CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Effect of Extraction Solvents on Phytochemical Contents, Antioxidant, Anti-inflammatory, and Anti-diabetic Activities of *Verbena tenuisecta* Extracts

by

Zainab Azad

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I dedicated this thesis to my supportive family and friends and all those who helped in completing my thesis from start to end.

CERTIFICATE OF APPROVAL

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(Zainab Azad)

Abstract

The study evaluated the impact of different extraction solvent on phytochemical properties and biological activities *Verbena tenuisecta* extract. This research evaluated the total phenolic and flavonoid contents and antioxidant and anti-inflammatory and anti-diabetic properties by utilizing spectrophotometric methods. The maceration method using water, methanol, and ethyl acetate produced extracts for evaluation. The Folin–Ciocalteu method determined total phenolic content while the aluminum chloride colorimetric assay investigated total flavonoid content and the DPPH radical scavenging method measured antioxidant activity. Protein denaturation inhibition examined anti-inflammatory properties whereas α-amylase enzyme inhibition calculated anti-diabetic potential. Results showed that maximum extraction yield was gained from the water solvent 7.15%, methanol was most effective solvent for total phenolic content determination by extracting 192.72 mg GAE/g phenolic, showed 25.50% scavenging at 50g/ml in anti-oxidant activity and exhibited 75.17% inhibition protein in antiinflammatory activity and ethyl acetate was most effective solvent by extracting 72.53±7.84 QE/g flavonoids in total flavonoid content determination assay. The study identifies methanol as the most effective solvent for extracting the phenols and for antidiabetic and anti-inflammatory activities, ethyl acetate proves superior for both flavonoid extraction and anti-diabetic effect and water extracted the highest yield percentage. The research demonstrates how solvent selection plays a vital role in obtaining optimal phytochemical yields and therapeutic outcomes while associating traditional practices to modern pharmacological analysis. Future research needs to focus on improving solvent selection methods as well as extraction technology development alongside bioactivity assay customization to achieve maximum benefits from natural products.

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Abbreviations

DPPH	2,2-diphenyl-1-picrylhydrazyl
DMEM	Dulbecco's Modified Eagle Medium
DNSA	Dinitro Salicylic Acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EU	European Union
FDA	Food and Drug Administration
FC	Folin–Ciocalteu
FT-IR	Fourier Transform Infrared
GAE	Gallic Acid Equivalents
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
IUCN	International Union for Conservation of Nature
PBS	Phosphate Buffer Saline
PDT	Photodynamic Therapy
RD	Rhabdomyosarcoma
SARS	Severe Acute Respiratory Syndrome
SDGs	Sustainable Development Goals
SWE	Subcritical Water Extraction
T&CM	Traditional and Complementary Medicine
TFC	Total Flavonoid Content
TLC	Thin Layer Chromatography
TPC	Total Phenolic Content
UHC	Universal Health Coverage

UK	United Kingdom
USD	United States Dollar
UV	Ultra Violet
WHO	World Health Organization

Chapter 1

Introduction

1.1 Medicinal Plant

Medicinal plant is termed as any plant that comprises of bioactive elements in its more than one part and utilized for therapeutic applications or serve as precursors for the development of pharmaceutical drugs. This definition allows for a clear differentiation between medicinal plants with scientifically established therapeutic properties and constituents and those that are considered medicinal but have yet to undergo comprehensive scientific investigation. Numerous plants have been employed in traditional medicine for extended periods. While some of these plants exhibit therapeutic effects, there may not be adequate scientific evidence, such as double-blind clinical trials, to validate their efficacy. Nevertheless, such plants can still be classified as medicinal plants [1].

Medicinal plants are integral components of indigenous medical systems worldwide. Ethnobotany serves as a valuable resource for the research and development of natural drugs [2]. The term "traditional" in herbal medicine denotes a significant historical context, particularly relevant to many products classified as "traditional herbal medicines." In numerous developing countries, a considerable portion of the population depends on traditional practitioners and their collection of medicinal plants to address healthcare needs [3]. While modern medicine often coexists with traditional practices, herbal remedies have retained their popularity

due to historical and cultural factors [4].

Natural products have played a crucial role in the treatment and prevention of human diseases globally. Medical products originating from natural substances derive from multiple resource types involving terrestrial plants [5], microorganisms and marine organisms and terrestrial and invertebrate animals. The significance of these natural products in contemporary medicine has been extensively discussed in numerous journals and reports [6].

The significance of natural products can be assessed through several criteria:

1. The number of new chemical entities defines the pace of discoveries that form diversity models for partial-synthetic and entire synthetic modifications.
2. The range of diseases that these substances can treat or prevent.
3. The frequency with which these products are utilized in disease management.

In recent years, there has been a renewed interest in utilizing traditional medicine information for plant research [7].

The remarkable diversity of chemical structures and biological activities of naturally occurring secondary metabolites, the unmet therapeutic needs, the potential of novel bioactive natural compounds as biochemical probes, improvements in sensitive techniques for detecting biologically active natural products, improved methods for isolating, purifying, and structurally characterizing these active components, and the progress made in meeting the demand for complex natural products are some of the factors driving this resurgence [8].

Traditional medicine has gained relevance to the World Health Organization (WHO) because the organization has developed guidelines to establish standards for botanical medicines. Agro-industrial technologies that have been established should guide the cultivation and processing of medicinal plants and the herbal medicine production process [9].

The discovery of new drugs depends heavily on medicinal plants since numerous

current pharmaceuticals find their origins in plant-based compounds. Analysis of the 250,000 known flowering plant species remains essential for determining plant poisons along with human and animal protection against natural toxins [10].

1.2 Overview and Historical Context of Traditional Herbal Medicine

The utilization of plants for medicinal purposes dates back to early humanity, with fossil records indicating that such practices emerged during the middle Paleolithic era. Scientists found proof of this historical bond in the grave of a Neanderthal individual who lived about 60,000 years ago. The analysis of pollen indicated that among the medicinal plants which were buried with the corpse there were several important species [11].

Archaeological records show that the oldest medical document was discovered in a Sumerian clay tablet from 4000 years ago which listed plant treatments for different illnesses. Extensive recordings of medicinal plant wisdom already existed during the time of ancient Egyptian civilization. Potable remedies from the time consisted of mandrake for pain relief and garlic for heart and circulatory disorders with papyrus texts recording these findings some 3,500 years ago [11].

Ancient China also offers rich historical insights into the early medicinal applications of plants [11]. The primary source of treatment stems from botanical sources even though animal and mineral substances have also been utilized. Traditional healers use more than 12,000 products with about 500 items classified as rare among them. Traditional botanical treatments need processing before use through procedures such as stir-frying and vinegar or wine-based soaking. Medical facilities begin using individualized complex remedies after performing traditional patient diagnoses. Traditional Chinese medical approaches remain popular in China as people use them frequently in rural areas and throughout more than fifty percent of the population. Traditional remedies in China total about 5,000 products with market representation at one-fifth of total pharmaceutical sales in the country [12].

Additionally, Herbal remedies originating from China made significant contributions to traditional Japanese healing practices as native Japanese plants appeared in the ninth-century pharmacopoeia of Japanese traditional medicine [13].

The history of herbal medicine in India spans multiple millennia where ancient knowledge originated from the Rig Veda collection of sacred Hindu texts. Snakeroot stands out among traditional Indian plants because it has been known since centuries for its sedative effects. Modern estimations of higher plant species on Earth span from 215,000 to 500,000 yet coincides with 250,000 as a mainstream figure. Of these, only around 6% have undergone biological activity screening, and approximately 15% have been phytochemically evaluated [14].

As high-throughput screening methods become more sophisticated and accessible, these figures are likely to change. The primary standard for comparing plant species derives from their methods to identify potential therapeutic candidates. The fundamental objective to acquire therapeutic plant materials includes multiple general aspects of selection and acquisition processes. Human usage of plants traces back numerous hundreds to thousands of years which provides plants with an advantageous position in this field [15].

