicrobial testing against selected bacterial strains. Gas Chromatography-Mass Spectrometry (GC-MS) and High-Performance Liquid Chromatography (HPLC) were employed to identify bioactive compounds in the most active fractions.

The results showed that the ethyl acetate fraction exhibited the highest antimicrobial activity, with significant inhibitory effects on a broad spectrum of bacteria. Key bioactive compounds identified include scopoletin, deoxyqinghaosu, naringenin, kaempferol, and sapogenin. Acute toxicity studies revealed a high safety margin for *A. annua* extract. These findings highlight *A. annua*'s potential as a natural antimicrobial agent, offering a sustainable and cost-effective alternative to synthetic antibiotics, particularly in resource-limited settings.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Artemisia annua, commonly referred to as sweet wormwood, belongs to the *Asteraceae* family, it is an annual fast-growing shrub, often attaining a height of 3 m and 1 m in width. It usually has a short fibrous root, a ribbed single-stem of alternate branches and a secondary stem with fine, silky grey-green hairs bearing the leaves (12 cm long) and the non-showy flowers and seeds (2-3 mm and 1 mm long, respectively) (Diemer, 2002; Orwa *et al.,* 2009). *A. annua* is native to Asia (particularly China, Vietnam, Japan and Korea) and is also widely cultivated across Europe, America and Africa. It is traditionally employed in treating and managing several disease conditions, including fevers, dysentery, scabies, tuberculosis, diabetes, inflammation and wound infections. The plant is prepared as decoction and served as tea extracts or juice to patients. Studies on the plant have confirmed its traditional use as cytotoxic, antioxidant, antidiabetic, anticancer, anti-inflammatory, antimicrobial, antimalarial and antiviral (Bhakuni *et al*., 2001; Bilia *et al*., 2014; Chougouo *et al*., 2016; Efferth, 2017; Lang *et al*., 2019; Lubbe *et al*., 2012).

During the search for effective drug-treatments against multi-drug resistant malaria, a compound, endoperoxide sesquiterpene lactone (*artemisinin*), was isolated from *A. annua* in 1971 and characterized as an active ingredient with remarkable potency against malaria. Thereon, being the principal commercial source of artemisinin, *A. annua* gained a widespread attention worldwide. Further investigations led to modifications that gave rise to several derivatives of artemisinin including (artesunate, artemether, arteather, artemiside, and dihydroartemisinin). Currently, the WHO recommends artemisinin combination based

derivatives as the primary therapy for the treatment of malaria.Interestingly, during the pandemic outbreak of the novel severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) in 2019, folk medicines made from *A. annua* extracts and artemisinin derivatives were used across many countries to prevent and treat COVID-19 infections (Nie *et al.,* 2021). Thus, there was a call for proper investigations into the potency of *A. annua* and its constituents in the management of SARS-CoV-2 (Fuzimoto, 2021). Although, the therapeutic potency of *A. annua* is traced to its antimalarial activity from which further investigations gave rise to the prominent sesquiterpene artemisinin, the plant has been widely investigated for other pharmacological activity based on reported ethnomedicinal uses. Known for its traditional use in treating fevers and malaria, this plant's bioactive compounds, such as artemisinin, have become crucial in modern medicine, particularly for malaria treatment. Beyond its antimalarial properties, *A. annua* has shown potential against various pathogens, indicating a broad-spectrum antimicrobial property that could address global health challenges, including rising antibiotic resistance.

Artemisia annua.

Scientific names: *Artemisia annua.*

Vernacular names

Chinese: Caohao, Cao Qinghao, Cao Haozi, Chouhao, Chou Qinghao, Haozi, Jiu Bingcao, Kuhao, San Gengcao, Xianghao, Xiang Qinghao, Xiang Sicao, Xiyehao

English: Annual wormwood, Sweet wormwood, Sweet annie

French: Armoise annuelle

Japanese: Kusoninjin

Korean: Chui-ho, Hwang-hwa-ho, Gae-tong-sook Vietnamese: Thanh cao hoa vàng.

Local names in Nigeria: Tazargade (Hausa), Ewe Egbin (Yoruba), Ocho-onye-ogwo (Igbo), Mkpatat (Ibibio).

Figure 1. 1: *Artemisia annua* in its natural habitat

1.2 Literature Review

1.2.1 Ethnomedicinal uses

It has been demonstrated that *Artemisia* plants contain antibacterial properties. Many varieties of *A. annua* have been used to treat a variety of illnesses in both Western and Chinese medicinal formulations. In the tropics, A*rtemisia* is a common and efficient antimalarial medication. Infusion made from *Artemisia* leaves is used to treat fevers, colds, and diarrhoea. *Artemisia* leaves are applied topically to boils, abscesses, and nose bleeds. Before the plant flowers in the summer, the leaves are collected and preserved for later use. Additionally, the seeds are used to cure indigestion, night sweats, and flatulence. Clinical investigations have shown that artemisinin is 90% more effective than conventional medications in treating malaria (Mueller *et al*., 2000). *A. annua*-derived terpenoids and flavonoids have cytotoxic properties in a number of human cancer cell lines. Artemisinin and artesunate, two constituents of Artemisia, have demonstrated efficacy as pharmaceuticals for the treatment of cancer (Dondorp *et al*., 2011). According to systematic evaluations, artemisinin is just as effective as quinine in treating both mild and severe cases of malaria. However, its elevated relapse risk could restrict its application.

1.2.2 Pharmacognostical Studies

1.2.2.1 Macroscopic Characteristics

A. annua is a scented annual herb with deeply grooved branches. Variation is more noticeable in the leaves and aerial parts. Although the leaf edges are not complete, the base is asymmetrical. The leaves range in colour from pale green to dark green and are arranged pinnately. Both the outside and interior surfaces are smooth. On both surfaces, there are glandular and non-glandular trichomes (Das, 2012).

1.2.2.2 Proximate Analysis

Physiochemical analysis has shown that *A. annua* contains 9.2 w/w moisture, 8.3 w/w total ash, 0.91% acid insoluble ash, 6.2 w/w alcohol extractive, and 3.8 v/w water extractive values. The leaves and inflorescence contain a high amount of protein, crude fat, and digestible fraction. Manganese and copper are abundant in the plant tissue. This plant has a rich amino acid and vitamin profile, which increases its nutritional value (Das, 2012).

1.2.3 Chemical Constituents

A. annua has turned into the subject of escalated phytochemical assessment following the discovery of the antimalarial drug artemisinin (Wang *et al*., 2011). Phytochemical analysis has recognized different phytoconstituents including steroids, coumarins, phenolics, flavonoids, purines, triterpenoids, lipids, and aliphatic mixtures, monoterpenoids (Emadi, 2013; Cafferata *et al*., 2010; Ferreira *et al*.,, 2010), essential oils, alkaloids, and glycoside (Zanjani *et al*., 2012). A couple of them are likewise present in essential oils (Verdian-rizi *et al*., 2008; Brown, 2010). Essential oils contain both non-volatile and volatile constituents. The volatile components of essential oils are camphene, 1-camphor, isoartemisia ketone, *β*-camphene, *β*-caryophyllene, *β*-pinene, artemisia ketone, 1, 8-cineole, camphene hydrate, cuminal (WHO Monographs, 2006; Willcox, 2009; Das, 2012), Artemisia ketone, 1.8-cineole camphor, germacrene D, camphene hydrate, and alpha-pinene, betacaryophyllene, myrcene, and artemisia liquor (Liao *et al*. 2006; Ferreira and Janick, 2009).

The non-volatile component of essential oil contains sesquiterpenoids (Brown, 2010), flavonoids and coumarins, *β*-galactosidase, *β*-glucosidase, *β*-sitosterol, and stigmasterol (Willcox, 2009; Cafferata *et al*., 2010; Das, 2012). It likewise contains erythritol (50.30 %), camphor (7.25 %), pinocarveol (4.13 %), and diethoxyethane (2.18 %) (Haghighian *et al.* 2008). Scopoletin (coumarin), scopoletin (coumarin glycoside), domesticoside

(phloroacetophenone), chrysosplenol-D (flavonoid), and norannuic corrosive (bisnor-cadinane) are crucial phytoconstituents (Emadi, 2013; Cafferata *et al*., 2010). First time, artemisinin (sesquiterpene lactone) was isolated from *A. annua* in 1972 (Geldre *et al*., 2000; Ogwang *et al*., 2012). Artemisinin is an uncommon sesquiterpene lactone endoperoxide of the cadinane series (Laughlin, 2002). Although there are roughly 400 types of *Artemisia* (Ferreira, 2004), artemisinin and essential oil levels in the leaves of *A. annua* went from 0.01 to 1.4 % and 0.04 to 1.9 %, separately (Damtew *et al*., 2011). The leaves of *A. annua* are just natural sources of artemisinin and other metabolites.

1.2.4 Ethnopharmacological History

Ethnopharmacological gathering named "52 prescriptions" (traces back to 168 BC) referenced *A. annua* (qinghao) as a medicinal herb. This record depicts it: as a remedy for hemorrhoids that look like "cow lice" (possibly ticks). Traditionally, it has been utilized as flavouring agent. In view of areas of strength for this utilization and characteristic fragrance later on, it turns into the potential source for essential oils for the perfume business (Ferreira and Janick, 2009; Huang *et al*., 2010; Liu *et al*., 2013). For more than 2000 years, the Chinese have utilized *A. annua* as a natural remedy to treat jungle fever (Geldre *et al*., 2000; Biesen, 2010). The Pharmacopeia of the People's Republic of China likewise portrays its utilization to cure consumptive fever and jaundice (WHO Monographs, 2006; Castilho *et al*., 2008; Ogwang *et al*., 2012) injury mending and to improve eye brightness (Liu *et al*., 2013). *A. annua* has likewise been utilized traditionally in Iran as a medicinal plant for babies as an antispasmodic, carminative, or sedative/hypnotic remedy (Emadi, 2013; Sharma *et al*., 2011). *A. annua* decoction has been utilized as antihemorrhage to cure the runs (Mirdeilami *et al*., 2011). The impact of *A. annua* L. on hemostasis is notable in traditional medicine (Wang *et al*.,

2011). Traditional medicinal practices include using different plant parts of *A. annua* to cure different sicknesses.

1.2.6 Pharmacological Activity

1. 2.6.1 Antimicrobial Activity

Methanol, ethanol, chloroform, acetone and petroleum ether extracts of *A. annua* have shown good antimicrobial activity against bacteria and fungi (Massiha *et al*., 2013; Tajehmiri *et al*., 2014; Rag *et al*., 2016; Rolta *et al*., 2020). Essential oils extracted from *A. annua* have been studied to have antimicrobial effects against *Klebsiella pneumoniae*, *Pseudomonas aeruginosa, E. coli*, *Streptococcus faecalis, Staphylococcus aureus*, *Bacillus licheniformis, Bacillus subtilis*, *Saccharomyces cerevisiae Aspergillus spp.* and *Candida albicans*, with microbial zone inhibitions ranging from 10-45 mm at for varied concentrations within periods of 24-48 hours (Rasooli *et al*., 2003; Sharopov *et al*., 2020). These oils are dominated by the monoterpenes and their oxygen derivatives, and reports suggests 1,8-cineole was responsible for the antibacterial activity in essential oils since it exhibited comparatively strong activity against Gram-negative and Gram-positive bacteria (Sharopov *et al*., 2015; Sharifi *et al*., 2017). The essential oil of *A. annua* showed weak antioxidant activity and average antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. Suggesting the antibacterial activity of *A. annua* essential oils is related to the presence of 1,8-cineole (Sharopov *et al*., 2020)

Staphylococcus aureus, Escherichia coli, and Enterococcus hirae were among the bacterial strains against which the oil was shown to have a significant inhibitory effect. *Pseudomonas aeruginosa*, however, did not exhibit any sensitivity to essential oils. Extracts from *A. annua* exhibit exceptional antibiotic activity against fungi, specifically *Candida albicans* and

Saccharomyces cerevisiae (Juteau *et al*., 2002; Das, 2012). This research also demonstrated that essential oils had stronger effects against fungal strains than they did against Gram-positive bacterial strains (Verdian-rizi *et al*., 2008). Research employing chemical analysis of plant extracts demonstrated that phytoconstituents are the cause of this antibacterial capacity. Sesquiterpene lactone endoperoxide artemisinin, scopoletin (Tzeng *et al*., 2007), and several other derivative chemicals are the most important molecules that have been investigated for this bioactive potential. The molecular mechanism of action of these compounds has been investigated in *Mycobacterium TB, Escherichia coli, and Mycobacterium smegmatis*. Research has shown that Arteether inhibits DNA-gyrase activity, which is resistant to quinolone, by acting at the nucleus level (Kumar *et al*., 2003). Another well-known precursor utilized in the semi-synthesis of artemisinin is artemisinic acid, as well as antibacterial properties have been investigated (Bhakuni *et al*., 2001; Muzemil, 2008; Huang *et al*., 2010).

1.2.6.2 Anti-inflammatory Activity

A combined treatment was done for hydroalcoholic *A. annua* extract and artemisinin with lipopolysaccharide LPS (500 ng/mL), an inflammatory stimulus. The LPS treatment showed a cytotoxic effect, reducing the cell vitality by 30%. The ethanol extracts showed a protective action of 25, 50 and 90% in a dose-dependent manner. In the same way, co-treatment with Artemisinin reduced the cytotoxic effect of LPS, but failed to protect against the action of LPS, at lower dose between 50 mg/mL to 5 mg/mL. Furthermore, in a second treatment with an anti-inflammatory cytokine mRNA gene regulator TNF, artemisinin and *A. annua* hydroalcoholic showed a significant reduction in the TNF-mRNA gene expression (at $p < 0.001$ and $p < 0.0001$ respectively). The comparably higher significance for the ethanol extract to that of artemisinin, was attributed to the synergy effects of other secondary metabolites present (Abate *et al*., 2021)

1.2.6.3 Anti-viral activity

A. annua plant extracts and isolated compounds including artemisinin and derivatives have exhibited antiviral activity against herpes, hepatitis, respiratory and human immunodeficiency syndrome viruses (Naesens *et al*., 2006; Efferth *et al*., 2008; Milbradt *et al*., 2009; Hakacova *et al*., 2013; Qi *et al*., 2013; Devaraj and Roelofson, 2015; Efferth *et al*., 2018). However, the more recent antiviral studies on *A. annua* have majorly focused on the severe acute respiratory coronavirus (SARS-CoV-2). Upon the outbreak of the novel SARS-CoV-2, the potent antimalarial drug(s) from *A. annua* (Artemisinin and derivatives) were considered for use as repurpose drugs, and thus, since have been investigated. Gendrot *et al.* (2020) conducted an *in vitro* inhibition test of the virus strain (IHUMI-3) in Vero E6 cells with 5 artemisinin-based combination therapies; mefloquine-artesunate exerted the highest antiviral activity of 72.1%. Hot water extracts inhibited SARS-CoV-2 infection, and the active component(s) in the extracts were attributed to the combination of different phytochemicals present not limited to artemisinin (Nair *et al*., 2021)

A different study on *A. annua* (ethanol and aqueous) and artesunate on the human isolate (SARS-CoV-2), revealed that the extracts suppressed the virus in a dose-dependent manner, particularly the ethanol extract. In a similar study with *A. annua* extracts (ethanol and aqueous), artemisinin and derivatives (artesunate, artemether) on a German and Danish SARS-CoV-2 strain, showed the highest activity observed was artesunate > artemether > ethanol extract > artemisinin > aqueous extract (Niel *et al*., 2020; Zhou *et al*., 2021).

Further studies can involve *in vivo* efficacies to assess whether *A. annua* might provide a cost-effective therapeutic to treat SARS-CoV-2 infections. However, researchers seemed to have overlooked *A. annua's* antiviral properties, as evidenced by the paucity of studies on the

cossac virus type A16 (CA16), human immunodeficiency virus (HIV), hepatitis A virus (HAV), Herpes Simplex Virus HSV-1 and HSV-2, and respiratory syncytial virus (RSV).

HAV is a non-enveloped RNA virus that can cause acute hepatitis. When HAV was co-treated with 50 g/mL *A. annua* extract, the titer of HAV was reduced by 2.33 logs (Seo *et al*., 2017). However, same anti-viral action was not detected when HAV was pre-treated with *A. annua* extract at the same dose, indicating that *A. annua* extract may exert anti-viral activity via direct virucidal activity or via inhibiting viral attachment to host cells.