1.3 Modern and Traditional Approaches to Herbal Medicine

The origins of pharmacological treatments for diseases can be tracked back to the early use of herbs. The practice of herbal medicine lost its prominence in the eighteenth and nineteenth centuries yet numerous successful treatments were adopted by medical practitioners during the period when scientists began studying therapeutic substances. The medical world remembered William Withering as a pioneering figure because he brought scientific methods to study traditional medicine. He established pharmaceutical chemistry through his research between 1775 and 1785 which demonstrated foxglove (*Digitalis purpurea*) effectiveness in dropsy (which is today known as congestive heart failure) treatment [16].

Science experts began extracting the active compounds from medicinal plants throughout the nineteenth century thus enabling breakthroughs in pharmaceutical chemistry. The achievement of Friedrich Sertürner brought major progress in 1806 by extracting morphine from (*Papaver somniferum*) opium poppy. German chemist Justus von Liebig emerged as a prominent figure after these developments which advanced the field of pharmacology. The growth of active chemical compound knowledge led to the creation of totally synthesized drugs from natural products during the middle of the nineteenth century [16].

Plant secondary metabolites known as bioactive compounds consist of alkaloids, steroids, tannins and phenolic compounds that demonstrate specific physiological responses on human bodies. Secondary metabolites along with essential oils of therapeutic significance make medicinal plants very rich in natural compounds [17].

Medicinal plants produce various benefits for medical treatment because they offer safe use and accessible prices along with efficient results and simple accessibility. Traditional medical practitioners incorporate medicinal plants into their everyday treatments because of this outcome [17].

Among the varied range of plant secondary metabolites, phenolic compounds represent a large and wide-ranging group known for their antioxidant properties. These phenolic compounds expressively contribute to the antioxidant potential of numerous plant species, underscoring their importance in both traditional and modern medicinal applications [18].

1.4 Preventive Strategies

One of the essential healthcare methods includes health promotion supplemented by disease prevention combined with chronic disease management which focuses on prevention across the healthcare sequence. Public health strategies for promoting wellness seek to defend good health before disease occurs and this process is known as primary prevention. According to the Commission on Chronic Illness in 1957

disease prevention includes three specific levels. These three levels primary, secondary, and tertiary prevention serve as a framework for reducing the impact of diseases on individuals and populations [1].

1.4.1 Primary Prevention

It is the first level of disease prevention, which focuses on reducing the occurrence of new cases of illnesses or disorders. It involves proactive measures that aim to maintain overall health and prevent the onset of disease. This includes strategies such as health education, public awareness campaigns, lifestyle modifications, and environmental changes that promote well-being. Additionally, specific protective measures, such as immunizations, proper sanitation, and nutritional supplementation, play a crucial role in preventing infections and other health conditions before they develop [1].

1.4.2 Secondary Prevention

It targets the early detection and prompt intervention of existing diseases to reduce their prevalence and severity within a population. This level of prevention emphasizes screening programs, routine medical check-ups, and diagnostic tests that help identify conditions in their initial stages. By diagnosing diseases early, healthcare providers can implement timely treatments, manage risk factors effectively, and prevent complications from developing. Examples include cancer screenings (such as mammograms and Pap smears), blood pressure monitoring for hypertension, and blood glucose testing for diabetes. Secondary prevention helps in slowing disease progression and improving health outcomes through early medical intervention [1].

1.4.3 Tertiary Prevention

It focuses on minimizing the long-term impact and disability associated with est-

ablished diseases. This level of prevention is crucial for individuals who are already affected by chronic illnesses or permanent conditions. It involves rehabilitation programs, physical therapy, occupational therapy, and other interventions designed to restore functionality and enhance the quality of life for affected individuals. Additionally, tertiary prevention includes strategies that help prevent further complications, manage symptoms effectively, and support individuals in adapting to their health conditions. Examples include cardiac rehabilitation for heart disease patients, physiotherapy for stroke survivors, and support groups for individuals with mental health disorders [1].

Overall, these three levels of disease prevention—primary, secondary, and tertiary—work mutually to promote the public health, reduce disease burden, and improve the quality of life by preventing illnesses, detecting them early, and mitigating their effects [1].

Medicinal plants are an important source of a wide variety of chemical compounds with diverse structures and functions, demonstrating vital biological activities associated with numerous beneficial effects. These effects encompass antimicrobial, anticancer, antiviral, antioxidant, enzyme inhibitory, anti-aging, anti-inflammatory, antihypertensive, neuroprotective, and anticoagulant properties [19, 20].

Globally, medicinal plants hold substantial importance, both as independant treatments and as supplements to conventional medications. The extensive literature documenting the therapeutic properties of medicinal plants, combined with centuries of experience in folk medicine, has fostered an increasing interest in the utilization of natural products for health benefits [21].

1.5 *Verbena tenuisecta*

Moss verbena (*Verbena tenuisecta*), also referred to as South American verbena or *Glandularia tenuisecta*, is a charming low-growing perennial that creates a dense mat of finely textured, lace-like foliage (Table 1.1). This delightful ground cover

showcases a continuous display of small, five-petaled flowers in various colors, including violet, lavender, pink, and white. With its trailing growth habit, moss verbena forms a vibrant carpet of color, typically growing to heights of 6 to 12 inches. It thrives in full sun and well-drained soil, demonstrating drought tolerance and making it an excellent choice for rock gardens, borders, and sunny landscapes. The nectar-rich flowers are highly alluring to butterflies, bees, and other pollinators, enhancing garden biodiversity. The species belongs to the Verbenaceae family and was formerly known as *Glandularia tenuisecta*. The attractive ornamental qualities of moss verbena allow gardens to gain lively charm when it flowers through late spring and into early autumn [22].

TABLE 1.1: Characteristics of *Verbena tenuisecta* [23]

S. No.	**Characteristics**	
1.	Scientific Name	*Verbena tenuisecta*
2.	Family	Verbenaceae
3.	Type	Perennial
4.	Zone	6-12 inches
5.	Height	12–18 Inches
6.	Bloom Time	Spring to Fall
7.	Bloom Description	Purple
8.	Sun	Full Sun to Partial Shade
9.	Color(s)	Purple
10.	Seed Type	Annual, Tender Perennial
11.	Sun	Full Sun
12.	Moisture	Dry, Moderate
13.	Height	12-18 inches
14.	Bloom Period	Spring, Summer
15.	Water	Low to Medium
16.	Maintenance	Minimal
17.	Flower	Showy
18.	Attracts	Butterflies
19.	Tolerates	Drought

Purple moss verbena represents a tender spreading perennial which shows attractive purple flowers and fern-like foliage types. The plant maintains a lengthy flowering season which functions excellently as a ground covering plant. The plant proves useful as a permanent solution to control erosion whenever conditions permit its growth. Only moss verbena ought to be treated as an annual plant when grown in regions with cold climate conditions. Since purple moss verbena maintains drought-tolerant characteristics it functions well in diverse landscape setups [24].

Moss verbena (*Verbena tenuisecta*) originates from South America as a perennial plant that is tender to temperature changes. Moss verbena seeds should be directly planted into outdoor soil because the plant develops perennial persistence within zones 7-11 but survives as an annual for all other USDA regions. The flowering herb known as cutleaf verbena or South American mock vervain (*Verbena tenuisecta*) displays its spike-shaped clusters of multiple 5 to 15 colored flowers that vary between blue and purple and lilac and violet during its spring and summer bloom. Flowering moss verbena functions as premium nectar nourishment for both honeybees and butterflies as well as hummingbirds [25].

Moss verbena is particularly well-suited for roadside plantings and land reclamation projects. Its low-growing, spreading habit makes it an ideal drought-resistant, evergreen ground cover, adorned with aromatic, finely cut fern-like foliage. This plant is also well-suited for cultivation in containers, pots, rock gardens, and meadows. Another botanical designation for this species is *Glandularia pulchella*. When grown from *V. tenuisecta* seeds, moss verbena establishes easily, demonstrating resilience to drought and tolerance for poor soils, while also re-seeding freely [25].

1.6 History

The moss variety of verbena originates from South American soils where later humans brought it to the south of the United States. Traditional preparations of moss verbena use the aerial parts of the plant that include dried and fresh stems.

These infusions are regarded as mild sedatives that help alleviate anxiety and headaches associated with stress or upper back tension. Additionally, moss verbena has been used to enhance milk production in nursing mothers [26].

1.7 Culture

A hillside can be transformed into a magnificent shimmering moss verbena-covered carpet. Moss verbena exhibits stunning blooming flowers that appear in purple, plum and white and create terminal clusters of tiny compound blossoms which rest above its fern-like leaves. Moss verbena shows great potential as a container species thus becoming an excellent butterfly attractor for your courtyard. These flowering plants have retained their status as decorative butterfly draws for several generations. This flowering plant grows effortlessly in typical, medium-watered earth when positioned under complete sun exposure. The best flowering results from moss verbena emerge in complete solar exposure although it demonstrates tolerance to mild shade. The low-maintenance aspect of moss verbena stems from its ability to handle heat conditions and periods of drought successfully [26].

1.8 Noteworthy Characteristics

Moss verbena, native to South America, thrives in sunny, hot, and dry conditions. This resilient ground cover adapts well to a variety of settings, including slopes, banks, rock gardens, and containers or hanging baskets. The plant produces tiny, colorful blooms that cluster in large, rounded formations at the tips of herbaceous branches. While purple varieties are the most prevalent, moss verbena can also be found in beautiful shades of pink and white. Regardless of their color, the abundant flowers are highly attractive to butterflies. The plant features deep green, fern-like foliage that remains particularly low to the ground when grown in full sun, typically reaching heights of up to one foot in shaded conditions. This versatility and appeal make moss verbena an excellent choice for enhancing any garden landscape [23].

1.9 Foliage of *Verbena tenuisecta*

The table 1.2 presents key characteristics of foliage of *V. tenuisecta*.

TABLE 1.2: Foliage of *Verbena tenuisecta* [27]

Characteristic	**Details**
Leaf Venation	Pinnate
Leaf Persistence	Semi Evergreen
Leaf Type	Simple
Leaf Blade	Less than 5 inches
Leaf Shape	Ovate
Leaf Margins	Entire
Leaf Textures	Hairy
Leaf Scent	No Fragrance
Color (growing season)	Green
Color (changing season)	Green

1.10 Flower

The table 1.3 presents key characteristics of flower of *V. tenuisecta*.

TABLE 1.3: Flower of *Verbena tenuisecta* [27]

Characteristic	**Description**
Flower Showiness	True
Flower Size Range	7 - 10 inches
Flower Type	Spike
Flower Scent	No Fragrance
Flower Color	Purple, Blue, White
Seasons	Summer, Fall

1.11 Fruit

The table 1.4 presents key characteristics of fruit of *V. tenuisecta*.

TABLE 1.4: Fruit of *Verbena tenuisecta* [27]

Characteristic	**Description**
Fruit Showiness	False
Fruit Size Range	0 - 1.5 inches
Seasons	Summer, Fall

1.12 Description

Moss verbena is a sprawling ornamental perennial in the southern regions but is treated as an annual in the north due to its intolerance to cold temperatures. This hardy plant typically reaches heights of 12 to 18 inches and features small, rich bluish-purple flowers that bloom in bouquet-like clusters. The drought-tolerant moss verbena needs full sunlight exposure and does well with occasional mowing without damage. Moss verbena produces flowers from March to July which provides continuous blooming over the spring into early summer months [26].

The South American ornamental plant *Verbena tenuisecta* known as *V. tenuisecta* operates successfully across different Libyan regions after its origination from South America. Scientists who perform phytochemical research on the Verbena family have identified different compounds found in these plants including volatile oils as well as sterols triterpenoids lipid classes iridoids alongside plenty of phenolic compounds. The Verbena genus provides traditional folk medicine with effective remedies to treat rheumatism while it also serves as a treatment for fever and tonsillitis and gastrointestinal problems and sexually transmitted diseases in South America. Modern medical research identifies Verbena as a treatment through its antimicrobial and antioxidant and anti-inflammatory and neuro sedative and diuretic properties [28].

1.13 Traditional Use

South and Central American traditional medicine integrates many different Verbena plant species to deal with fever as well as diarrhea and STIs along with digestive system issues and skin inflammation treatment. The primary chemical compounds that appear in Verbena genera are the iridoid glycosides including verbascosides coupled with the flavonoids kaempferol and luteolin and additionally volatile oils, ursolic acid and dihydrochalcones and sterols phenylethanoids and anthocyanidins as well as verbenalin together with its derived products [29].

1.14 Current Medical Uses

Medical research about *Verbena tenuisecta* remains very limited which leads to its status as primarily an ornamental plant instead of a clinical usage plant because valid clinical evidence is lacking [26].

1.15 Reported Biological Activities

1.15.1 Total Phenolic Content

Researchers examined *V. tenuisecta* extract phenolic compounds by using Folin–Ciocalteu reagent analysis [30]. The analyses confirmed that *Verbena tenuisecta* extracts hold high amounts of phenolic compounds in a quantity of 18.5 ± 1.6 mg GAE/g (dried sample weight basis in terms of gallic acid equivalents). Phenolic compounds often function as antioxidants based on the measured high concentration levels of these compounds in the tissue [31].

1.15.2 Total Flavonoid Content

The scientists analyzed the flavonoid concentration in *Verbena tenuisecta* extracts

by processing the samples with aluminum chloride in colorimetric analysis [30]. The testing revealed *Verbena tenuisecta* extracts hold noteworthy flavonoid concentrations which amount to 3.63 ± 1.2 mg RE/g (rutin equivalents per gram of dried sample). The high flavonoids level demonstrates that *Verbena tenuisecta* shows promise as a bioactive source that might provide both antioxidant and additional therapeutic properties [31].

1.15.3 Antibacterial Assay

Tests on antibacterial activities of *Verbena tenuisecta* extracts were performed using the well diffusion assessment method [32]. Antibacterial tests showed that plant extracts had moderate levels of inhibition capacity against studied microorganisms. The antibacterial potential of plant extracts increased through the preparation of nanoparticles with silver nitrate concentrations ranging from 1 mM to 5 mM and 10 mM. The antibacterial effect increased after synthesizing silver nanoparticles with a 5 mM silver nitrate concentration indicating that this level may provide the best antibacterial properties for *Verbena tenuisecta*-derived silver nanoparticles [31].

1.15.4 Antifungal Assay

The evaluation of antifungal activities for *Verbena tenuisecta* crude extract and synthesized nanoparticles occurred through the well diffusion testing method [32]. The research demonstrated that both crude extracts alongside nanoparticles showed moderate antifungal effectiveness against Candida strains thus validating their potential use as antifungal substances in fungal infection treatment [31].

1.15.5 DPPH Free Radical Scavenging Activity

DPPH tests were used to evaluate the antioxidant properties of crude extracts from *Verbena tenuisecta* and nanoparticles produced through synthesis [30]. The

V. tenuisecta crude extracts exhibited weak antioxidant behavior which signifies limited antioxidant potential of this plant species. The data indicates that *V. tenuisecta* contains bioactive substances although its antioxidant effects remain constrained [31].

1.15.6 Cytotoxicity Assay and Photodynamic Therapy

Scientists employed the MTT assay to evaluate cytotoxic effects using *Rhabdomyosarcoma* RD (ATCC# CCL-136) cell line in Dulbecco's Modified Eagle Medium (DMEM) culture medium. The experimental results demonstrated that both the purified plant extracts effectively killed RD cells so they exhibited cytotoxic properties. A photodynamic treatment utilizing merged plant extract combination with metallic nanoparticles and photosensitizers occurred. A decrease in cell survival rate occurred during the variation of these mixtures while nanoparticles tested alone led to 27.0% cell survival. The cell viability measurement reached 12.6% after adding plant extract and drug together with photosensitizer to the experimental system. The growth of cells treated with plant extracts remained high until nanoparticles and photosensitive compounds were added. Introduction of nanoparticles alongside treatment ingredients increases the toxicity level that affects RD cells [31].

1.16 Reported Phytochemicals

Verbena tenuisecta is native to South America and is now cultivated in Libya. Several species from the genus Verbena are currently utilized in traditional folk medicine in Central and South America to treat diarrhea, fever, gastrointestinal disorders, and certain sexually transmitted diseases, as well as for anti-inflammatory topical applications. Phytochemical studies of various Verbena species have resulted in the isolation of numerous constituents, including volatile oils, sterols, triterpenoids, iridoids, and phenolic compounds such as flavonoids, dihydrochalcones, anthocyanidins, and phenylethanoids [33].