HSV-1 and HSV-2 are enveloped DNA viruses that cause a variety of illnesses ranging from Herpes labialis to severe encephalitis. In HeLa cells, *A. annua* methanol extraction shown potential anti-viral activity against HSV-1 that was more effective than acyclovir at concentrations of 3.125, 6.25, 12.5, and 25 g/mL (Karamoddini *et al*., 2011). In another work, *A. annua* aqueous extract demonstrated anti-viral activity against HSV-2 in Vero cells that was comparable to acyclovir (Zhang *et al*., 2003). Nevertheless, the extraction of *A. annua* in petroleum ether, ethyl acetate, and n-butanol did not show any antiviral activity against HSV-2. Subsequent examination of the aqueous extract of *A. annua* revealed that the primary components were polyphenols and carbohydrates. These findings led to the isolation of a condensed tannin with promising anti-HSV-2 action from the *A. annua* aqueous extract (Zhang *et al*., 2004). Condensed tannin, in addition to HSV-2, demonstrated anti-hepatitis B virus (HBV) efficacy by suppressing the hepatitis B e-antigen (HBeAg) production of HepG2. cells, a persistently infected HepG2 cell line derived from HBV.

1.2.6.4 Antioxidant Activity

A. annua is a strong source of antioxidants and other nutritious components (Das, 2012). Research shows that the high antioxidant activity of crude organic extracts of aerial portions

is most likely caused by the large concentration and diversity of flavonoids found in the leaf, including the recently discovered C-glycosyl flavonoid, which may be an antioxidant component. *A. annua* contains flavonoids and essential oils, both of which have antioxidant potential. Accordingly, these investigations placed *A. annua* at the top of the list of therapeutic plants because of its highest potential for antioxidants (Juteau *et al*., 2002; Ferreira and Janick, 2009). According to Ferreira *et al*. (2010), major groups of hydroxylated and polymethoxylated flavonoids have been found, and they additionally include eupatin, chrysosplenol-D, cirsilineol, chrysoplenetin, casticin, and artemetin.

The five bioactive flavonoids have been found through studies and are now undergoing structural analysis. According to Yang *et al*. (2009), they include quercetin, blumeatin, 5-hydroxy-3,7,40-trimethoxyflavone, 5-hydroxy-6,7,30,40tetramethoxyflavonol, and 5,40-dihydroxy-3,7,30-trimethoxyflavone.

Oxygen radical species causes oxidative damage in the human body and are often associated with various diseases, including cancer, diabetes, arteriosclerosis cardiovascular disease, inflammations and aging Antioxidants are chemical constituents (flavonoids, polyphenols, alkaloids) that reduces the risk of these diseases, inhibits cellular damage and improve cellular defence (Kim *et al*., 2014; Imieje *et al*., 2017). Several studies have reported the presence of these phytoconstituents and have further confirmed the antioxidant activity of the plant. (Song *et al*., 2015; Zhigzhitzhapova *et al*., 2019; Guo *et al*., 2020; Sembiring *et al*., 2022).

1.2.6.5 Immunosuppressive Activity

An evaluation of *A. annua*'s immunosuppressive activity has been conducted. Concanavalin A (Con A) and lipopolysaccharide (LPS)-stimulated splenocyte proliferation were strongly

inhibited in vitro by an ethanolic extract of *A. annua*, and this action increased with dosage. Additionally, studies have demonstrated that *A. annua* ethanol extract can inhibit the humoral and cellular responses (Das, 2012). Flavonoids found in leaves have been related to immunosuppressive potential because they have the ability to alter the immune response (Ferreira *et al*., 2010).

1.2.6.6 Antiarthritis Activity

According to experimental research, the artemisinin derivative SM905 (obtained from *A. annua*) reduces Th17 and inflammatory responses, which improves arthritis brought on by collagen. These investigations have used the type II bovine collagen model (CII) to generate collagen-induced arthritis (CIA) in DBA/1 mice by oral administration of the artemisinin derivative SM905. The frequency and severity of the condition were routinely monitored. The level of T helper (Th) 17/Th1/Th2 type cytokine production as well as gene expression have been studied. The results of this trial showed that the SM905 chemical was important since it postponed the beginning of the disease, which decreased the incidence of arthritis. Additionally, it lessens the overexpression of certain chemokines and pro-inflammatory cytokines (Das, 2012).

1.2.6.7 Antimalarial Activity

The discovery of novel, extremely efficient substitutes has been made possible by the extraction of artemisinins from *A. annua* (Ferreira, 2004; Ridder *et al*., 2008; Ferreira and Janick, 2009). Today, *A. annua* L is well known around the world (Liu *et al*., 2009; Willcox, 2009), and it is used as a potent medication alternative against malaria, especially chloroquine-resistant malaria (Ferreira *et al*., 2006), in over 50 countries. Flavonoids that have been shown to exhibit antiplasmodial activity include artemetin, casticin, chrysoplenetin,

chrysoplenol-D, cirsilineol, and eupatorin (Lubbe *et al*., 2012). Methoxylated flavonoids play a crucial role in the production of artemisinin peroxide by facilitating the interaction between artemisinin and plasmodial haemoglobin through the catabolic route. This mechanism was linked to the activation of artemisinin. Moreover, artemisinin peroxide suppresses heme polymerization, which in turn grants antimalarial properties against the protozoan Plasmodium species falciparum vivax, malariae, and ovale. Flavonoids may also prevent Plasmodium from incorporating hypoxanthine, according to a different mode of action (Laughlin, 2002; Muzemil, 2008; Das, 2012).

1.2.6.8 Antiparasitic Activity

Artemisinin medications appear to have strong antiparasitic capability for Leishmania, Trypanosoma Babesia, Eimeria or coccidiosis, trematodal blood fluke Schistosoma spp., Schistosoma japonicum, Schistosoma mansoni, and Schistosoma haematobium, according to research. As a result, its application in the cattle sector is expanding (Kumar *et al*., 2003; Ferreira and Janick, 2009). A study was carried out on Neospora canum, a protozoal infection of animals. Artemisinin was infected into cultured Vero cells or mice peritoneal macrophages for 14 days. After 11 days, all microscopic foci of *N. caninum* were entirely eradicated at concentrations of 20 or 10 g/mL, and the same findings were achieved at concentrations of 0.1 g/mL. As a result, artemisinin has the ability to inhibit *N. caninum* tachyzoites intracellular proliferation. Artemether was also tested against the larval stages of *Schistosoma mansoni* in another investigation. It was discovered that artemether therapy did not cause schistosomiasis in animals. The parasite's susceptibility was highly apparent as compared to untreated controls (Das, 2012).

1.2.6.9 Cytotoxic and Anticancer activity

Artemisinin selectively induced the ubiquitin-26S proteasome-mediated degradation of the human androgen receptor protein and disrupted androgen responsiveness of human prostate cancer cells (Adrea *et al*., 2017), suggesting its potential in preventing prostate cancer progression from an androgen-responsive state to a phenotype in which the artemisinin is activated in a ligand-independent manner. Dihydroartemisinin was demonstrated (Liu *et al*., 2018) to inhibit cell viability, migration, and invasion, as well as induces apoptosis in epithelial ovarian cancer through suppression of the hedgehog signalling pathway. It was also shown to enhance osteogenic differentiation via the signalling pathways of Wnt/β and ERK1/2. (Licheng *et al*., 2019)

Aside the sesquiterpenes (Artemisinin and derivatives), other isolated compounds of polyphenols (Ko *et al*, 2016) were shown to exhibit anticancer properties. In a study by Jung *et al*. (2020**),** polyphenol compounds isolated from 70% methanol *A. annua* whole plant extract were shown to induce propidium iodide uptake, reactive oxygen species (ROS) production, nuclear structure change and acidic vesicles in a p53-independent manner in p53—null HCT116 cells. The p53 (tumour protein) augments polyphenolics-induced anticancer effect by activating p53-dependent signalling and inducing cleavage of PARP1 and lamin A/C in HCT116 human colorectal cancer cells.

1.2.6.10 Immunomodulatory effects

Artemisinin derivative Dihydroartemisinin, was found to reverse weight loss and decrease disease activity and mortality rates observed in an oxazolone and 2,4,6-trinitro-benzene sulfonic acid (TNBS) induced colitis in animal models. In particular, the oxazolone-induced mice which recorded higher mortalities was significantly reduced in mice administered with

DHA. It was demonstrated, that DHA inhibits Th9 and Th22 cells in oxazolone-induced colitis and Th17 cells in TNBS-mediated colitis, and increased Tregs in both models. This it does by exerting colitis and regulating the Th/Treg balance in a mechanism similar to the induction of activated CD4+ T cell apoptosis via the regulation of HO-1 (Yan *et al*., 2019)

1.2.7 Phytochemistry

Phytochemical screening of *A. annua* has revealed the presence of flavonoids, alkaloids, saponins, alkaloids, phenolics and Terpenoids. Investigations have led to the isolation of predominant secondary metabolites of the monoterpenes, sesquiterpenes, coumarins, flavonoids and phenolics. The monoterpenes are the primary constituents of *A. annua* essential oils with good antimicrobial, anti-inflammatory and antioxidant, analgesic and antidiabetic properties (Yang *et al*., 2016; Bejeshk *et al*., 2019; Quiroga *et al*., 2019). Most Sesquiterpenes present have a characteristic Lactone moiety of an endoperoxide bridge, typically the Artemisinin and derivatives which have been demonstrated to have good antimalarial, anticancer and antiviral activities (Flobinus *et al*., 2014; Wong *et al*., 2017; Idowu *et al*., 2018). The flavonoids, phenolics and coumarins have been shown to exhibit antioxidant, anticancer, anti-inflammatory, antipyretic and antiviral activities (Shaw *et al*., 2003; Kaur *et al*., 2008; Pan *et al*., 2009; Bian *et al*., 2017).

Table 1.1: Some major constituents isolated from *Atermisia annua*

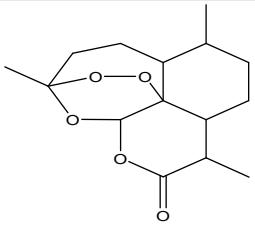
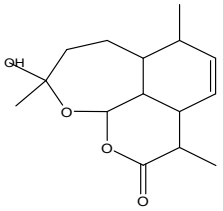
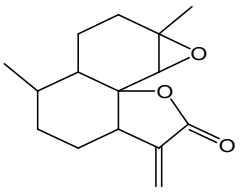
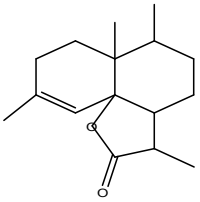
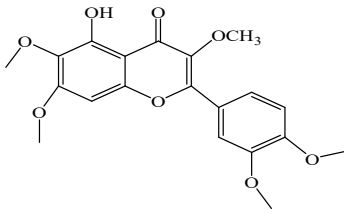
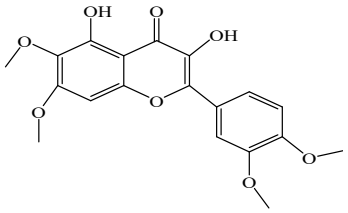
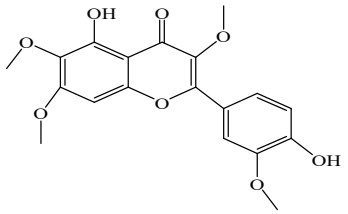
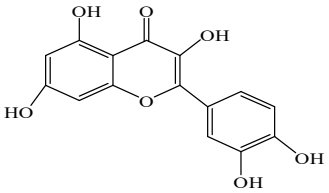
Phytochemical class	Compound	Activity	Reference(s)
Sesquiterpenoids	 artemisinin	Antimalarial, anti-inflammatory, antiviral, and cytotoxicity	Zhenng *et al*., 1993; Qin *et al*., 2017
	 deoxyartemisinin		
	 arteannuin B		
	 dihydro-epideoxyarteannuin		

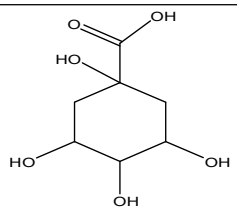
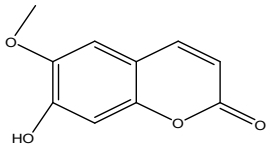
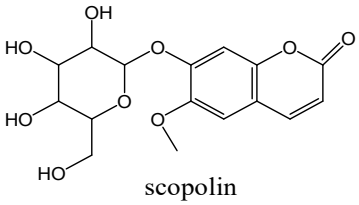
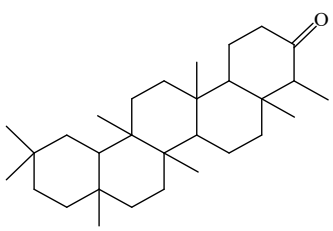
Table 1.1 Cont'd: Some major constituents isolated from *Atermisia annua*

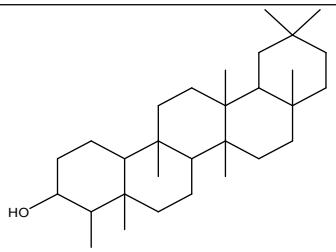
Phytochemical class	Compound	Activity	Reference(s)
Flavonoids	 artemetin	Anti-inflammatory, antioxidant, anticancer, antiviral	Zheng *et al*., 1993; Chougouo *et al*., 2016; Malaterre *et al*., 2020
	 quercetagetin-6,7,3',4'-tetramethyl e		
	 chrysosplenetin		



quercetin

Table 1.1 Cont'd: Some major constituents isolated from *Atermisia annua*

Phytochemical class	Compound	Activity	Reference(s)
Phenolics	 quinic acid		
Coumarins	 scopoletin	Anti-inflammatory, antioxidant, antipyretic	Chougouo *et al*., 2016; Malaterre *et al*., 2020
	 scopolin		
Steroid	 friedelin	Anti-inflammatory	Chougouo *et al*., 2016;

 friedelan-3*B*-ol	Anti-inflammatory

1.2.7.1 Phenolic Acids

Rosmarinic and chlorogenic acids, which have recently been discovered in a wide range of *A. annua* cultivars, are powerful antioxidants (De Magalhes *et al*. 2012). These acids dramatically inhibited the release of the inflammatory cytokines IL-6 and IL-8 in Caco-2 trials. They also reduced inflammation while inhibiting CYP3A4 activity and increasing antimalarial action.

1.2.7.2 Flavonoids

About forty flavonoids have been found in *A. annua* (Ferreira *et al*.,, 2010), and no less than ten, including artemetin, casticin, chrysoplenetin, chrysoplenol-D, circilineol, eupatorin, kaempferol, luteolin, myricetin, and quercetin, show some feeble *invitro* therapeutic management against plasmodium falciparum (Lehane and Saliba, 2008).

The flavone, luteolin, was found in Artemisia species containing up to 0.0023 % of dry weight (Bhakuni *et al*., 2001). Ethnobotanical utilization of luteolin incorporates treatment for hack, the runs, loose bowels, diabetes, cancer, and intestinal diseases. Contrasted and different flavones, for example, kaempferol, myricetin, quercetin, isoquercitrin, acacetin, apigenin, baicalein, and chrysin, luteolin was viewed as the most dynamic with IC_{50} values around 11 lM (Lehane and Saliba, 2008).

1.2.7.3 Saponins

Saponins are found in many plants and have a vital role in human and animal nutrition. They are said to be present in *A. annua*, however only in the non-quantitative foaming test of alcoholic extracts (Ashok and Upadhyaya, 2013; Massiha *et al.,* 2013; Weathers, unpublished). Saponins are amphiphilic (lyophilic and hydrophilic) soaplike bioactive chemicals mostly generated by plants. Unexpected interest in the clinical use of saponins as chemotherapeutic agents has recently emerged (Podolak *et al.,* 2010).

1.2.7.4 Coumarins

The coumarin scopoletin is found in the majority of *Artemisia* species. The concentration of scopoletin in numerous *A. annua* plant samples tested in Luxembourg is around 0.2% (W/W). Scopoletin has anti-inflammatory, antioxidant, and hepatoprotective properties (Malik *et al*. 2011). Scavenging ability for hydroxyl radical, DPPH, superoxide anion, hydrogen peroxide, and Fe2+ chelating activity is nearly equal to a-tocopherol (Vitamin E) (Malik *et al*. 2011). Scoploletin suppresses TNF-*a*, IL-6, and IL-8 at mM doses and is thus likely one of the key anti-inflammatory and antipyretic ingredients of *A. annua* (Moon *et al*. 2007). Scopoletin has antinociceptive effects as well (Meotti *et al*., 2006; Chang *et al*., 2012).