1.17 Effect of Solvents

The well-chosen solvent in the extraction process significantly influences the type and quantity of secondary metabolites obtained from medicinal plants, impacting their pharmacological properties. Polar solvents like water and methanol are typically effective for extracting phenolic compounds, while non-polar solvents like ethyl acetate are more suited for isolating fatty acids and steroids [34]. Numerous studies indicate that both the solvent used and the extraction technique whether conventional (e.g., maceration, percolation, infusion) or non-conventional (e.g., microwave-assisted, ultrasonic-assisted, supercritical fluid extraction) affect the yield and activity of phytochemicals. Hence, careful selection of extraction solvents and methods is essential to achieve the desired bioactivity in medicinal plant extracts [35]. In developing countries, medicinal plants are widely employed as an accessible and affordable means of healthcare [36]. In Sudan, for example, traditional medicine plays a key role, supported by the country's vast diversity of flora, which includes approximately 3000 flowering plant species, around 15% of which are endemic. Most of these plants are traditionally used in food and medicine for disease prevention and treatment [37]. This study will investigate *Verbena tenuisecta* by extracting it with three solvents of varying polarity: ethyl acetate, methanol, and water. The goal is to analyze the effects of these solvents on the phytochemical content as well as the antioxidant, anti-inflammatory, and anti-diabetic activities of *Verbena tenuisecta* extract.

1.18 Hypothesis

Different extraction solvents will affect phytochemical content and biological activities of *Verbena tenuisecta* extracts.

1.19 Aim

Determination of effects of extraction solvents on phytochemical content, antiox-

idant, anti-inflammatory, and anti-diabetic activities of *Verbena tenuisecta* extracts.

1.20 Objectives

1. To determine total phenolic contents and total flavonoid contents of *Verbena tenuisecta* extracts.

2. To determine the antioxidant, anti-inflammatory, and anti-diabetic activities of *Verbena tenuisecta* extracts.

1.21 Scope of Study

The study investigates the impact of different solvents (Ethyl Acetate, Methanol, and Water) on the extraction yield and concentration of phytochemicals from *Verbena tenuisecta*. Key phytochemicals, including flavonoids, phenolics, and tannins, are identified and quantified using analytical techniques.

The bioactivity of the extracts is evaluated through various assays, including antioxidant capacity, which measures their ability to neutralize free radicals, anti-inflammatory potential assessed via enzyme inhibition assays, and anti-diabetic activity determined by enzyme inhibition relevant to diabetes management. A comparative analysis is conducted to identify the solvent that produces the most bioactive and phytochemically rich extract. Ultimately, the study aims to determine the optimal solvent for maximizing the health benefits of *Verbena tenuisecta*.

1.22 Impact on Society

The study has the potential to influence medicinal research by identifying the most effective solvent for extracting health-promoting bioactive compounds from *Verbena tenuisecta*. Optimizing extraction methods could enhance the plant's the-

rapeutic potential, strengthening its antioxidant, anti-inflammatory, and anti-diabetic properties. These bioactivities are crucial for managing chronic conditions such as oxidative stress-related diseases, inflammatory disorders, and diabetes. Improved extraction techniques may also contribute to the development of potent natural remedies or supplements, making affordable plant-based medicinal options more accessible, particularly in regions that rely on herbal medicine. Furthermore, the study's findings could drive pharmaceutical advancements and support the evidence-based use of traditional medicinal plants. By bridging modern science with traditional medicine, this research promotes the integration and acceptance of herbal remedies in healthcare.

Chapter 2

Literature Review

2.1 Medicinal Plants

Plants serve as a crucial source of numerous pharmaceuticals that are widely employed as therapeutics. For example, morphine is utilized for pain management; vincristine is used in the treatment of various cancers; penicillin is effective against bacterial and fungal infections; and warfarin is commonly prescribed for several heart conditions [38].

In underdeveloped regions with limited access to healthcare, plant-based traditional medicines serve as crucial, life-saving resources. Plants showcase significant chemical diversity and can produce complex phytomolecules with various functionalities. Their secondary metabolites enhance the flavor and quality of plant-derived foods and beverages while providing notable health benefits. Often termed "nutraceuticals," these products are widely used for preventing and treating various diseases. As a result, extensive research is being conducted on the functional properties of different plant extracts to explore their potential as innovative nutraceuticals and functional foods [39].

However, the total of plants with recognized therapeutic uses is likely underestimated, primarily because of lack of publications in ethnobotanical field in certain areas [40]

2.2 Current Trends in Traditional and Complementary Medicines

The global utilization of herbal medicines encompassing herbal pharmaceuticals, dietary supplements, and functional foods is experiencing significant growth. With approximately 6% expansion rate annually in global sales are projected to reach USD 130 billion by 2023. Notably, the main segment, herbal medicines, accounted for sales around USD 51 billion in 2017 [41]. Several factors contribute to the growth of herbal medicine, including the increasing occurrence of chronic diseases and ongoing therapeutic searches in areas with insufficient conventional treatments. The pharmaceutical herbs in Europe counting UK are classified as having a well-established use which was standardized by EU Directive 2004/24/EC. According to this directive, pharmaceutical herbal products must demonstrate a long custom of uses in medicine specifically, 30 years at least with fifteen of those years within the Union such as European. Additionally, these products must come across specified standards for quality and safety; they are required to comply with the monographs of relevant European or other pharmacopoeias. However, there is no requirement for demonstrating efficacy [42]. United States Food and Drug Administration (FDA) in 2004 established a pipeline botanical for drug development that includes materials from both plants and fungi. Two botanical drugs only to date have received approval through this pathway: Sinecatechins, sourced as of green tea *Camellia sinensis*, and crofelemer, derived from (*Croton lechleri)* dragon's blood [43]. In various regions globally, certain fungi and plants are utilized as folk medicines, although they often lack formal regulation through legislation. Traditional medicines are key elements of WHO's vision to strengthen their contribution in health promotion and population well-being [44]. Traditional medicines typically consist of mixtures of compounds, setting them apart from therapeutic drugs that contain a single ingredient which is active.

Traditionally, in the health care system around world herbal medicines have a vital role where a large share of healthcare expenses is paid out-of-pocket, leading many individuals to seek medications in private markets. For millions of people living

in rural regions, traditional therapists are their primary healthcare providers and sources of medicine. In Africa, the ratio of traditional therapists is 80 times higher to the population than that of conventional medical doctors, with nearly 4 billion people around world relying on herbal drugs as their main basis of healthcare [44, 45]. In all healthcare services offered represented about 40% are from herbal medicines.

Historically, the World Health Organization (WHO) has given limited attention to herbal medicines. However, recent global initiatives aimed at promoting Universal Health Coverage (UHC), particularly in light of increasing health maintenance costs and constrained resources, have provoked a reassessment of this stance. The recognition that of conventional medications are often unaffordable and unreachable in many regions, though herbal drugs are widely accessible, reasonable, and traditionally accepted, and has driven the World Health Organization to advocate for the integration of Traditional and Complementary Medicine (T&CM) into healthcare systems [38]. There is a growing focus on preventing and managing lifestyle-related chronic diseases, as well as addressing the health needs of aging populations. In response to these challenges, World Health Organization (WHO) implemented initiatives to address concerns regarding the efficacy, quality, standardization and safety of herbal medicines. The WHO is dedicated to assisting Member States in providing safe, qualified, and effective Traditional and Complementary Medicine (T&CM) services, while also promoting their proper integration into healthcare systems to achieve Universal Health Coverage (UHC) and the Sustainable Development Goals (SDGs) [46]. Almost 64% of WHO Member States by 2018 had established nationwide regulations for herbal medicines, with (34) of these countries including herbal or traditional drugs in their National Essential Medicines Lists, which comprise drugs essential for meeting the healthcare necessities of their populations. For example, for malaria the primary combination remedies are based on artemisinin (from *Artemisia annua* L.) or its byproducts to enhance patient devotion and reduce the risk of drug resistance. However, due to concerns that the unrestrained use of artemisinin could lead to drug resistance of malaria, the WHO has opted not to endorse non-medicinal forms (i.e., plant material) from *A. annua* for treatment of malaria [47] .

Growing integration of conventional and traditional scientific approaches in systems such as healthcare has not however been reflected in development initiatives and research. Key drawbacks include developing methods to effectively gather and synthesize existing knowledge regarding the use and scientific understanding of fungi and therapeutic plants, distinguishing between verified efficacy and anecdotal claims, and facilitating the discovery of possible new medications. Additionally, the worldwide plea for natural sourced medicines poses significant risks to certain species [38].

2.3 Threatened Medicinal Plants and Fungi

Research aimed at improving the medicinal species in prospect of survival is common in areas where basic healthcare depends on the plants. However, plant preservation literature typically centers on species endemic to a single country and those classified as threatened. In contrast, the literature on medicinal plant conservation often downplays the importance of plant endemism, prioritizing the preservation of medicinal plant populations within national boundaries, even when these species may flourish in other regions. This focus also emphasizes maintaining genetic diversity, which is not adequately reflected in existing extinction risk classifications [48]. These measures could improve the ongoing availability of important therapeutic compounds and help mitigate loss of biodiversity. Species that haven't been classified as Threatened or Near Threatened under the global IUCN Red List criteria may still face a significant extinction risk in the wild and warrant conservation attention, as well as research and prompt intervention. In fact, among five Chinese medicinal plants one of these deemed high priority for preservation do not meet the IUCN Red List criteria for being endangered [38].

2.4 Future Strategies for Utilizing Natural Products in Therapeutics

Recent advancements in, combinatorial chemistry, high-throughput screening and

molecular biology, along with changes in medicinal strategies driven by developing biological agents, have coincided through essential legislature aimed at biodiversity protection. Altogether, these factors have led to a decline in the discovery of drugs derived from natural products in recent decades [49, 50].

The contribution of plants and fungi to medicine today goes beyond the discovery of newly active tiny molecules and have continuously evolving role. One of the approaches to this evolution is drug repurposing, which involves assessing drugs approved for one therapeutic use for their potential applications in other areas. For instance, aspirin, which was originally derived from salicylates found in willow bark (*Salix spp.*), [51] serves as an analgesic, anti-inflammatory, antipyretic, and anti-platelet agent. It is now being explored for its potential in cancer therapies [52].

Compounds which are naturally derived are utilized in pharmaceutical manufacturing include shikimic acid, which serve as a precursor for the anti-influenza medication oseltamivir semi-synthesis [53] . Future medicine manufacturing may leverage additional fungal molecules and plant as precursors for the synthesis of drugs, complementing various medicinal strategies to address present and evolving global health challenges, for example the COVID-19 pandemic of 2020. Additionally, traditional herbal drugs had been utilized in 2003 to help and manage severe acute respiratory syndrome (SARS), which is another coronavirus, in China [41]. However, further studies are necessary to better assess their observed effects, as the clinical trials evaluating the efficiency of Chinese herbal medicines for severe acute respiratory syndrome faced methodological challenges [54, 55]. In this perspective, the role of traditional drugs in addressing worldwide health challenges warrants closer examination, encompassing their chemistry, pharmacology, safety, authentication and efficacy. The latter should be evaluated through clinical controlled trials to meet standards analogous to that of pharmaceutical drugs.

Furthermore, the untapped potential or "waste" of fungi and plants a currently utilized in non-medical industries might offer valuable molecules for pharmaceutical manufacturing, contributing to Sustainable Development Goal 12 (SDG12) on the efficient use and sustainable management of natural resources. A major example

includes sisal (*Agave sisalana* Perrine), whose plants parts such as leaves are used in the textile industry for fiber. And steroid compounds are obtained from the leftover waste (such as hecogenin), which make available the starting material for producing approximately globally 5% steroids to use in pharmaceutical industry, thereby enhancing the efficiency of this natural resource [56].

2.5 Advances in Biosynthetic Pathways of Medicinal Compounds

For some specialized metabolites plant biosynthetic pathways are often complex, lengthy, and extremely branched, with their control and modulation influenced via various factors that remain poorly understood. Recent progress in mapping the vinblastine pathway has significantly transformed our comprehension of specialized metabolism. Building on this knowledge, researchers have also delineated the metabolic pathways of other indole alkaloids, which has been studied for its potential impact on drug addiction [56] such as ibogaine from iboga (*Tabernanthe iboga Baill.*), and ajmaline from snakeroot (*Rauvolfia serpentina* (L.)), known for its antiarrhythmic properties. These investigations lay a crucial groundwork for future developments in synthetic biology, particularly in revealing the metabolic pathways of medically important indole alkaloids like the antimalarial quinine [38].

Himalayan mayapple (*Podophyllum hexandrum* Royle) is preferred for podophyllotoxin extraction for semi-synthesizing anticancer drugs like etoposide due to its higher podophyllotoxin content compared to American mayapple (*P. peltatum* L.). Though, trade in *P. hexandrum* is limited since its uninhabited populations are endangered. Identifying the genes involved in podophyllo-toxin biosynthesis and metabolic pathway reconstruction in *Nicotiana benthamiana* Domin. offers novel avenues for sustainable manufacture in fungi or plants [38].

Producing bioactive compounds using engineered fungal strains presents a sustainable and safe approach. Utilizing fungi like *Aspergillus oryzae* which is food grade addresses concerns about harmful mycotoxins associated with wild-type fungi dur-

ing fermentation. However, scaling up the production of new compounds in these fungal "cell factories" which are bioactive, demands important effort to achieve economic viability. A key challenge is developing tools to accurately predict enzyme specificities biosynthetic pathways. An alternative, commercial strategy may involve integrating this knowledge into semi-synthesis methods that combine fermentation-derived precursors with chemical modifications [57] .

2.6 Morphology of *Verbena tenuisecta*

The plant described is an erect or semi-erect perennial herb, standing between 20 to 35 cm tall, characterized by glabrescent, cylindrical stems. The leaves measure 1.5 to 3.5 cm in length and are tripinnate, divided into narrow linear segments with appressed hairs on the nerves underneath. The terminal spikes are simple and range from 6 to 12 cm in length, appearing congested when in flower but becoming elongated and loose in fruit. The flowers are purple and sessile, with lanceolate-linear bracts approximately 3 mm long. The calyx tube is about 8 mm long, cylindrical, with five teeth and appressed hairs. The corolla tube measures around 10 mm long, with a limb that is five-lobed, featuring obovate and emarginate lobes. The anthers are distinguished by appendaged connectives. The fruits are approximately half the length of the calyx [58].

Purple Moss Verbena plants grow as spreading perennials which display aromatic foliage along with eye-catching purple flowers. Its long blooming season creates an ideal choice to fulfill both purposes of ground cover usage and persistent erosion prevention. Colder growing areas allow gardeners to grow Purple Moss Verbena as merely an annual plant. When planted in containers or located at the front of mixed borders Verbena displays its vigorous nature. The historical traditions link Moss Verbena to mystical abilities and describe its use in Jesus's burial preparations. The drought-tolerance of Verbena makes it highly suitable as low-maintenance addition to any garden [59].

Verbena tenuisecta presents itself as moss verbena and exhibits hardy behavior

while displaying spreading properties with fern-like leaves that gracefully cover the ground. The plant presents plentiful purple flowers over the period between spring and late autumn. Due to its drought tolerance along with versatile soil adaptability this plant makes a superior choice as a garden addition. The vivid flowers of this plant attract many bees because they create a helpful ecosystem for pollinators in the area [60].