Coumarins have the ability to activate lymphocytes, hence boosting immune processes. Scoploletin has an immunomodulatory impact and stimulates cell proliferation in normal lymphocytes, according to Moon *et al*. (2007).

1.2.8 Acute and sub-acute toxicity

The aqueous and methanol extracts of *A. annua* in an animal model studies were demonstrated to cause no death or any significant changes at LD_{50} of 72 mg/kg and 2000 mg/kg respectively in an acute toxicity study (Souleymane *et al*., 2018; Li *et al*., 2021). In the

subacute toxicity studies, no significant changes were observed in the body weights, their haematological parameters and biochemical indices were not affected in comparison to the control groups and histological evaluation of vital organs (heart, lung, spleen, duodenum, stomach, kidney ovary and testis) all appeared normal suggesting the plant safety for use.

1.3 Rationale of the Study

Due to the rise in antimicrobial resistance, the search for alternative agents is critical. *Artemisia annua*, is notable for its production of artemisinin, a compound crucial in the treatment of malaria. However, the plant's broader antimicrobial potential has drawn significant research interest. The following points highlight the rationale for studying *A.annua*:

A.annua contains a variety of secondary metabolites that have demonstrated activity against bacteria, fungi, and viruses. These natural compounds could serve as alternatives to synthetic drugs, contributing to the fight against drug-resistant pathogens (Willcox, 2009; Efferth, 2017).

Research has suggested that *A.annua* extracts may enhance the effectiveness of existing antibiotics through synergistic effects, potentially lowering the dose needed and reducing resistance development.

As a plant that can be cultivated in diverse regions, *A.annua* offers a sustainable and cost-effective option for developing countries, where access to modern antibiotics may be limited. Its use in low-cost treatments could address the healthcare challenges faced by resource-poor communities (Van der kooy and Sullivan, 2013).

The bioactive components of *A.annua* may present toxicological risks if not properly evaluated. It is important to determine the plant's safety, effective dosages, and potential side effects to ensure it is safe for human use in therapeutic formulations (Liu *et al.,* 2010).

1.4 Aim

The study aimed to evaluate the antimicrobial potential of *Artemisia annua* and its bioactive compounds, with the objective of advancing the search for natural, plant-based antimicrobial agents effective against drug-resistant pathogens.

1.5 Objectives

The objectives are to;

- Conduct a phytochemical screening of *Artemisia annua* extract and fractions
- Investigate the antimicrobial activity of extract and fractions of *A. annua* against multidrug-resistant bacteria.
- Determine the acute toxicity profile of *A. annua* in experimental animals.
- Identify the bioactive compounds potentially responsible for antimicrobial activity using GC-MS and HPLC.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Glassware

Measuring cylinders, conical flasks, volumetric flasks, separating funnel, beakers, graduated pipette, test tube rack, test tube, micropipette, sterile pipettes

2.1.2 Equipment

Heating mantle, Clevenger type apparatus, UV- Visible Spectrophotometer (T80 PG Instrument), Rotary evaporator (Stuart® digital water bath RE300B), Incubator, GC-MS (Shimadzu QP-2010 Plus), HPLC (Shimadzu LC-10AD dual binary pumps, Shimadzu CTO-10AS column oven, and Shimadzu Prominence SPD-20A UV/Vis detector).

2.1.3 Reagents

Methanol (99.8%; Laba Chemie PVT Ltd India), Ethyl acetate, Dichloromethane (98% ; Molychem India), n.hexane (95%; CDH India), Dragrndorff's, Mayer, Hagar and Wagner's reagent, 1% alcoholic naphthol, concentrated sulphuric acid, Benedict's reagent, 1% gelatin, 10% sodium chloride, chloroform, iron chloride, 20% sodium hydroxide, dilute hydrochloric acid, lead acetate solution, distilled water, Ferric Chloride, Sulphuric Acid (H_2SO_4), Fehlings Solution, DPPH (2,2-diphenyl-1-picrylhydrazyl), TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine), Ferric Chloride($FeCl_3$), Ascorbic Acid, Phosphate Buffer, Quercetin, garlic acid, sodium bicarbonate, Test microorganisms (bacteria), Sterile Mueller-Hinton Broth (MHB),

ammonium molybdate. The above chemicals and reagents used to conduct the research work were all of analytical grade.

2.2 Methods

2.2.1 Collection of Plant Materials

Leaves of *A. annua* were sourced from Iwajowa, Ogun State, Nigeria, and authenticated by the Forestry Research Institute of Nigeria Ibadan, Oyo State, Nigeria; with the herbarium voucher number FHI113652.

2.2.2 Extraction Procedure

A 2.3 kg powdered sample was macerated with 10 L of methanol at room temperature for 48 hours. After which the extract was filtered from the marc. This was again macerated with recovered methanol solvent. This was done to get as many constituents as possible from the plant sample. After 48 hours (second maceration), the extract was filtered from the marc. The combined extract was concentrated to dryness using a rotary evaporator at 45^0C under reduced pressure. The dried extract was spread on a stainless steel plate to allow complete removal of residual solvent. The dried extract was weighed and transferred to an air-tight container and stored in the refrigerator at 4^0C.

Fractionation of A. annua extract Using Solvent Partitioning

A portion of the dried methanol extract of *Artemisia annua* (68g) was subjected to solvent-solvent partitioning to fractionate its phytoconstituents based on differences in polarity. The extract was dissolved in a 300mL mixture of methanol and distilled water at a 4:1 (v/v) ratio, forming a medium-polar solvent system.

The methanol-water solution was transferred into a 1000mL separating funnel and subjected to liquid-liquid extraction using organic solvents of increasing polarity, namely n-hexane, dichloromethane (DCM), ethyl acetate, and methanol.

The fractionation process began with 500 mL of n-hexane, a non-polar solvent. The separating funnel was gently agitated and allowed to stand until two distinct layers formed. The n-hexane (upper) layer was carefully separated, collected, and the process was repeated multiple times to ensure exhaustive extraction of non-polar substances. 4.3 L total volume of n-hexane was used for this process

Following this, 500mL of dichloromethane was added to the aqueous methanol layer. After gentle mixing and settling, the lower DCM layer was collected. This process was repeated to optimize the recovery of moderately non-polar phytochemicals. 2.2 L total volume of DCM was used for this process.

Subsequently, ethyl acetate (500mL) was employed. Several rounds of extraction were carried out to ensure the efficient transfer of these compounds into the ethyl acetate layer, which was then separated and concentrated. 1.5 L total volume of ethyl acetate was used for this process.

Finally, pure methanol (500 mL) was used to re-extract the remaining highly polar constituents from the residual aqueous phase. 800 mL total volume of methanol was used for this process.

Each organic layer (n-hexane, DCM, ethyl acetate, and methanol) was separately collected and subjected to rotary evaporation under reduced pressure to remove residual solvents. The dried residues now concentrated fractions were further air-dried to obtain semi-solid extracts.

These fractions were stored in labeled amber containers for subsequent phytochemical screening, antimicrobial testing, and GC-MS/HPLC profiling.

2.2.3 Phytochemical Screening

The plant crude extract and fractions were subjected to qualitative phytochemical tests for alkaloids, tannins, saponins, terpenoids, steroids, anthraquinones, flavonoids and other phenolic compounds using standard methods (Evans, 2002). Each test identifies specific groups based on observable reactions such as colour changes.

Test for Alkaloids

Dragendorff's reagent: The plant crude extract and fractions (2 mL) were shaken with 1% HCl for 2 mins. The mixture was filtered and a few drops of Dragendorff's reagent were added. The formation of an orange precipitate indicated the presence of alkaloids.

Wagner's reagent: The plant crude extract and fractions (2 mL) were shaken with 1% HCl for 2 mins. The mixture was filtered and a few drops of Wagner's reagent were added. The formation of reddish-brown precipitate indicated the presence of alkaloids.

Mayer's reagent: The plant crude extract and fractions (2 mL) were shaken with 1% HCl for 2 mins. The mixture was filtered and a few drops of Mayer's reagent were added. The formation of cream precipitate indicated the presence of alkaloids.

Hager's reagent: The plant crude extract and fractions (2 mL) were shaken with 1% HCl for 2 mins. The mixture was filtered and a few drops of Hager's reagent were added. The formation of yellow crystalline precipitate indicated the presence of alkaloids.

Test for Saponins

***Frothing test*:** The plant crude extract and fractions (1 mL) were shaken with 5 mL of water. The formation of stable froth was a positive result, indicating the presence of saponins.

Fehling test: 5 mL of diluted H_2SO_4 was added to 10 mL of the plant crude extract and fraction. The mixture was boiled for 15 mins, filtered and cooled. 2.5 mL of the filtrate as made alkaline with 20% NaOH solution an boiled with 0.1 mL each of Fehling"s solution A and B for 2 mins. Formation of blue black precipitation indicated the presence of saponins.

Test for Tannins (*Gelatin Test)*: 1% gelatin solution in 10% NaCl was added to 2 mL of the plant crude extract and fractions. White buff precipitate indicates the presence of tannins

Test for Terpenoids (*Salkowski Test):* 5 mL of the extract and different fractions with 2 mL of chloroform and concentrated H_2SO_4 were carefully added dropwise to form a layer. Formation of ring layer, a reddish-brown colour indicates the presence of terpenoids

Test for Phenolic Compounds (*Ferric Chloride Test):* 2 mL of distilled water, followed by 2 drops of 5% ferric chloride solution, was added to 2 mL of extract and different fractions. A blue-green colour shows a positive test for phenols.

Test for Flavonoids

Lead Acetate Test: A few drops of lead acetate solution were added to 2 mL of the plant crude extract and fractions. A yellow coloration indicates the presence of flavonoids.

Aluminium Chloride test: 3 mL of the plant crude extract and fractions were shaken with 0.1 mL each of 1% $Alcl_3$ solution and 1 M CH_3COOK solution. The mixture was allowed to stand for 30 minutes. Formation of white precipitate indicated the presence of Flavonoids.

Test for coumarins: 1 mL of 10% sodium hydroxide solution was added to 1 mL of the plant crude extract and fractions. Colour change indicates the presence of coumarins.

2.2.4 Acute Toxicity Screening

Experimental animals

Adult Swiss albino mice of either sex were obtained from the animal house of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin.

The animals were kept under a 12-h light/dark cycle in clean and well-maintained cages for two weeks to acclimatize to the laboratory environment. The animals were fed with standard rodent pellets and allowed access to water *ad libitum*.

Ethical consideration

The animals were handled following the U.K. Animals (Scientific Procedures) Act, 1986, and associated guidelines, EU directive 2010/63/EU for animal experiments.

Ethical approval was obtained from the Faculty of Pharmacy, University of Benin ethical committee with the approval number EC/FP/020/12.

Acute toxicity screening

The acute toxicity screening was carried out according to Lorke's method (Lorke, 1983) using twelve mice of either sex, divided into two phases. The plant crude extracts were

administered orally at different doses. General symptoms of toxicity and mortality in each group were observed within 24 hours and then for another 14 days.

Phase 1: Nine mice were divided into three groups of three mice per group. The three groups were administered orally with graded doses (10, 100 and 1000 mg/kg) of the plant crude extract.

Phase 2: Another three mice were divided into three groups of one mouse per group, which received graded doses (1600, 2900 and 5000 mg/kg) of the plant crude extract, respectively.

Animals were observed during the first 30 minutes of treatment and then occasionally within 24 hours, then daily for 14 days. Animals were monitored for signs of toxicity including tremors, convulsions, salivation, diarrhoea, behavioural changes, coma, and death. The number of deaths in each group within 24 h was recorded and the median lethal dose (LD_{50}) was calculated as the geometric mean of the highest non-lethal dose (with no deaths) and the lowest lethal dose (where deaths occurred).

LD_{50} (Geometric Mean Method) Formula:

$LD_{50} = \sqrt{D_0 \times D_1}$

Where:

D_0 = the highest dose at which no deaths occurred

D_1 = the lowest dose at which deaths occurred

$\sqrt{}$ = square root (to calculate the geometric mean)

2.2.5 Antimicrobial Assay

Selection and Source of Test Microorganisms

The bacterial strains used in this study were clinical isolates of both Gram-positive and Gram-negative bacteria, selected for their medical relevance and resistance profiles. The microorganisms included *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa, Enterobacter cloacae,* and *Bacillus subtilis*. These strains were isolated and identified in the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Benin, Benin City, Nigeria**,** using standard microbiological and biochemical methods. The selection was based on their frequent implication in human infections and their suitability for antimicrobial susceptibility testing. The isolates were maintained on nutrient agar slants at 4°C and subcultured prior to use to ensure viability and purity.

Preparation of microbial inoculum

The microorganisms were grown in suitable culture media (i.e. Mueller-Hinton Agar (RDM-MHA-01) for bacteria). The inoculum was standardized using the 0.5 McFarland standard to ensure consistency in microbial density, equivalent to approximately 1.5×10^8 CFU/mL.

Preparation of test compound

Preparation of *Artemisia annua* extract and different fractions at varying concentrations and sterilization using appropriate methods was made.

Antimicrobial Susceptibility Testing Agar Well Diffusion Method *(for zone of inhibition measurement)*

The crude extract and several solvent fractions of *Artemisia annua* were tested for antibacterial activity using the agar well diffusion method, a common in vitro methodology

for determining the antimicrobial potential of plant-derived compounds (CLSI, 2012; Perez *et al*., 1990).

Mueller-Hinton Agar (MHA) was prepared according to the manufacturer's specifications. The medium was sterilized by autoclaving at 121°C for 15 minutes and allowed to cool to approximately 45–50°C before being aseptically dispensed (~20 mL per plate) into sterile Petri dishes. The agar was left to solidify at room temperature, forming a smooth and uniform surface suitable for microbial inoculation.

The agar surface was inoculated with the test microorganisms after it had solidified. These included clinical isolates *of Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa, Enterobacter cloacae,* and *Bacillus subtilis*.

Using a sterile cotton swab, the standardized microbial suspensions were uniformLy spread across the entire agar surface to create a lawn culture. This homogeneous distribution is necessary for accurate measurement of inhibition zones following therapy.

Wells were then aseptically introduced into the agar using a sterile cork borer (typically 6 mm in diameter). Care was taken to ensure uniformity in the size and spacing of the wells. Each well was gently cleared of the agar plug using sterile forceps.

Subsequently, a fixed volume (usually 50–100 µL) of the plant crude extract and each solvent fractions was dispensed into the wells using a micropipette. A positive control, consisting of Ciprofloxacin (10 µg/mL), was included in separate wells to serve as a standard reference for antimicrobial efficacy.

Following 18 to 24 hours of aerobic incubation at 37°C, the plates were inspected for the existence of zones of inhibition surrounding the wells. Using a clear ruler, the diameter of

each inhibitory zone was measured in millimetres, and the findings were noted as a measure of antimicrobial activity.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

To determine the Minimum Inhibitory Concentration (MIC) of the crude extract and solvent fractions of *Artemisia annua*, the agar dilution method was employed following established guidelines (CLSI, 2012; Andrews, 2001). This method allows for the quantification of the lowest concentration of an antimicrobial agent that inhibits visible microbial growth.

Minimum inhibitory concentration determination

Two-fold serial dilutions of the plant extract and its respective fractions were prepared in Mueller-Hinton broth, a standard nutrient-rich medium used for antimicrobial susceptibility testing. Serial dilutions were carried out to obtain a range of concentrations, typically starting from a high concentration (100 mg) and halving with each step down to the lowest concentration (3.002 mg).

Each dilution dish was inoculated with a standardized microbial suspension previously adjusted to match the 0.5 McFarland standard, equivalent to approximately 1.5×10^8 CFU/mL. The standardization ensures consistent bacterial load across all test concentrations (Andrews, 2001).

Following inoculation, the dishes were incubated at 37°C for 18–24 hours under aerobic conditions. After incubation, the dishes were visually examined for turbidity (cloudiness), which indicates microbial growth. The MIC was recorded as the lowest concentration of the

extract or fraction at which no visible turbidity (i.e., a clear solution) was observed, indicating that microbial growth had been inhibited (Balouiri *et al*., 2016).

Minimum Bactericidal Concentration Determination (For Bactericidal Activity)

To evaluate the bactericidal activity of the extract and fractions, samples were collected from all non-turbid MIC dishes (i.e., those that showed no visible growth) and sub-cultured onto freshly prepared Mueller-Hinton agar plates. A sterile loop was used to transfer aliquots from each clear dish to the agar surface, and the samples were streaked aseptically.