2.7 Pollen Characteristics of *Verbena tenuisecta*

- Type: Tricolpate
- Shape in Polar view: Semi-angular
- Shape in Equatorial view: Sub-prolate
- Polar diameter: 28.75μm-30µm)
- Equatorial diameter: (25- 30μm) 23.4μm (20-25μm)
- P/E ratio: 1.229 μm
- Length of colpi: 13.75 μm
- Width of colpi: 13.75 μm
- Exine thickness: 2.5μm
- Sculpturing : Reticulate [27]

2.8 Pharmacochemical Investigations on *Verbena tenuisecta*

The plant volatile oil assessment by GC-MS revealed a total of thirteen different compounds. The volatile oil contained 60% alcohol besides 16.55% bicyclic monoterpenes and 11.95% monocyclic monoterpenes and aromatics (6.69%) and

aldehydes (3.25%) along with a cyclic monoterpene (0.59%). 1-octen-3-ol accounts for 52.87% of the volatile compounds in *V. officinalis* volatile oil with bicycle [3.2.0] heptan-2-one-6-hydroxy-5-methyl-6-vinyl at 13.89% and limonene making up 9.33%. Researchers of 2003 detected 1-octen-3-ol as the primary substance in *V. officinalis* volatile oil yet the 1996 investigation revealed limonene as the main component in the volatile fraction of the same plant [33].

2.9 Solvent Extraction

Extraction is the primary method for obtaining bioactive compounds from biomass materials Extraction efforts focus on obtaining the highest possible quantity of target compounds and attaining outstanding biological features in the extracted substances. The extraction yield with biological efficiency of extracts heavily depends on the combination of extraction method with solvent selection choice [61].

The extraction of bioactive compounds from plant-based substances requires scientists to use different solvents which include ethanol, water, methanol and acetone. Plants alongside their particular extractable compounds need specialized solvent solutions to achieve maximum extraction because compounds demonstrate different solubility patterns during extraction with different solvents. The process of choosing proper solvents for plant material extraction becomes an difficult task because of these conditions [61].

The purification method known as solvent extraction uses the fundamental principle of molecular solubility differences between two non-mixable fluids to achieve separation of metabolites or metal compounds. The target substances separate efficiently through solvent-based distinction regarding their solubility levels [62].

Solvent extraction can be utilized to selectively remove or extract specific materials. It is also an effective method for enriching a particular component, whether in the extracted phase or in the residual phase (the material left behind). To achieve optimal results, it is essential to choose a solvent with a high affection for the target component(s) and a minimal affinity for the remaining materials. Selecting

a solvent that has an intermediate ability to extract the desired ingredient is not advisable, as this can lead to a situation where the solvent has affinities for both phases, increasing the risk of forming a stable emulsion [63].

The basis of solvent extraction relies on the fundamental chemical understanding that "similar substances will dissolve each other". The analyst employs an appropriate solvent for dissolving all ignitable liquids contained within the testing sample. Low-cost solvent extraction stands as a basic method requiring low-cost tools and it executes quick procedures. The method has demonstrated efficacy in extracted accelerants from fire debris through laboratory examinations. Among solvent selection criteria stands the effectiveness of extracting common ignitable liquids. The common origin of accelerants from crude oil hydrocarbon sources makes nonpolar solvents the most suitable extraction agents during analysis. The most frequently used solvents for separating mixtures consist of pentane, carbon disulfide alongside diethyl ether and various chlorinated solvents [64].

A wide variety of bioactive compounds have traditionally been extracted from natural sources using organic solvents such as hexane, ether, chloroform, acetonitrile, and benzene, often in various ratios with water. These organic solvents are effective for extracting both polar and nonpolar secondary compounds, including alkaloids, phenols, aromatic hydrocarbons, and pigments [65].

The advantages of solvent extraction surpass alternative processes because they provide both economical operation and affordable processing needs. The extraction method comes with disadvantages including the requirement of toxic solvents as well as extract recovery needing evaporation or concentration steps while using a significant amount of solvent for an extended extraction period. The prolonged extraction period leads to thermal compound degradation of target components despite the risks.

Conventional solvent extraction techniques include:

1. **Soxhlet Extraction**: Bioactive compounds extraction takes place through solid-liquid extraction inside a Soxhlet extractor by using volatile nonpolar solvents.

2. **Maceration**: The maceration procedure uses an economical blending method that involves mixing solids with solvents through grinding after which filtration separate essential oils and bioactive compounds using centrifugation.

3. **Hydro-distillation**: The hydro-diffusion of bioactive polar components happens through methods of water and steam distillation or direct steam distillation under the hydro-distillation process.

4. **Liquid-Liquid Extraction**: This technique relies on the segregating of compounds between two immiscible solvents based on their differential distribution according to polarity. Typically, one phase is aqueous (water-based) while the other contains an organic solvent, referred to as the organic phase [65].

Medicinal plants require specific solvents during extraction because these solvents determine both the available secondary metabolites as well as the obtained pharmacological activity. Numerous studies highlight how different solvents affect the substances of secondary metabolites and their antioxidant activity. Additionally, the extraction technique itself plays a critical role in the recovery of phytochemicals, with methods broadly categorized into conventional methods (such as maceration, percolation, and infusion) and advanced methods (such as microwave-assisted extraction, ultrasonic-assisted extraction, and supercritical fluid extraction). The solvent choice can further influence secondary metabolite content when advanced extraction methods are employed. Therefore, careful selection of both the extraction solvent and technique is essential to maximize the pharmacological activity of the resulting extracts [66].

Several factors determine the extraction of bioactive compounds from medicinal plants such as extraction time, temperature along with dried plant material size and plant-to-solvent ratio during extraction. To promote herbal medicine and enhance its acceptance within modern Western integrative medicine, it is essential to investigate and optimize extraction protocols. This optimization will ensure that a consistent daily intake of active components can be achieved in a reproducible manner, meeting the growing societal demand for herbal remedies [67].

The discovery of therapeutically active phytoconstituents begins with exploring medicinal plants and extracting bioactive compounds from them. Substantial progress has been achieved in the extraction, purification, and isolation of bioactive compounds based on their activities. Conventional solvent extractions are widely used to produce plant extracts due to their ease, efficiency, and broad applicability. Typically, plant extracts are prepared using a variety of solvents, each chosen to yield specific types of phytomolecules depending on the solvent's polarity. To optimize the biological properties of phytoconstituents, careful selection of extraction solvents and techniques is essential. Comparative studies examining the same plant extract using different solvents provide valuable insights into these variations; for example, water was found to be the most effective solvent for producing bioactive constituents from *Quercus infectoria* (manjakani) when compared to other solvents [68].

2.10 Ethyl Acetate as Extraction Solvent

The industrial applications of ethyl acetate extend because this environmentally friendly carboxylate ester solvent functions as a polar solvent able to dissolve nonpolar and polar compounds. The carboxylate ester contains an ethyl group that offers nonpolarity as well as a polar carbonyl group and oxygen atom which create polarity. This combination allows effective balancing between these two properties. Analysis centers rely on ethyl acetate for extracting and purifying compounds within challenging sample environments including biological solutions along with environmental and food samples. Its low boiling temperature makes solvent evaporation and final analyte recovery process operationally efficient [69].

2.11 Ethyl Acetate as a Solvent in Biosynthesis

Scientists use two types of ethyl acetate (Biosynthesis OmniSolv® and biotech grade) for extraction and purification of small molecules including natural products and metabolites derived from biological materials. The chemical serves as an opti-

mal reaction solvent because it maintains both the reaction process and enzyme stability intact. Beyond this, ethyl acetate finds applications in protein sequencing, as well as in peptide and oligonucleotide synthesis [69].

Ethyl acetate is an ideal solvent for extraction due to its low boiling point, which allows for easy evaporation and compound purification, and its low toxicity, making it a safer choice compared to other solvents. Its moderate polarity enables it to dissolve a broad range of polar compounds, making it versatile for various extraction needs. Ethyl acetate is also highly selective, capable of forming hydrogen bonds with certain functional groups to isolate specific compounds from mixtures. Additionally, it can dissolve both lipophilic and hydrophilic substances, making it suitable for extractions from diverse sources. Its affordability and recyclability further enhance its value as an efficient extraction solvent [70].

2.12 Methanol as Extraction Solvent

Methanol functions as a polar and universal solvent because it demonstrates powerful extraction properties towards several compositions. Methanol efficiently dissolves acidic and polar substances as well as organic compounds because of its dual polar and non-polar chemical properties. Due to its dual character methanol shows high flexibility in extraction applications because it extracts both polar substances along with non-polar components from diverse matrices starting from plant materials up to biological samples. The extraction of bioactive compounds relies on methanol because it enables the complete recovery of phytochemicals with different chemical natures. Methanol demonstrates universal extraction properties which make it vital in academic research as well as industrial applications [71].