The infected agar plates were incubated at 37°C for an extra 18 to 24 hours. The plates were examined to see whether any microbial colony formation had taken place after incubation. The MBC is the lowest concentration of the extract or fraction at which no microbial colonies were observed, indicating complete microbial death, according to CLSI (2012) and Wiegand *et al*. (2008).

This two-step process MIC followed by MBC helps distinguish between bacteriostatic (growth-inhibiting) and bactericidal (killing) effects of the tested substances.

2.2.6 Quantitative Phytochemical Screening

Determination of Total Phenolic Content *A. annua*

Total phenol contents in the dichloromethane and ethyl acetate fractions were determined by the method described by Kim *et al*., 2003. The fractions solution (0.5 mL) with a concentration of 1000 µg/mL was added to 4.5 mL of deionized distilled water and 0.5 mL of Folin Ciocalteu's reagent (previously diluted with water 1:10, v/v) which was then added to the solution. After mixing the tubes, they were maintained at room temperature for 5 minutes followed by the addition of 5 mL of 7% sodium carbonate and 2 mL of deionized distilled

water. After mixing the samples, the samples were incubated for 90 minutes at room temperature. The absorbance was measured by a spectrophotometer at 750 nm. The total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram of extract (mg GAE/g extract). The standard curve was prepared by gallic acid in six different concentrations (12.5, 25, 50, 75, 100 and 150 mg/L).

Determination of Total Flavonoid of *A. annua*

Total flavonoid contents were estimated using the method described by Ebrahimzadeh *et al*., 2008. 0.5 mL of dichloromethane and ethyl acetate fractions (1 mg/mL) was mixed with 1.5 mL of methanol and then, 0.1 mL of 10 % aluminium chloride was added, followed by 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. The mixture was incubated at room temperature for 30 minutes. The absorbance was measured by a spectrophotometer at 415 nm. The results were expressed as milligrams of quercetin equivalents (QE) per gram of extract (mg QE/g extract). The standard curve was prepared by quercetin in six different concentrations (12.5, 25, 50, 75, 100 and 150 mg/L).

Gas Chromatography-Mass Spectrometry (GC-MS)

The chromatographic (GC-MS) analysis of the dichloromethane and ethyl acetate fractions was carried out using a Shimadzu QP-2010 Plus GC-MS system with an EI mode (70 eV), a scan range of 45–400 amu, and a scan rate of 3.99 scans/sec. Separation was achieved on an HP-5MS fused silica capillary column (30 m × 0.25 mm, 0.25 μm film thickness) with helium (99.99%) as the carrier gas at a flow rate of 1.61 mL/min. The oven temperature was programmed from 60°C to 180°C at 10°C/min (held for 2 min), then from 180°C to 280°C at 15°C/min (held for 4 min). The injector, ion source, and interface temperatures were set at 250°C, while the detector was at 280°C. 0.30 μL of the sample was injected in the split mode.

Components were identified by comparing mass spectra with the NIST Mass Spectral Library, and their relative abundances were reported based on total ion current (Okhale *et al.,* 2018)

High-Performance Liquid Chromatography (HPLC) Analysis

The extract (0.2 g) was weighed and transferred in a test tube and 15mL ethanol and 10 mL of 50%m/v potassium hydroxide was added. The test tube was allowed to react in a water bath at 60^0c for 3hrs/mins. After the reaction time, the reaction product contained in the test tube was transferred to a separatory funnel. The tube was washed successfully with 20 mL of ethanol, 10 mL of cold water, 10 mL of hot water and 3 mL of hexane, which was all transferred to the funnel. This extracts were combined and washed three times with 10 mL of 10%v/v ethanol aqueous solution. The ethanol solvent was evaporated. The sample was solubilized in 1000 µL of pyridine of which 200 µl was transferred to a vial for analysis.

High-performance liquid chromatography (HPLC) analysis was performed using Shimadzu LC-10AD dual binary pumps, Shimadzu CTO-10AS column oven, and Shimadzu Prominence SPD-20A UV/Vis detector. The analysis was performed using a C-12 normal phase column (Phenomenex, Gemini 5 µ, 200 mm length × 4.8 mm internal diameter). The mobile phase consisted of acetic acid-acidified deionized water (pH 2.8) as solvent A and acetonitrile as solvent B at a flow rate of 0.8 mL/min. The column was equilibrated with 5% solvent B for 20 min after each injection of samples. The column temperature was set to 38°C and the injection volume was 20 µL. The wavelengths were set to 280 nm for the detection of phenolics, Phenolic compound identification and quantification were performed by comparing respective retention times and peak areas with pure standard compounds utilizing the method of external standards to construct a calibration curve. Gradient elution was executed as follows: 0-5 min, 5-9% solvent B; 5-15 min, 9% solvent B; 15-22 min, 9-11%

solvent B; 22-38 min, 11-18% solvent B; 38-43 min, 18-23% solvent B; 43-44 min 23-90% solvent B; 44-45 min, 90-80%, solvent B; 45-55 min

Statistical Analysis

Data from antimicrobial studies, including zones of inhibition (mm), Minimum Inhibitory Concentration (MIC), and Minimum Bactericidal Concentration (MBC), were also analysed. MIC and MBC values were determined using the broth dilution method and expressed in mg.

All experiments were conducted in triplicate (n = 3) and the results were expressed as mean ± standard deviation (SD). Quantitative phytochemical analyses, including Total Phenolic Content (TPC) and Total Flavonoid Content (TFC), were statistically evaluated using descriptive statistics. TPC was expressed as milligrams of gallic acid equivalents per gram of extract (mg GAE/g), while TFC was expressed as milligrams of quercetin equivalents per gram of extract (mg QE/g).

Chromatographic data obtained from Gas Chromatography–Mass Spectrometry (GC-MS) were presented as relative peak areas (%) to indicate the abundance of identified compounds. High-Performance Liquid Chromatography (HPLC) data were analyzed by calculating the concentration of each compound based on calibration curves, and results were expressed in µg/mL or ppm.

CHAPTER THREE

RESULTS

3.1 Organoleptic Properties

Odour: Strong, aromatic, and slightly camphor-like with herbal and earthy undertones.

Taste: Bitter, slightly astringent, with a lingering herbal aftertaste.

Colour: Green to yellowish-green (fresh leaves); pale green to brownish-green (dried leaves).

Texture: Soft and delicate when fresh; dry, brittle, and slightly fibrous when dried.

3.2 Extract and Fractions of *A. annua*

Percentage Yield: Percentage yield of the extract and fractions was calculated using the formula below and result presented in Table 3.1

$$\text{Percentage yield of Crude extract} = \frac{Weight\ of\ extract}{Sample\ weight} \times 100$$

$$\text{Percentage yield of Fraction} = \frac{Weight\ of\ fraction}{Crude\ extract\ weight} \times 100$$

3.3 Phytochemical Constituents of *A. annua*

Tables summarizing positive (+) or negative (-) results for each phytochemical test across the extract and fractions. This section indicates that the ethyl acetate fraction showed the highest number of active compounds associated with antimicrobial properties. Table 3.2 shows the Phytoconstituents of *Artemisia annua* leaf extract and different fractions

3.4 Acute Toxicity of *A. annua* crude extract

Table 3.3 shows acute toxicity of *A. annua* crude extract, displaying observed symptoms and dose-response relationships.

3.5 Antimicrobial Activity Results

Zone of Inhibition: Quantitative data on zones of inhibition (in mm) for each extract, highlighting that ethyl acetate displayed the most significant antibacterial activity shown in table 3.4 below

MIC and MBC: Results are presented in mg (table 3.5, 3.6, 3.7, 3.8, 3.9 and 3.10), showing the lowest effective concentrations against specific bacteria for each extract. Ethyl acetate, for instance, could show lower MIC/MBC values against multi-drug-resistant strains, indicating higher potency.

3.6 Quantitative Phytochemical Constituents

Total phenolic and Flavonoids Content of *A. annua* dichloromethane and ethyl acetate fractions: Total Phenolic and Flavonoid content of *A. annua* results are presented in table 3.11 and 3.12 below. Figure 3.2 and 3.3 showed the graph of absorbance of standard against concentration (TPC and TFC) of *Artemisia annua* dichloromethane and ethyl acetate fractions

Gas Chromatography-Mass Spectrometry (GC-MS) and High-Performance Liquid Chromatography (HPLC) Analysis

Figure 3.2, 3.3, 3.4 and 3.5 shows the GC-MS and HPLC Chromatogram of *Artemisia annua* dichloromethane and ethyl acetate fractions. The results of bioactive compounds of *Artemisia*

Figure 3. 1: Percentage Yield of A. annua Extract and Fractions

Sample	Weight (g)	Percentage Yield (%)
N-Hexane Fraction	24.64	36.24
DCM Fraction	20.22	29.74
Ethyl Acetate Fraction	1.59	2.34
Methanol Fraction	21.30	31.32
Crude Extract	136	5.91

annua dichloromethane and ethyl acetate fractions identified using the GC-MS and HPLC analyses is presented in table 3.11 and 3.12 below.

Table 3. 2: Phytoconstituents of Artemisia annua extract and fractions

Sample	Alkaloids	Coumaris	Flavonoids	Phenolic	Saponins	Tannins	Terpenoids
N-Hexane fraction	-	-	-	-	-	-	+
DCM fraction	+	+	+	+	-	-	+
Ethyl Acetate fraction	+	+	+	+	+	-	+
Methanol fraction	+	+	+	+	+	-	+
Crude extract	+	+	+	+	+	-	+

Table 3.3: Acute toxicity profile of *Artemisia annua* leaf extracts

	Dose (mg/kg)	Number of mice	Number of Death recorded
Phase 1			
	10	3	0/3
	100	3	0/3
	1000	3	0/3
Phase 2			
	1600	1	0/1
	2900	1	0/1
	5000	1	0/1

Table 3.4: Antibacterial activity of *Artemisia annua* leaf different fractions extract

Multidrug resistance Bacteria	**Control (Ciprofloxacin) (20mg)**	**N-Hexane fraction (100mg)**	**Dichloromethane fraction (100mg)**	**Ethyl acetate fraction (100mg)**	**Methanol fraction (100mg)**	**Crude extract (100mg)**
Bacillus subtilis	30 mm	*	20 mm	20 mm	15 mm	14 mm
Enterobacter cloacae	No Zone	*	12 mm	18 mm	15 mm	20 mm
Escherichia coli	No Zone	*	20 mm	15 mm	No Zone	18 mm
Klebsiella pneumonia	No Zone	*	No Zone	18 mm	No Zone	No Zone
Pseudomonas aeruginosa	30 mm	*	20 mm	15 mm	No Zone	15 mm
Staphylococcus aureus	23 mm	*	19 mm	19 mm	19 mm	No Zone

Table 3.5: Minimum inhibitory concentration of *Artemisia annua* dichloromethane fraction

Concentration (mg)	**50**	**25**	**12.5**	**6.125**	**3.002**
Multidrug-resistance Bacteria					
Bacillus subtilis	NG	NG	NG	NG	G
Enterobacter cloacae	NG	G	G	G	G
Escherichia coli	G	G	G	G	G
Pseudomonas aeruginosa	G	G	G	G	G
Staphylococcus aureus	G	G	G	G	G

***NG** : Inhibits the growth of the corresponding bacterial (i.e No growth of the bacteria on the extract)*
***G** : Did not inhibit of the growth the corresponding bacterial (i.e Growth of the bacteria on the extract)*

Concentration (mg)	**50**	**25**	**12.5**	**6.125**	**3.002**	**0.5**
Multidrug-resistance Bacteria						
Bacillus subtilis	NG	NG	NG	NG	NG	G
Enterobacter cloacae	NG	NG	NG	NG	NG	G
Escherichia coli	NG	NG	NG	NG	NG	G
klebsiella pneumoniae	NG	NG	NG	NG	NG	G
Pseudomonas aeruginosa	NG	NG	NG	NG	NG	G
Staphylococcus aureus	NG	NG	NG	NG	NG	G

Table 3.6: Minimum inhibitory concentration of *Artemisia annua* ethyl acetate fraction

NG : *Inhibits the growth of the corresponding bacterial (i.e No growth of the bacteria on the extract)*
G : *Did not inhibit of the growth the corresponding bacterial (i.e Growth of the bacteria on the extract)*

Concentration (mg)	**50**
Multidrug-resistance Bacteria	
Bacillus subtilis	G
Enterobacter cloacae	G
Escherichia coli	G
Pseudomonas aeruginosa	G

Table 3.7: **Minimum inhibitory concentration of *Artemisia annua* methanol fraction**

G *: Did not inhibit the growth of the corresponding bacterial (i.e Growth of the bacteria on the extract)*

Table 3.8: Minimum inhibitory concentration of *Artemisia annua* crude extract

Concentration (mg)	**50**
Multidrug resistance Bacteria	
Bacillus subtilis	G
Enterobacter cloacae	G
Staphylococcus aureus	G

G: *Did not inhibit the growth of the corresponding bacterial (i.e Growth of the bacteria on the extract)*

Table 3.9: Minimum bactericidal concentration of *Artemisia annua* dichloromethane fraction

Concentration (mg)	**50**	**25**	**12.5**
Multidrug resistance Bacteria			
Bacillus subtilis	NG	G	G
Enterobacter cloacae	NG	G	G

***NG** : Kills the corresponding bacterial (i.e No growth of the bacteria on the extract)*

G: Did not kill the corresponding bacterial (i.e Growth of the bacteria on the extract)

Table 3.10: Minimum bactericidal concentration of *Artemisia annua* ethyl acetate fraction

Concentration (mg)	**50**	**25**	**12.5**	**6.125**	**3.002**
Multidrug resistance Bacteria					
Bacillus subtilis	G	G	G	G	G
Enterobacter cloacae	NG	NG	NG	NG	NG
Escherichia coli	NG	NG	NG	NG	NG
klebsiella pneumoniae	NG	NG	NG	NG	NG
Pseudomonas aeruginosa	NG	NG	NG	NG	NG
Staphylococcus aureus	NG	NG	NG	NG	NG

***NG** : Kills the corresponding bacterial (i.e No growth of the bacteria on the extract)*

G: Did not kill the corresponding bacterial (i.e Growth of the bacteria on the extract)

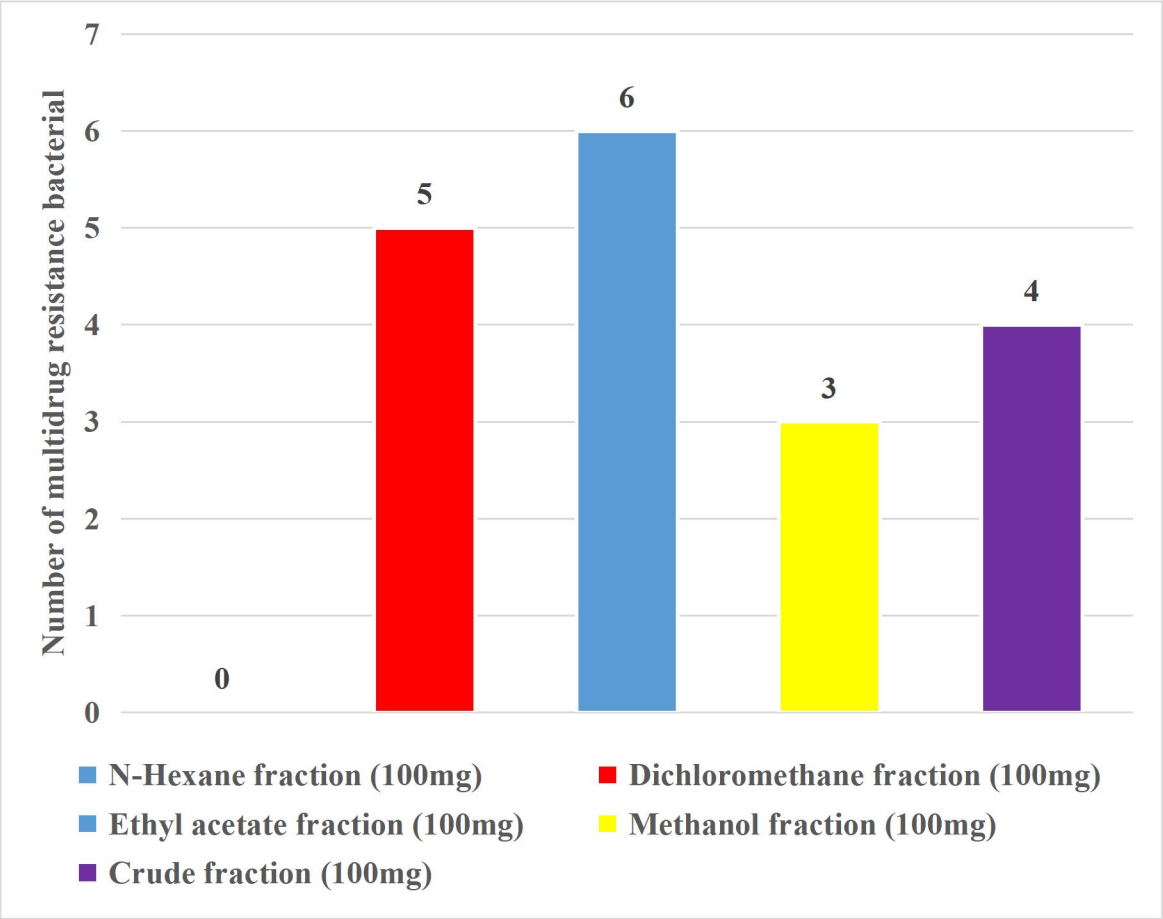


Figure 3.2: Rank of extract and different fractions based on the number of bacterial they are active against

Concentration (µg/mL)	Absorbance			Mean Absorbance
	1	2	3	
12.5	0.082	0.093	0.070	0.080
25	0.156	0.102	0.121	0.130
50	0.201	0.172	0.205	0.190
75	0.336	0.291	0.259	0.300
100	0.359	0.352	0.343	0.350
150	0.393	0.370	0.380	0.380
Blank	0.050			
DCM	0.695	0.754	0.893	0.780
Ethyl Acetate	2.460	2.414	2.871	2.580

Table 3. 11: Total Phenolic Content of *A. annua* dichloromethane and ethyl acetate fractions (Absorbance of Gallic acid at different concentration)

Equation of line; $Y = 0.0024X + 0.07$

$R^2 = 0.9301$

TPC of *A. annua* Dicholoromethane Fraction = 295.83 mg/Gallic acid

TPC of *A. annua* Ethyl Acetate Fraction = 212.20 mg/Gallic acid

Table 3. 12: Total Flavoniods Content of *A. annua* dichloromethane and ethyl acetate fractions (Absorbance (415nm) of Quercetin acid at different concentration)

Concentration (µg/mL)	**Absorbance**			**Mean Absorbance**
	1	2	3	
12.5	0.082	0.093	0.070	0.080
25	0.156	0.102	0.121	0.130
50	0.201	0.172	0.205	0.190
75	0.336	0.291	0.259	0.300
100	0.359	0.352	0.343	0.350
150	0.393	0.370	0.380	0.380
Blank	0.050			
DCM	0.695	0.754	0.893	0.780
Ethyl Acetate	2.460	2.414	2.871	2.580

Equation of line; $Y = 0.0041X + 0.1066$

$R^2 = 0.9878$

TFC of *A. annua* Dichloromethane Fraction = 69.12 mg/Quercetin

TFC of *A. annua* Ethyl Acetate Fraction = 83.76 mg/Quercetin

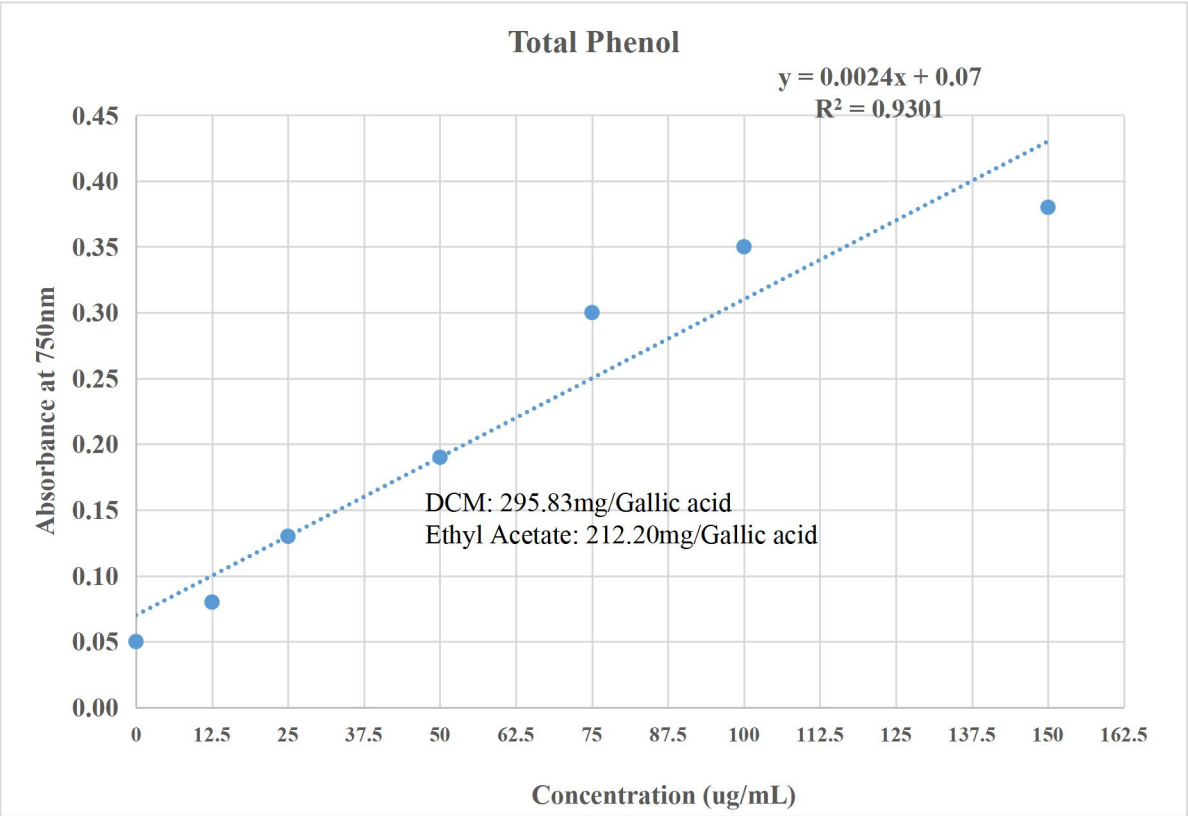


Figure 3.3: Graph of absorbance against concentration (Total Phenolic Content) of *Artemisia annua* dichloromethane and ethyl acetate fractions

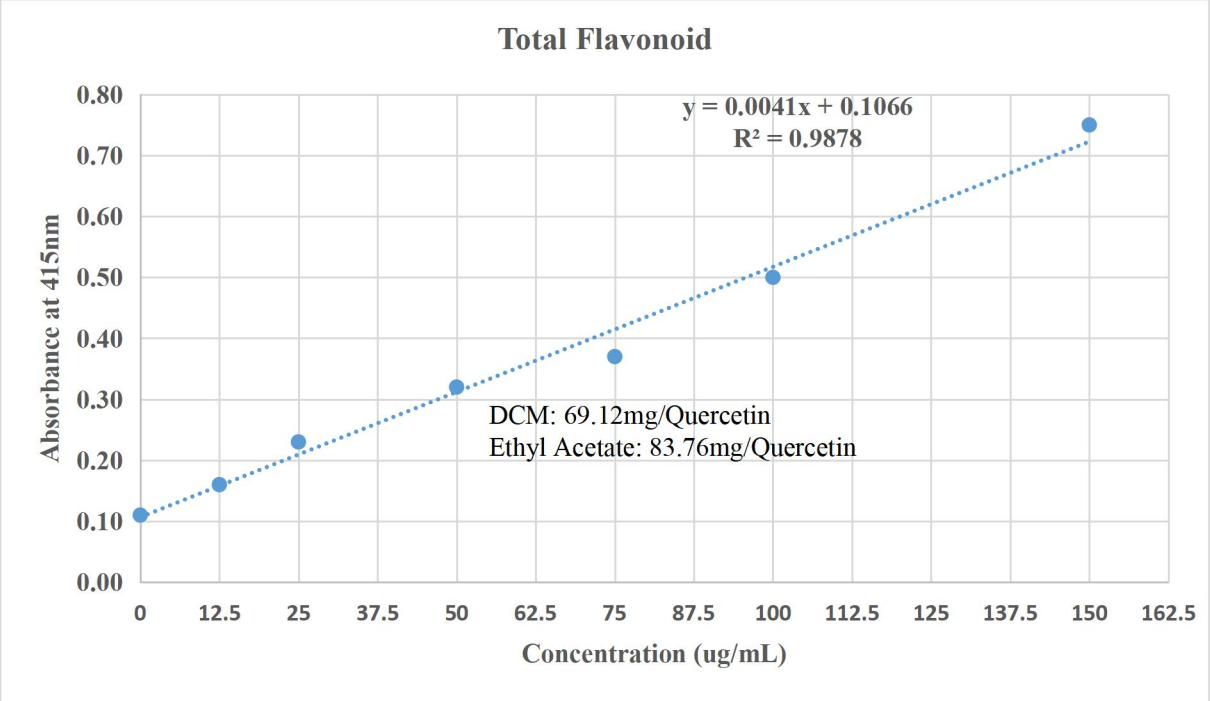


Figure 3.4: Graph of absorbance against concentration (Total Flavonoid content) of *Artemisia annua* dichloromethane and ethyl acetate fraction