The chemical compound known as methanol functions as wood alcohol and methyl alcohol because manufacturers employ it mainly to produce fuel and solvents along with antifreeze materials. Methanol exists as a colorless volatile substance which poses high toxicity to human beings when taken orally. Methanol displays value as a universal solvent within industry because it enables the production of numerous items such as inks and resins and adhesives and dyes. Methanol functions as a

pharmaceutical solvent for producing crucial pharmaceutical elements and ingredients including cholesterol and streptomycin and hormones and vitamins. The dissolving ability of methanol across broad chemical compounds makes it essential to chemical manufacturing together with pharmaceutical production lines [72].

Methanol is indeed a polar solvent and is highly soluble in water. Although it is not as polar as water, it still possesses significant polarity due to its hydroxyl (-OH) group, which can form hydrogen bonds with water molecules. This property allows methanol to dissolve a variety of polar and some non-polar compounds, making it a useful solvent in various chemical and industrial applications. Its solubility in water enhances its versatility, particularly in extraction and reaction processes where mixing with aqueous solutions is necessary [73].

Methanol is widely used for extracting secondary metabolites from medicinal plants due to its low boiling point, high volatility, and effectiveness. It also serves as a co-solvent to enhance extraction in supercritical fluid processes. While high-dose toxicity of methanol is well-known, the risks associated with lower-level exposure from herbal extracts are less clear. Notably, methanol is a natural byproduct of protein metabolism in the human body, with typical blood concentrations ranging from 2 to 30 mg/L. This underscores the need to balance the benefits and risks of methanol in herbal medicine [74].

2.13 Water as Extraction Solvent

Water is often referred to as the "solvent of life," essential for all known organisms and potentially crucial for unknown life forms elsewhere in the universe. It facilitates numerous physiological functions by dissolving various molecules, participating in vital metabolic pathways, maintaining acid-base balance, and supporting enzyme activity, thus playing a central role in cellular processes. Beyond its biological significance, water is also highly valued in laboratory and industrial applications, with many researchers deeming it the greenest solvent in chemistry due to its accessibility, low cost, and environmentally friendly attributes [75].

Since the 1980s scientific communities have increasingly adopted water-based solubilization and extraction methods as part of their sustainable chemical practices that obey green chemistry principles. Users find water attractive as an environment-friendly solvent because it is renewable and non-hazardous while being easy to manage and treat and handling-easily [75].

The extraction of phenolic compounds through water demonstrates preference because water offers safety combined with accessibility and affordability as a solvent. Aqueous solutions effectively break up phenolic compounds because its polarity bonds to many phenolic compounds recognized for their antioxidant properties along with potential health advantages. Heating water to 100°C increases compound yield but the industrial process becomes more expensive because it needs additional energy and complex temperature-controlling equipment. Efficient extraction needs to achieve cost-effective results during large-scale processes because temperature optimization remains crucial for both expense management and yield optimization [76]

Due to its low viscosity property water promotes more efficient extractions because it allows quick diffusion of extractable compounds including polyphenols through plant cell structures. The quick mass transfer action of this property permits the solvent to access plant material easily and dissolve target compounds successfully. Water is effective at extracting polar compounds with therapeutic and antioxidant characteristics because of its distinct hydrogen-bonding abilities. Its low viscosity not only speeds up the extraction process but also simplifies the handling and processing of large volumes of plant material, making water a practical choice for both laboratory and industrial extractions, where efficiency and cost-effectiveness are critical [77].

Water is regarded as the most environmentally friendly solvent due to its non-flammability, non-toxicity, and abundance. Although its natural dielectric properties restrict its extraction capacity to polar compounds, subcritical water extraction (SWE) overcomes this limitation by modifying water's properties under elevated temperatures (100°C to 374°C) and pressures. As the temperature rises, the dielectric constant of water decreases significantly, reaching levels similar to

ethanol at approximately 250°C, thereby enabling the dissolution of less polar compounds that would typically be inaccessible at standard conditions. Additionally, elevated temperatures reduce water's viscosity, enhancing its penetration into plant matrices, while increasing its ionic product above 10^{-11}, which facilitates the breakdown of complex molecular structures. This increased reactivity in the subcritical state supports the depolymerization of larger compounds, such as lignin and polysaccharides, allowing for the extraction of a wider range of bioactive compounds. Consequently, subcritical water extraction broadens the applicability of water as a solvent while preserving its environmentally friendly properties, making it a valuable method for the sustainable extraction of both polar and non-polar compounds from plant materials [78].

The increasing environmental pollution resulting from the extensive use of volatile and harmful organic solvents in the chemical industry has prompted chemists to prioritize green solvents that minimize their environmental impact. A "green" solvent is characterized by its low toxicity to health and the environment, affordability, and renewability, with water emerging as an ideal candidate due to its non-toxic, safe, and economical nature [79].

A distinctive advantage of water is its tunable properties under varying temperatures, which has led to heightened interest in its use as an extraction solvent. When employed in subcritical or near-critical states (ranging from 100°C to 374°C under controlled pressure), water's properties—such as polarity, viscosity, and ionic product—can be adjusted to dissolve both polar and less polar compounds. This versatility has fostered a growing body of research exploring water as a sustainable extraction solvent for a wide array of bioactive compounds from plant and other natural matrices. Consequently, water not only meets the criteria for a green solvent but also enhances its functionality through temperature-controlled property adjustments, making it a promising alternative in greener chemical processes [79].

The use of water as a solvent in the synthesis, purification, or extraction of natural products is garnering significant attention in various fields of modern chemistry, primarily due to its numerous advantages that promote sustainable practices. Firstly, water reduces environmental impact as it is non-toxic, non-flammable, rea-

dily available, and environmentally benign, making it a green alternative to traditional organic solvents. Its polarity, along with tunable properties under subcritical conditions, facilitates selective extraction of specific compounds through temperature and pressure adjustments, thereby enhancing extraction efficiency. Additionally, water typically requires simpler and less expensive equipment, which reduces the need for special handling protocols associated with hazardous solvents. Safety is another crucial factor, as water is non-toxic and non-volatile, posing no explosion or inhalation hazards during extraction processes. Furthermore, the properties of water, especially under subcritical conditions, enable rapid setup, reduced processing times, and a streamlined workflow that often eliminates the need for additional purification steps. Therefore, incorporating water as an extraction solvent not only aligns with green chemistry principles but also enhances the efficiency, safety, and cost-effectiveness of extraction processes in natural product chemistry [80].

Chapter 3

Materials and Method

3.1 Apparatus and Equipment

The apparatus and equipment used in this research include a spectrophotometer, rotatory flask shaker, incubator, electronic analytical balance, mechanical grinder, water bath, eppendorf tubes, test tubes, pipettes, muslin cloth, filter paper, funnel, beakers, vials and flasks.

3.2 Chemicals

Ethyl Acetate, Methanol, Distilled water, DPPH (2,2′−diphenyl−1−picrylhydrazyl), Gallic Acid, Ascorbic Acid, Folin−Ciocalteu reagent, Quercetin, Ammonium Molybdate, Phosphate Buffer Saline, Aluminium Chloride and Dimethyl Sulfoxide.

3.3 Sample Collection and Authentication

An intact herbaceous plant sample of *Verbena tenuisecta* originated from the Rawalpindi Region during June 2024 (Fig 3.1). The authenticity of the plant was verified by Plant Museum of Natural History, Islamabad. The voucher specimens with voucher number 047156 were also deposited in Plant Museum of Natural His-

tory, Islamabad (Fig 3.2).

FIGURE 3.1: *Verbena tenuisecta* collected plant sample.