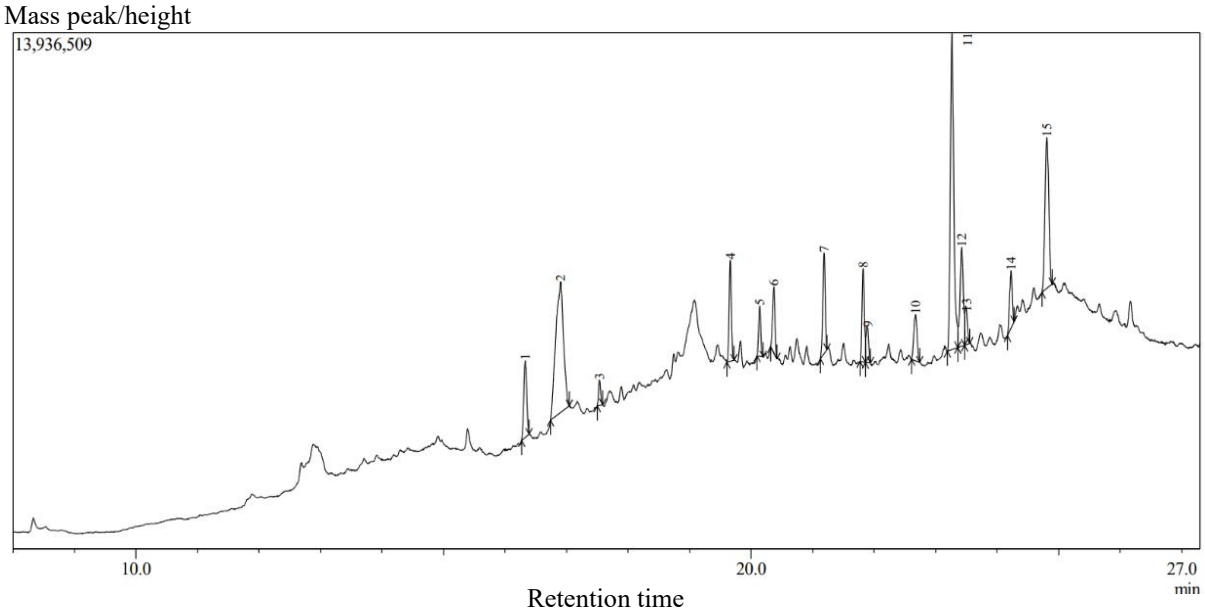


Figure 3.5: GC-MS Chromatogram of *Artemisia annua* ethyl acetate fraction

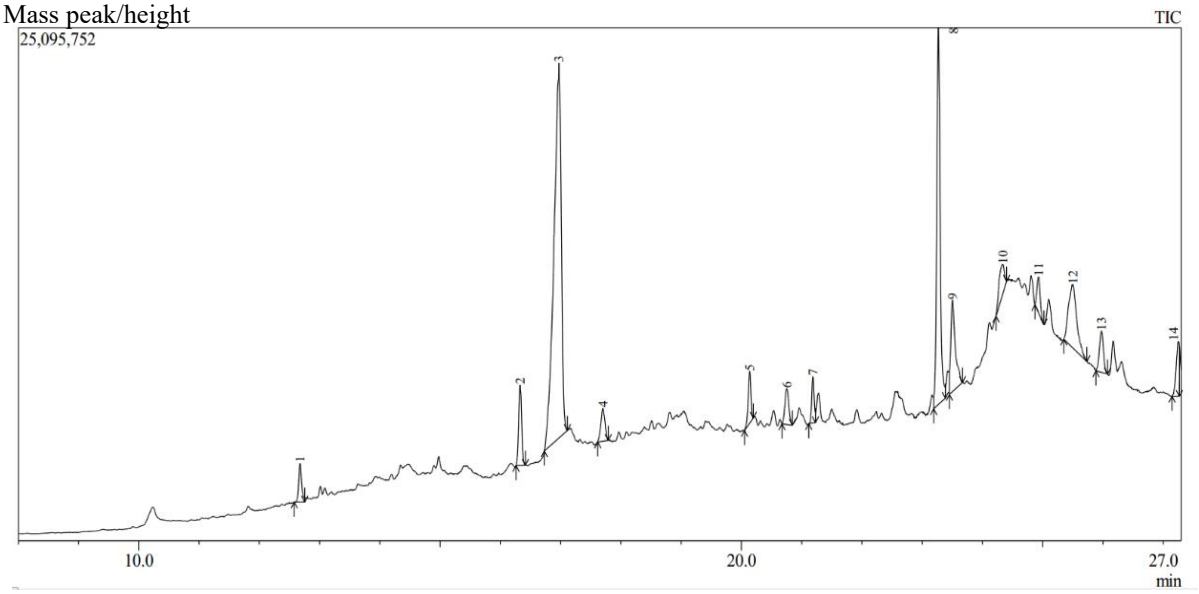


Figure 3.6: GC-MS Chromatogram of *Artemisia annua* dichloromethane fraction

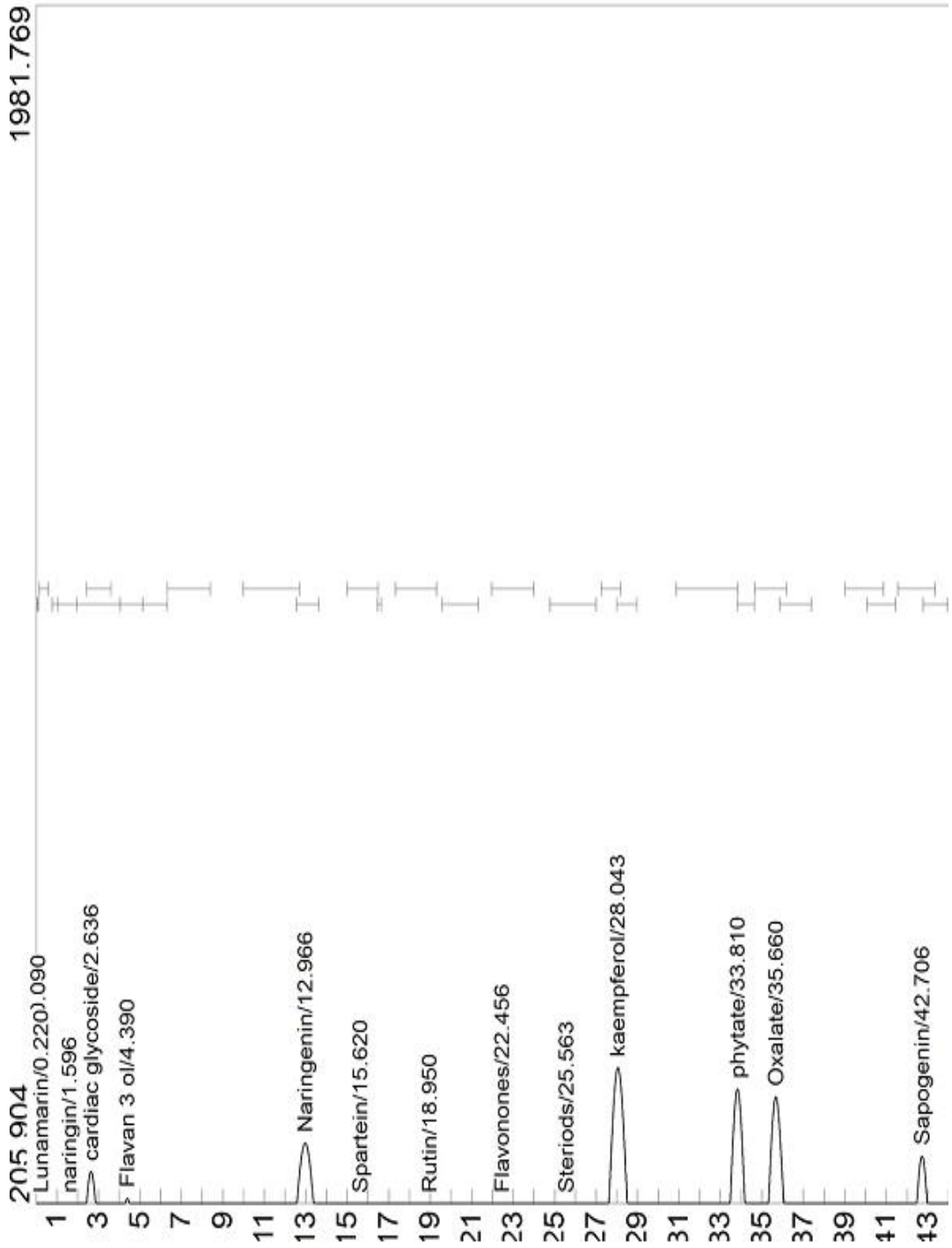


Figure 3.7: HPLC Chromatogram of *Artemisia annua* ethyl acetate fraction

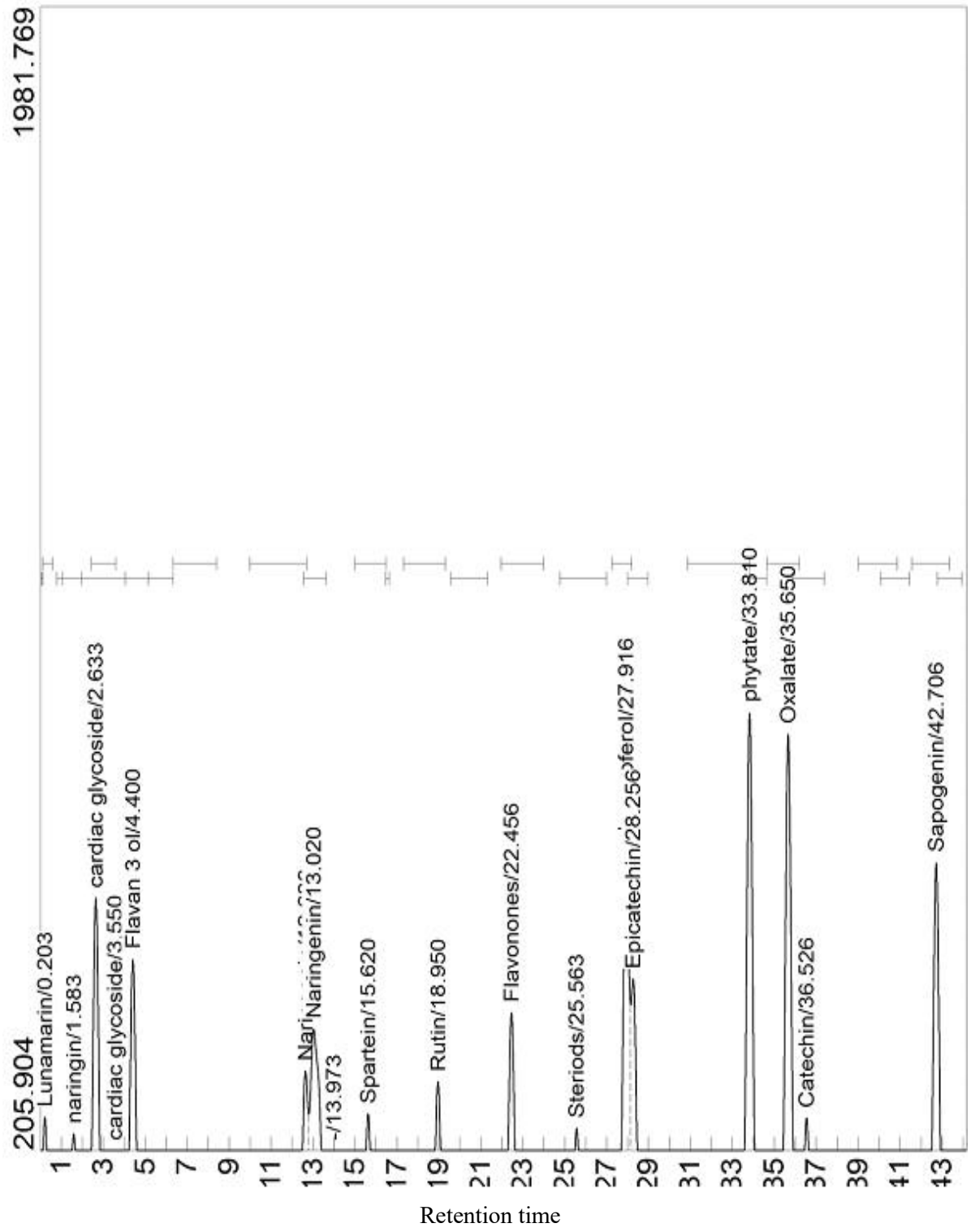


Figure 3.8: HPLC Chromatogram of *Artemisia annua* dichloromethane fraction

Table 3.13: Bioactive compounds of *Artemisia annua* ethyl acetate fraction identified using the GC-MS analysis

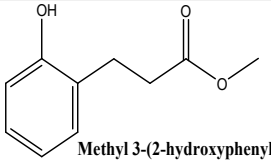
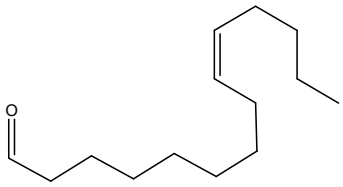
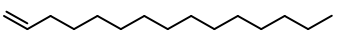
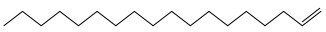
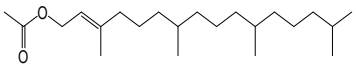
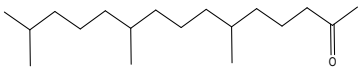
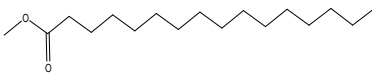
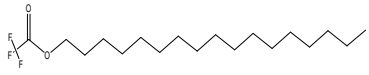
Peak	RT	Compounds	Mol. Formula/ Phytochemical class	Mol. Weight	Area %	Pharmacological Activity
1	16.329	 Methyl 3-(2-hydroxyphenyl)propionate	$C_{10}H_{12}O_3$ Coumarin	180.20	4.64	Antioxidant, Anti-inflammatory, Auxin Perception
2	16.900	 9-Tetradecenal, (Z)	$C_{14}H_{26}O$ Aldehyde	210.36	21.75	Insect Pheromone, Potential Plant Bioactivity, Antimicrobial, Antioxidant, Anti-inflammatory
3	17.536	 1-Pentadecene	$C_{15}H_{30}$ Lipids	210.48	1.15	Insect Defence
4	19.663	 1-Octadecene	$C_{18}H_{36}$ Lipids, specifically fatty acyls	252.48	5.09	Potential Biomedical Applications
5	20.140	 Phytol, acetate	$C_{22}H_{42}O_2$ Diterpenoid	338.57	2.38	Antimicrobial, Anti-inflammatory, Antioxidant, Antidiabetic, Antinociceptive, Antitumor/Anticancer, Antispasmodic, Antidepressant
6	20.369	 2-Pentadecanone, 6,10,14-trimethyl- Hexahydrofarnesylacetone	$C_{18}H_{36}O_2$ sesquiterpenes	268.48	3.18	Antibacterial, Anti-inflammatory, Anti-nociceptive, Antioxidant, Insecticidal
7	21.187	 Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$ Fatty acid	270.45	5.32	Anti-inflammatory, Cancer-preventive, Hepatoprotective
8	21.821	 Heptadecyl trifluoroacetate	$C_{19}H_{35}F_3O_2$ Fatty acid	358.50	4.96	Antioxidant

Table 3.13 Cont'd: Bioactive compounds of *Artemisia annua* ethyl acetate fraction identified using the GC-MS analysis

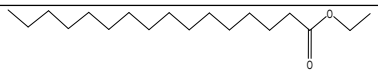
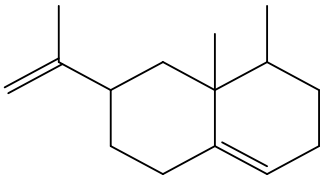
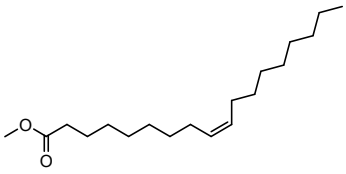
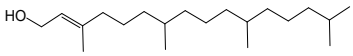
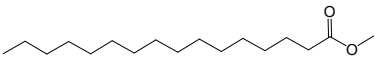
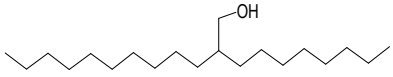
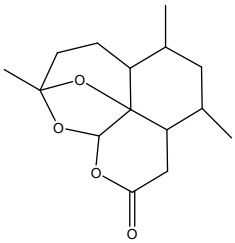
Peak	RT	Compounds	Mol. Formula/ Phytochemical class	Mol. Weight	Area %	Pharmacological Activity
9	21.895	 Palmitic acid, ethyl ester (Hexadecanoic acid, ethyl ester)	$C_{18}H_{36}O_2$ Fatty acid ester	284.49	1.83	Antioxidant, Anti-inflammatory, Hypocholesterolemic, Nematocidal and Pesticidal, Potential Anticancer, Hair and Skin Conditioning, Antimicrobial
10	22.671	 1,2,3,5,6,7,8,8a-octahydro-1,8a Naphthalene	$C_{15}H_{24}$ bicyclic hydrocarbon	204.34	3.14	-
11	23.267	 Oleic acid, methyl ester (9-Octadecenoic acid (Z)-, methyl ester)	$C_{19}H_{36}O_2$ Fatty acid ester	296.53	22.58	Antioxidant, Anti-inflammatory, Antimicrobial
12	23.425	 Phytol (3,7,11,15-tetramethyl -2-Hexadecen-1-ol)	$C_{20}H_{40}O$ Diterpene	296.53	6.84	Antimicrobial, Antidiabetic, Antioxidative
13	23.505	 Stearic acid, methyl ester Methyl stearate (Octadecanoic acid, methyl ester)	$C_{19}H_{28}O_2$ Fatty acid methyl ester	298.50	2.01	Antiviral, Anti-inflammatory, Antioxidant
14	24.225	 Eutanol ; Standamul ; (2-Octyl-1-dodecanol)	$C_{20}H_{42}O$ Fatty acid	298.55	3.20	Emollient, Emulsifier and Stabilizer, Moisturizer
15	24.810	 Deoxyqinghaosu	$C_{15}H_{22}O_4$ Sesquiterpene lactone	266.33	11.92	Antimalarial, Antiviral, Anti-inflammatory

Table 3. 14: Bioactive compounds of *Artemisia annua* dichloromethane fraction identified using the GC-MS analysis

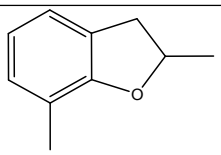
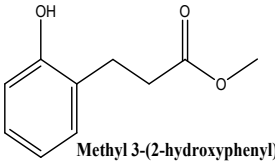
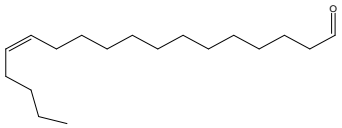
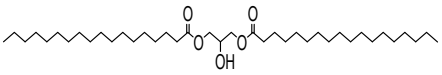
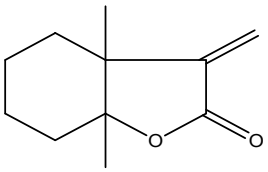
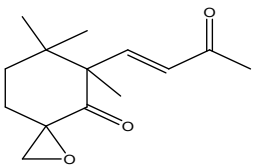
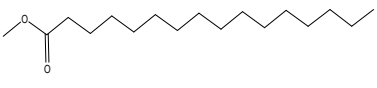
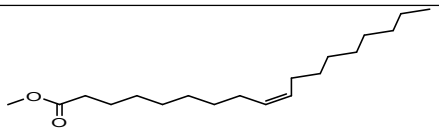
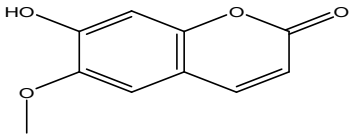
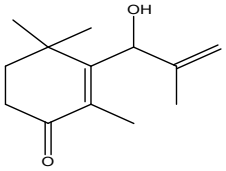
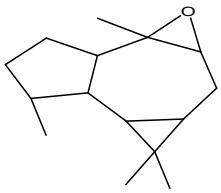
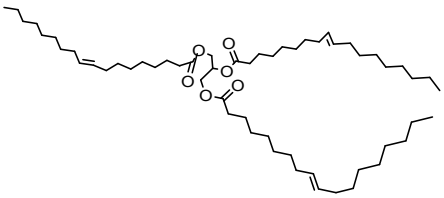
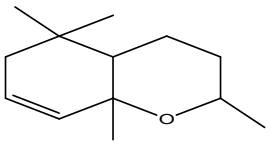
Peak	RT	Compounds	Mol. Formula/ Phytochemical class	Mol. Weight	Area %	Pharmacological Activity
1	12.675	 Coumaran (Benzofuran, 2,3-dihydro)	C_8H_8O **Benzofuran**	120.15	1.85	Antioxidant, Anti-inflammatory, Antimicrobial, Anticancer
2	16.328	 Methyl 3-(2-hydroxyphenyl)propionate	$C_{10}H_{12}O_3$ Coumarin	180.20	4.64	Antioxidant, Anti-inflammatory, Auxin Perception
3	16.970	 13-Octadecenal, (Z	$C_{18}H_{34}O$ Lipids	266.45	42.35	Antibacterial/Antimicrobial, Pheromone
4	17.701	 Stearic acid (Octadecanoic acid, 2-hydroxy l-1,3-propanediyl ester)	$C_{39}H_{76}O_5$ Fatty acids	624	2.06	Emulsifying and Lubricating Agent, Skin Protectant, Binding Agent, Cosmetics and Soaps, Food Industry, Rubber Manufacturing
5	20.138	 Hexahydro-3a,7a-dimethyl-3-methylene, 2(3H)-Benzofuranone	$C_{11}H_{16}O_2$	180	2.26	-
6	20.752	 5,6,6-Trimethyl-5-(3-oxobut-1-enyl)-1-oxaspiro[2.5]octan-4-one	$C_{14}H_{20}O_3$	236	2.22	AMPA receptor modulator
7	21.186	 Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$ Fatty acid	270.45	5.32	Anti-inflammatory, Cancer-preventive, Hepatoprotective

Table 3.14 Cont'd: Bioactive compounds of *Artemisia annua* dichloromethane fraction identified using the GC-MS analysis

Peak	RT	Compounds	Mol. Formula/ Phytochemical class	Mol. Weight	Area %	Pharmacological Activity
8	23.270	 Oleic acid, methyl ester (9-Octadecenoic acid (Z)-, methyl ester)	$C_{19}H_{36}O_2$ Fatty acid ester	296.53	22.58	Antioxidant, Anti-inflammatory, Antimicrobial
9	23.503	 Scopoletin	$C_{10}H_8O_4$ Coumarin	192.17	6.61	Antimicrobial, Anticancer, Anti-inflammatory, Antioxidant, Antidiabetic, Neuroprotective
10	24.340	 4-Oxo-.beta.-isodamascol; 3-(1-Hydroxy-2-methyl-2-propenyl)-2,4,4-trimethyl-2-cyclohexen-1-one	$C_{13}H_{20}O_2$ -	208	2.71	-
11	24.933	 Isoaromadendrene epoxide (1,3b,6,6-Tetramethyldecahydro-1H-cyclopropa[7,8]azuleno[4,5-b]oxirene)	$C_{15}H_{24}O$ Sesquiterpene epoxide	220.35	1.84	Antimicrobial, Anti-inflammatory, Anticancer
12	25.975	 9-Octadecenoic acid, 1,2,3-propanetriyl ester	$C_{57}H_{104}O_6$ Lipid, Fatty Acid Ester	884	8.03	Antioxidant, Anti-inflammatory, Anticancer
13	25.975	 Dihydroedulan IA 2,5,5,8a-Tetramethyl-3,4,4a,5,6,8a-hexahydro-2H-chromene	$C_{13}H_{22}O$ Chromene	194	2.69	Antioxidant

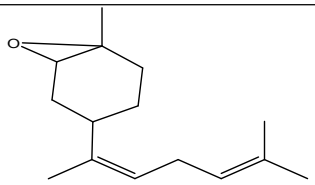
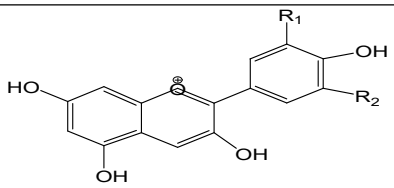
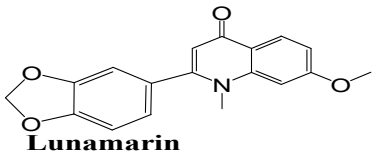
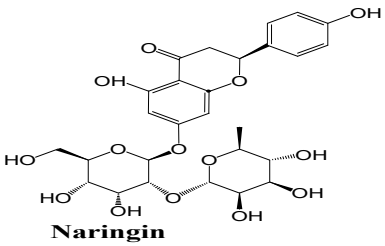
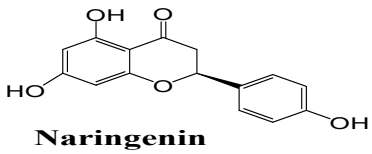
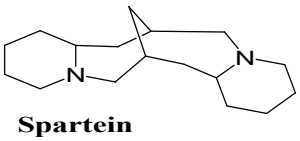
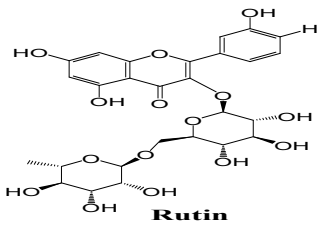
14	27.256	 Cis-Z-.alpha.-Bisabolene epoxide 4-[(1Z)-1,5-Dimethyl-1,4-hexadienyl]-1-methyl-7-oxabicyclo[4.1.0]heptane	$C_{15}H_{24}O$ Sesquiterpene	220.35	2.75	Pregnane X Receptor (PXR) Ligand, Blood-Brain Barrier Penetration, Hepatic Metabolism

Table 3.15: Bioactive compounds of *Artemisia annua* ethyl acetate fraction identified using the HPLC analysis

Peak	RT	Compounds	Mol. Formula/ Phytochemical class	Mol. Weight	Area %	Pharmacological Activity
1	0.090 -	 Proanthocyanin	$C_{15}H_{14}O_6$ Polyphenols	578.53	0.20 -	Antioxidant, Anti-inflammatory, Anticancer, Antidiabetic, Neuroprotective, Antimicrobial, Cardiovascular
2	0.220 0.203	 Lunamarin	$C_{16}H_{12}O_8$ quinolone alkaloid	332.26	1.79 2.74	lower arterial blood pressure
3	1.596 1.583	 Naringin	$C_{27}H_{32}O_{14}$ Flavonoid	580.54	2.72 3.01	Antioxidant, Anti-inflammatory, Anticancer
6	12.966 13.020	 Naringenin	$C_{15}H_{12}O_5$	272.25	14.41 12.31	Flavonoid, Antioxidant, Anti-inflammatory, Anticancer, Cardioprotective
7	15.620 15.620	 Spartein	$C_{15}H_{26}N_2$ Alkaloid	234.38	3.36 3.43	Antiarrhythmic, Hypoglycemic, Diuretic, Anti-inflammatory
8	18.950 18.950	 Rutin	$C_{27}H_{30}O_{16}$ Flavonoid	610.52	4.00 4.08	Antioxidant, Anti-inflammatory, Anti-cancer, Cardiovascular Health, Antimicrobial Neuroprotective, Antidiabetic, Antigout

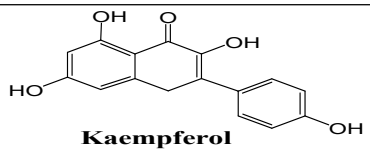
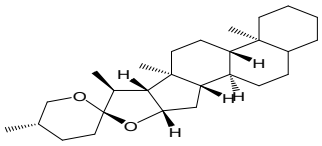
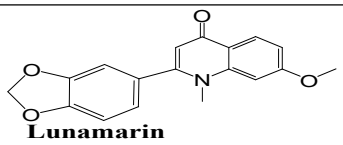
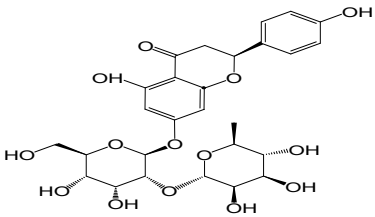
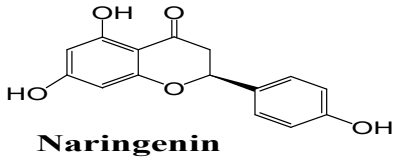
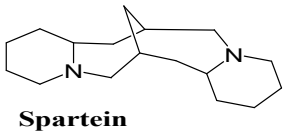
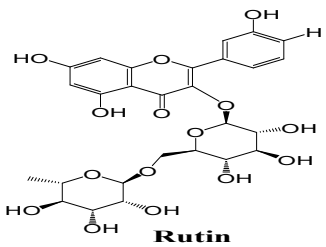
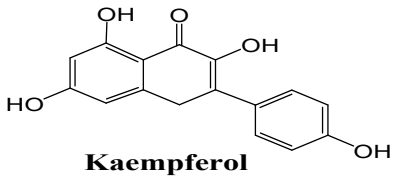
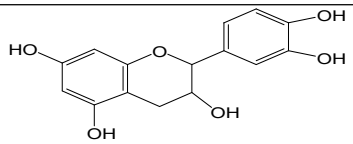
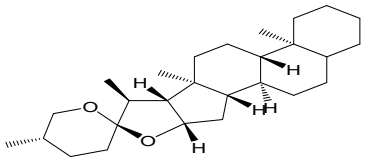
11	28.043 27.916	 Kaempferol	$C_{15}H_{10}O_6$ Flavonoid	286.24	14.39 5.48	Antioxidant, Anti-inflammatory, Antimicrobial, Anticancer, Antidiabetic, Cardioprotective, Neuroprotective
14	42.706 42.706	 Sapogenin	$C_{24}H_{42}O_3$ Triterpenoid Sapogenins	400.6	8.33 8.37	Antimicrobial, Anti-inflammatory, Anticancer, Antioxidant, Cardioprotective, Immunomodulatory

Table 3.16: Bioactive compounds of *Artemisia annua* dichloromethane fraction identified using the HPLC analysis

Peak	RT	Compounds	Mol. Formula/ Phytochemical class	Mol. Weight	Area %	Pharmacological Activity
1	0.220 0.203	 Lunamarin	$C_{16}H_{12}O_8$ quinolone alkaloid	332.26	1.79 2.74	lower arterial blood pressure
2	1.596 1.583	 Naringin	$C_{27}H_{32}O_{14}$ Flavonoid	580.54	2.72 3.01	Antioxidant, Anti-inflammatory, Anticancer
6	12.966 13.020	 Naringenin	$C_{15}H_{12}O_5$	272.25	14.41 12.31	Flavonoid, Antioxidant, Anti-inflammatory, Anticancer, Cardioprotective
8	15.620 15.620	 Spartein	$C_{15}H_{26}N_2$ Alkaloid	234.38	3.36 3.43	Antiarrhythmic, Hypoglycemic, Diuretic, Anti-inflammatory
9	18.950 18.950	 Rutin	$C_{27}H_{30}O_{16}$ Flavonoid	610.52	4.00 4.08	Antioxidant, Anti-inflammatory, Anti-cancer, Cardiovascular Health, Antimicrobial Neuroprotective, Antidiabetic, Antigout
12	28.043 27.916	 Kaempferol	$C_{15}H_{10}O_6$ Flavonoid	286.24	14.39 5.48	Antioxidant, Anti-inflammatory, Antimicrobial, Anticancer, Antidiabetic, Cardioprotective, Neuroprotective

13	- 36.526	 Catechin Epicatechin	$C_{15}H_{14}O_6$ Flavonoids, Polyphenols	290.27	- 8.64	Antioxidant, Anti-inflammatory, Antimicrobial, Cardiovascular Health, Neuroprotection
17	42.706 42.706	 Sapogenin	$C_{24}H_{42}O_3$ Triterpenoid Sapogenins	400.6	8.33 8.37	Antimicrobial, Anti-inflammatory, Anticancer, Antioxidant, Cardioprotective, Immunomodulatory

CHAPTER FOUR

DISCUSSION

4.1 Extraction

The purpose of sequential solvent extraction is to fractionate plant constituents based on their differential solubilities, enabling the concentration of chemically similar compounds into distinct solvent layers (Cowan, 1999; Doughari, 2012).

Mixture of methanol and water at 4:1 (v/v) ratio was chosen to enable separation of both hydrophilic and lipophilic compounds during partitioning process (Sasidharan *et al*., 2011).

N-hexane, a non-polar solvent, which was used to remove lipophilic compounds, such as waxes, chlorophyll, essential oils, and non-polar terpenes. DCM, being moderately polar and denser than water, is effective for extracting compounds such as sesquiterpene lactones, alkaloids, sapogenins, and steroids (Hamburger and Hostettmann, 1991). Subsequently, ethyl acetate was employed a moderately polar solvent is known for its affinity for flavonoids, phenolic acids, and some glycosides (Harborne, 1998).

Re-extraction with pure methanol at the final step targeted tannins, saponins, polar alkaloids, sugars, and amino acids that were not partitioned into the less polar solvents. Methanol is

highly effective for dissolving these polar compounds and is often the final solvent in polarity-based extraction schemes (Sasidharan *et al*., 2011).

4.2 Phytochemical composition

Secondary metabolites including flavonoids, terpenoids, alkaloids, phenolics, and saponins were found in *Artemisia annua* (*A. annua*) during phytochemical screening. The pharmacological advantages of these substances, especially their antibacterial, antioxidant, and anti-inflammatory properties, have been extensively researched.

This is consistent with the findings of Bhakuni *et al*. (2001) and Willcox (2009), who found that *A. annua* contains more than 600 phytochemicals, including sesquiterpenes, coumarins, and polyphenols, which contribute to its therapeutic qualities. Similarly, Marinas *et al*. (2015) discovered that the chemical composition of *A. annua* varies depending on climatic circumstances, which might explain variances in bioactivity seen in different investigations.

The ethyl acetate fraction contained the highest number of active compounds, suggesting that medium-polarity solvents are most effective in extracting bioactive constituents from *A. annua*. This observation agrees with the findings of Ferreira *et al*. (2010), who reported that solvent polarity plays a crucial role in maximizing the extraction of antimicrobial compounds from medicinal plants.

4.3 Toxicity Assessment and Safety Profile

Acute Toxicity screening of *A. anna* crude extract shows no mortality or severe adverse effects observed at doses up to 5000 mg/kg, indicating that *A. annua* has a high safety margin for medicinal use. This is consistent with study by Liu *et al*. (2010), who reported that *A. annua* extracts exhibit low toxicity when administered orally but may require further investigation for long-term use.

4.4 Antimicrobial Activity of *A. annua*

The antimicrobial analysis demonstrated that *A. annua* extracts possess significant antibacterial activity, particularly the ethyl acetate fraction. The extract exhibited broad-spectrum activity against both Gram-positive and Gram-negative bacteria, including multidrug-resistant (*MDR*) strains such as *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumonia, Enterobacter cloacae* and *Bacillus subtilis*

Ethyl acetate extract showed the largest zones of inhibition, indicating its potential as a broad-spectrum antimicrobial agent.

Dichloromethane extract displayed moderate activity, indicating the presence of some antimicrobial compounds. Whereas methanol and n-hexane extracts had lower efficacy, reinforcing that flavonoids and terpenoids (which are best extracted with ethyl acetate) are key antimicrobial agents, suggesting that highly polar and non-polar solvents were less efficient in extracting bioactive antimicrobial agents.

A. annua extracts and ciprofloxacin, a popular antibiotic for bacterial infections, were contrasted in the study. The findings demonstrated that for a variety of bacterial strains, the ethyl acetate extract exhibited inhibitory zones that were either similar to or marginally lower than those of ciprofloxacin. The MIC and MBC values, on the other hand, were remarkably low, suggesting strong antibacterial activity at low concentrations.

These findings are consistent with Mishina *et al*. (2007), which found that *A. annua* extracts could inhibit bacterial growth at low MIC values, suggesting their potential as alternatives to conventional antibiotics. Similarly, Suberu *et al*. (2013) reported that *A. annua* could enhance antibiotic efficacy through synergistic interactions, reducing bacterial resistance.

4.5 Total Phenolic and Total Flavonoid content

Total Phenolic Content: Phenolic compounds are vital secondary metabolites in plants, widely recognized for their antioxidant and antimicrobial properties. Their strong radical-scavenging abilities enable them to protect cells from oxidative stress, while their ability to disrupt microbial cell membranes contributes to their antimicrobial activity (Ferreira *et al*., 2010).

The dichloromethane and ethyl acetate fractions of *A. annua* exhibited high total phenolic content (TPC), measured at 295.83 mg/g and 212.20 mg/g gallic acid equivalents (GAE), respectively in this study. These values suggest a direct correlation between TPC levels and the antimicrobial potency of the extracts, as phenolics have been shown to interfere with microbial growth, protein synthesis, and enzyme activity (Bhakuni *et al*. 2001).

The TPC values obtained in this study align with findings from previous research; Marinas *et al*. (2015) reported higher TPC values (~350 mg GAE/g) in *A. annua* methanolic extracts, which they associated with strong antioxidant activity. A study by Lubbe *et al*. (2012) found that the phenolic content of ethanol extracts from *A. annua* showed antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*.

Also, Tao *et al*. (2020) reported TPC values in hexane and chloroform fractions of *A. annua* to be significantly lower (~150 mg GAE/g), suggesting that solvent polarity plays a key role in extracting bioactive phenolics.

These comparisons indicate that the high phenolic content observed in the dichloromethane and ethyl acetate fractions in this study supports their enhanced antimicrobial activity, consistent with literature findings that phenolic contribute to bacterial inhibition by disrupting

cell walls, altering membrane permeability, and chelating essential metal ions required for microbial survival (Isani *et al*. 2019).

Total Flavonoid Content: Flavonoids are another major class of bioactive phytochemicals with potent antimicrobial, antioxidant, and anti-inflammatory properties. Their mechanism of antimicrobial action includes inhibiting nucleic acid synthesis, disrupting bacterial membranes, and interfering with energy metabolism (Suberu *et al*. 2013).

The dichloromethane and ethyl acetate fractions of *A. annua* were found to have total flavonoid contents (TFCs) of 69.12 mg/g and 83.76 mg/g quercetin equivalents (QE), respectively, in this investigation. The extracts' antibacterial activity is supported by these values, which show a significant presence of flavonoids.

Lang *et al*. (2019) observed higher flavonoid content (~95 mg QE/g) in ethanol extracts of *A. annua*, correlating with its strong antimicrobial activity against MDR bacteria. Similarly, Foglio *et al*. (2002) found that flavonoid-rich fractions of *A. annua* exhibited antifungal activity, particularly against *Candida albicans*, reinforcing the role of flavonoids in microbial inhibition. In addition, Nair *et al*. (2021) demonstrated that flavonoids in *A. annua* extracts inhibited viral replication, suggesting potential antiviral benefits in addition to antibacterial effects.

According to Feng *et al.* (2020), these comparisons show that the flavonoid content found in the current study is in line with earlier findings, emphasising the function of flavonoids in upsetting the cell structures of bacteria and fungi, preventing the formation of biofilms, and boosting immune responses against infections.

4.5 Chemical Constituents of *A. annua* dichloromethane and ethyl acetate fractions from GC-MS and HPLC analyses

Key bioactive substances found in the ethyl acetate and dichloromethane fractions of *A. annua* were determined by GC-MS and HPLC analyses. These include:

1. Deoxyqinghaosu is a notable compound a structural variant of artemisinin, but it lacks the endo-peroxide bridge. This absence reduces its antimalarial potency but may influence other biological activities such as antimicrobial or anticancer effects.
2. Scopoletin – Known for antimicrobial, antioxidant, and anti-inflammatory properties, a Notable Compound, commonly found in *Artemisia annua*, has been highlighted in previous studies for its role in plant defence mechanisms and its synergistic antimicrobial effects when combined with other compounds.
3. Naringenin – A flavonoid with antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*.
4. Kaempferol – Exhibits strong antibacterial effects against MDR pathogens.
5. Sapogenin – Known to have antibacterial and antifungal properties.
6. Phytol – Demonstrates moderate antimicrobial effects and is commonly found in medicinal plants.

A study by Marinas *et al*. (2015) also identified scopoletin in *A. annua*, associating it with antibacterial and antifungal activities. Similarly, Isani *et al*. (2019) confirmed that flavonoids like naringenin and kaempferol disrupt bacterial cell walls and inhibit enzyme activity, which could explain their effectiveness against MDR bacteria.

CHAPTER FIVE

CONCLUSION

5.1 Conclusion

This study provided evidence that *A. annua* possesses significant antimicrobial properties, with the ethyl acetate fraction exhibiting the highest potency against MDR bacteria**.** The presence of flavonoids, terpenoids, and coumarins explains its broad-spectrum antibacterial activity, while toxicity studies confirm its safety for medicinal applications.

Given its low toxicity, high antimicrobial efficacy, and potential synergy with conventional antibiotics, *A. annua* could serve as a natural alternative for treating drug-resistant infections. Further clinical trials are necessary to assess its therapeutic effectiveness in human bacterial infections.

The results of this study confirm that the antimicrobial activity of *A. annua* is strongly linked to its phenolic and flavonoid contents, as observed in the dichloromethane and ethyl acetate fractions. The findings align with previous studies, reinforcing the role of phenolic and flavonoid compounds in plant-based antimicrobial therapies.

Given that phenolics and flavonoids can work synergistically with conventional antibiotics, further research should explore their potential use in combination therapies to combat antibiotic-resistant pathogens. Additionally, optimizing extraction techniques could further enhance the bioavailability and pharmacological efficacy of these bioactive compounds for therapeutic applications.

This study explored the phytochemical profile, antimicrobial potency, and toxicity of *A. annua* extracts.

GC-MS and HPLC analyses confirmed the presence of key antimicrobial compounds such as Deoxyqinghaosu, Sapogenin, Scopoletin, Naringenin, and Kaempferol which may be responsible for its antimicrobial properties.

These findings support the potential integration of *A. annua* into antimicrobial drug development.

5.2 Contribution to Knowledge

The study has contributed to knowledge in the following ways:

1. This study revealed the broad-spectrum antimicrobial potential of *Artemisia annua* fractions against multi-drug resistant bacteria.
2. The ethyl acetate fraction was identified as the most potent antimicrobial agent, with broad-spectrum bactericidal activity.
3. This study identified distinct bioactive compounds in dichloromethane fraction and ethyl acetate fraction that may be responsible for the observed antimicrobial activity.
4. Established a Safety Profile for *A. annua.* Toxicity studies showed that *A. annua* has a high safety margin, making it a viable candidate for pharmaceutical development.

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APPENDICES

ETHICS COMMITTEE
FACULTY OF PHARMACY
UNIVERSITY OF BENIN, BENIN CITY, NIGERIA

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5 August 202

Our Ref: EC/FP/020/12

Prof. Abiodun Falodun
Department of Pharmaceutical Chemistry
Faculty of Pharmacy
University of Benin
P. M. B 1154
Benin City

Dear Sir.

RE – APPLICATION FOR ETHICAL APPROVAL FOR THE INVESTIGATION OF POTENTIAL THERAPEUTIC CANDIDATES FROM NIGERIAN MEDICINAL PLANTS AGAINST CORONA VIRUS DISEASE.

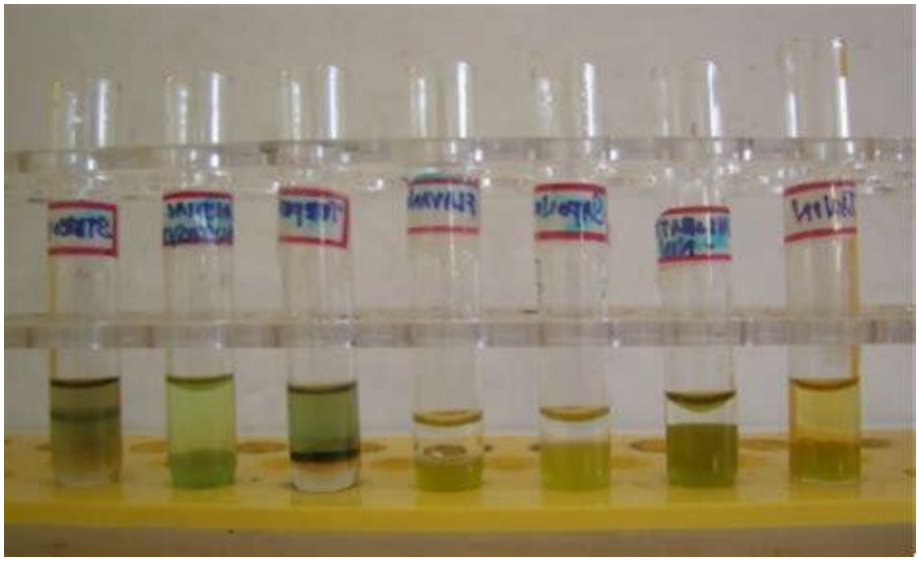
Your application for ethical approval of your proposal for the investigation of potential therapeutic candidates from Nigerian medicinal plants against corona virus (COVID 19) disease has been reviewed by the Ethics Committee and you are hereby granted approval for the study.

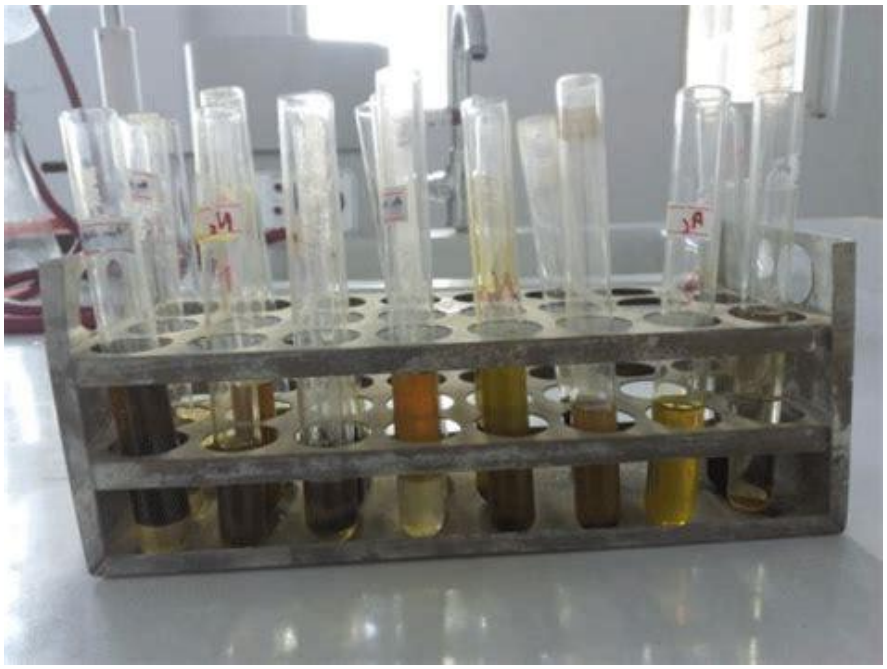
Note that you are to adhere strictly to the methods and guidelines specified in your proposal. Anv need for protocol variation should be submitted to the Committee for further consideration.

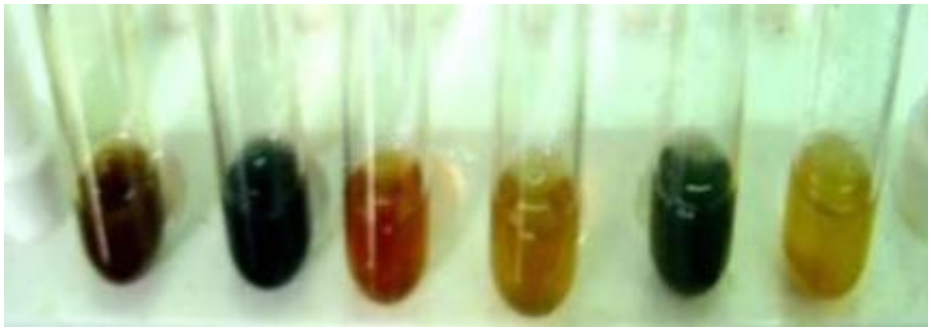
Yours faithfully,

Prof. E. K. I. Omogbai
Chairman, Ethics Committee

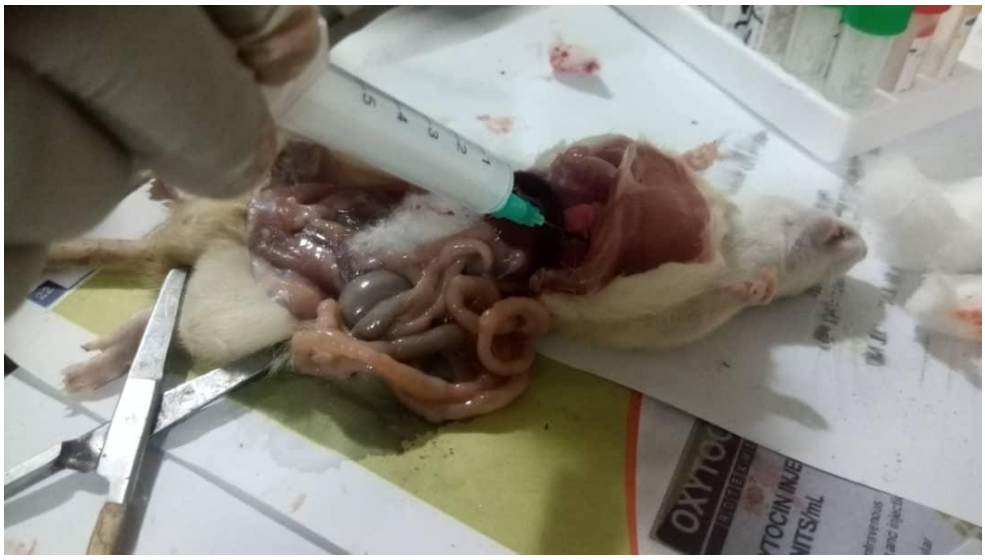
Appendix 1: Ethical Approval

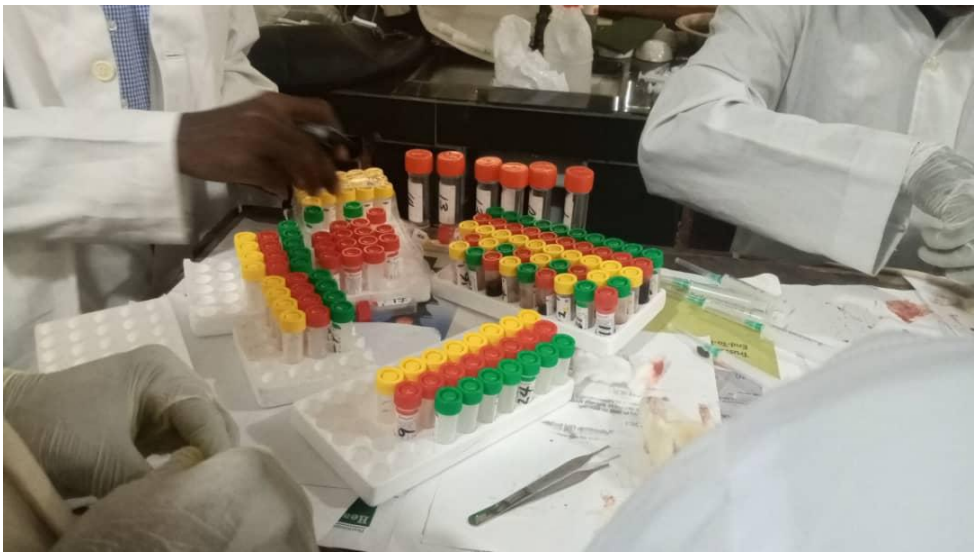




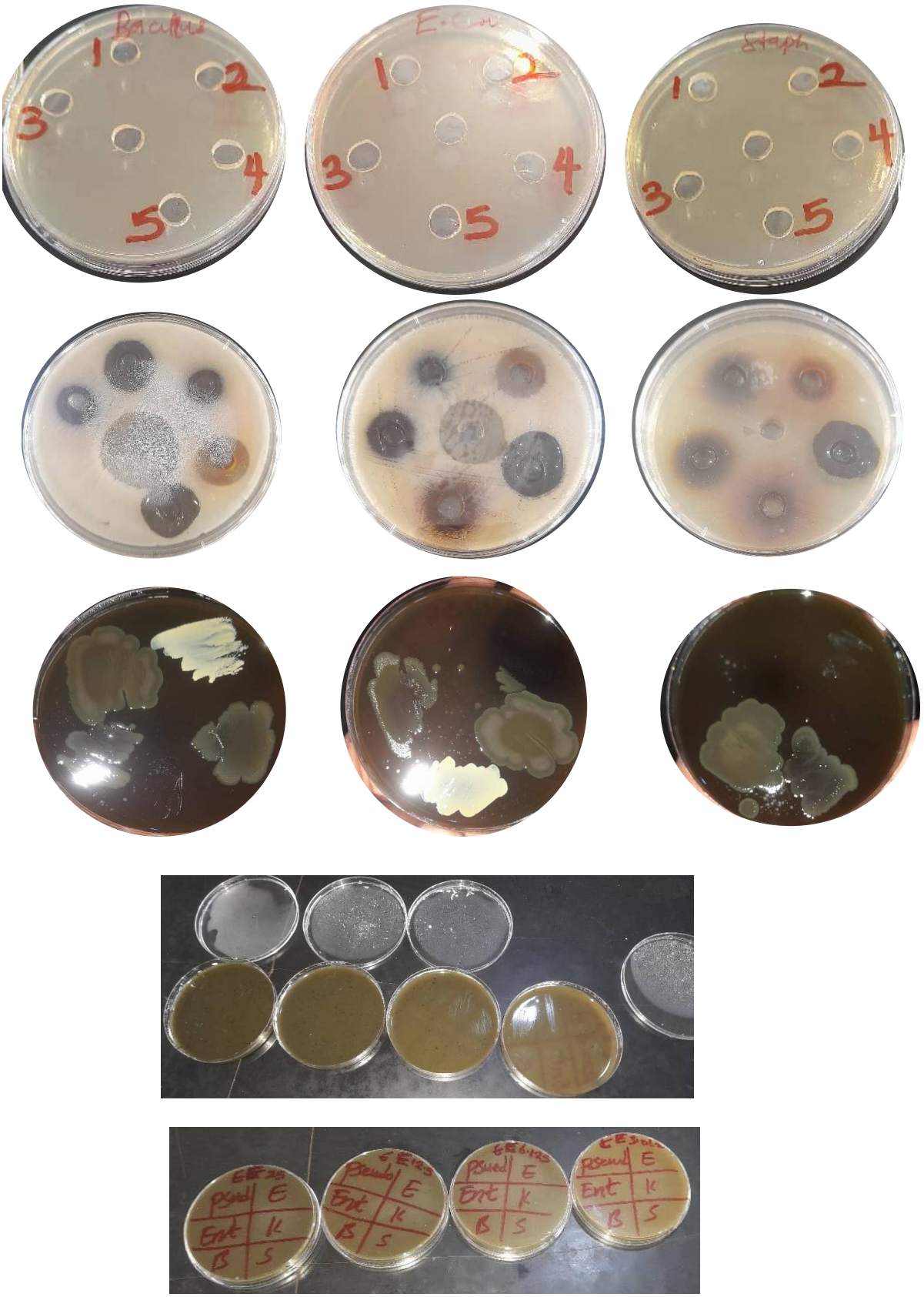


Appendix 2: Phytochemical screening of *A. annua* extract and fractions





Appendix 3: Toxicity Study of *A. annua* crude extract



Appendix 4: Antimicrobial study of *A. annua* extract and fraction