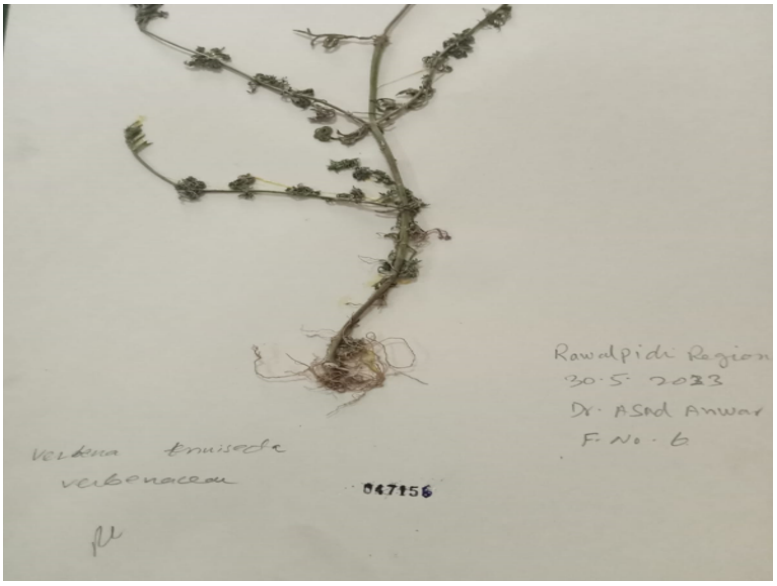

FIGURE 3.2: Voucher Specimen 047156.

3.4 Preparation of Sample

The fresh sample of *Verbena tenuisecta* was cleaned to eliminate any foreign residue in it and allowed for shade-dry for 7 days at room temperature. Then the dried sample was grinded into fine powder by using a mechanical grinder. The powdered material was stored in sterile bag for preservation in dry location.

The maceration method was used for extraction of plants. Ethyl Acetate, Methanol and Water was used as extraction solvents in ratio of 1: 5 for plant sample. In each 500ml bottle, 20g of plant powder and 100 ml of solvent was added and put them on the shaker for 7 days. The extracts were collected by passing them though muslin cloth followed by the filter paper. The ethyl acetate, and methanol extracts were collected in the beakers and left them to dry while the water extract was placed in water bath at 55°C for the solvent to evaporate. The resultant extracts were weighed using analytical weighing balance to calculate the percentage yield.

To determine the solvent efficiency, the percentage yield [81] will be calculated from the extracts by using Eq 3.1.

$$\text{Yield } (\%) = \frac{\text{Weight of dry extract}}{\text{Weight of dry sample}} \times 100 \tag{3.1}$$

3.5 Total Phenolic Content Determination

The Total Phenolic Content (TPC) was determined using a spectrophotometric method, beginning with the preparation of the sample and Folin-Ciocalteu reagent addition. First, a 200μl aliquot of the sample with a concentration of 1 mg/ml was mixed with 1.5 ml of Folin-Ciocalteu's phenol reagent and placed them in dark for 5 minutes. After allowing the mixture to sit, 1.5ml from 10ml of 6% sodium carbonate was added. The reaction mixture was then incubated in the dark for 90 minutes at room temperature to ensure complete color development. Following incubation, the absorbance of the solution was measured at 720 nm using a spectrophotometer [14].

To quantify the TPC, the absorbance values were compared to a calibration curve prepared from Gallic acid solutions of known concentrations, and the results were expressed as milligrams of Gallic acid equivalents (GAE) per gram of dried sample. To ensure accuracy and reproducibility, this procedure was performed in triplicate.

The Folin-Ciocalteu method is widely used for a reliable estimation of phenolic content, providing valuable insight into the antioxidant potential and other health-related properties of plant extracts [14].

3.6 Total Flavonoid Content Determination

The Total Flavonoid Content (TFC) was assessed using the following procedure. First, 1.5ml of methanol was added to a 10 ml test tube followed by the addition of 200μl of extracts, 100μl of 10% $AlCl_3$, 100μl of Potassium Acetate and 2.8ml distilled water. The reaction mixture was allowed to stand for 30 minutes at room temperature. The absorbance of the resulting solution was then measured at 415 nm against a reagent blank. To ensure accuracy and reproducibility, this procedure was performed in triplicate. A standard curve for total flavonoids were generated using a quercetin solution prepared in the same manner. The TFC was subsequently be calculated and expressed as milligrams of quercetin equivalents per gram of dried sample [82].

3.7 DPPH Radical Scavenging Assay

The free radical scavenging activity of the fractions was evaluated in vitro using the DPPH assay. To begin, a stock solution of DPPH was prepared by dissolving 3 mg of DPPH in 100 ml of methanol, which was then stored at 20°C until needed. A working solution was made by diluting the DPPH stock with methanol to reach an absorbance of approximately 0.7–0.8 at 517 nm, measured with a spectrophotometer. For the assay, 2 ml of the working DPPH solution was combined with 200 μl of the extracts sample at varying concentrations (1 μg/ml, 5 μg/ml, and 50 μg/ml). After extensive mixing of the reaction solution, it was placed in an incubator under dark conditions at room temperature for thirty minutes. The spectrophotometer measured absorbance of solutions at 517 nm following incubation time. A negative control sample was prepared similarly but without any added extract, was used for comparison. For the positive control ascorbic acid was

used at the same varying concentrations. The scavenging activity was estimated based on the percentage of DPPH radical scavenged as the following Eq 3.2 [83].

$$\text{Scavenging Effect } (\%) = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \times 100 \tag{3.2}$$

3.8 Protein Denaturation Assay

Protein denaturation assay was carried out using a following method. The plant extracts dilutions consist of varying concentrations (0.5mg/ml,1mg/ml and 1.5 mg/mL) and reference drug quercetin was used with same concentrations while Distilled water was used as control. From each concentration 200μl of extract was added followed by the addition of 2ml Albumin protein. The reaction mixture was incubated at 37°C for 20 min and then temperature was increased to keep the samples at 70°C for 5 min. After cooling, turbidity was measured at 660 nm using UV-visible spectrophotometer. The control represents 100% protein denaturation. The percentage inhibition of protein denaturation was calculated Using Eq 3.3.

$$\%\ \text{Inhibition of Protein Denaturation} = 100 \times \left[1 - \frac{A_2}{A_1}\right] \tag{3.3}$$

Where A1 = absorbance of the control, and A2 = absorbance of the test sample [84].

3.9 α-Amylase Inhibition Assay

The α-amylase inhibition assay was conducted using the 3,5-dinitrosalicylic acid (DNSA) method. Plant extracts were first dissolved in a minimal amount of 1ml DMSO and then further diluted it to 10, 50,200 μg/ml. To initiate the reaction, 20 μl of the prepared extracts was mixed with 200μl of PBS solution, 160μl of starch solution and 800μl of enzyme solution then incubated for 5 minutes at 27°C. The reaction was halted by adding 100 μl of DNS color reagent, which consists of 12 g of

sodium potassium tartrate tetrahydrate dissolved in 8.0 mL of 2 M NaOH and 20 mL of a 96 mM 3,5-dinitrosalicylic acid solution, followed by boiling for 15 minutes in a water bath maintained at 85–90°C. After cooling to room temperature, the mixture was diluted with 900 μl of PBS and the absorbance was measured at 540 nm using a UV-Visible spectrophotometer. A positive control was established using Berberine with same concentrations and following the same procedure as with the plant extracts. The α-amylase inhibitory activity was expressed as percent inhibition, calculated using the specified equation 3.4 [85, 86].

$$\alpha\text{-Amylase Inhibition } (\%) = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Control}}} \times 100 \tag{3.4}$$

3.10 Statistical Analysis

The experiments for total phenolic content, total flavonoid content, antioxidant (DPPH assay), anti-inflammatory (protein denaturation inhibition) and anti-diabetic (α-amylase inhibition) activities were carried out three times to guarantee precision and reliability. Each experimental data set was recorded and analyzed through Microsoft Excel software. The data representation included mean values along with standard deviation (SD) to show central trends and replicate variability. The evaluation of extract effectiveness for antioxidant and anti-inflammatory and anti-diabetic activities used percentage inhibition measurements. The IC_{50} values were determined through regression analysis of both DPPH and α-amylase inhibition data to quantify extract potency. Statistical methods enabled the researchers to assess different solvent extracts and determine which extraction method produced the most potent results [86].

Chapter 4

Results

4.1 Extraction Yield (%)

Total extraction yield (%) from all three extracts (Ethyl acetate, Methanol, and Water) is expressed in the Table No. 4.1. Percentage shows the total yield extracted from the extracts.

Table 4.1: Effect of solvents (Ethyl acetate, Methanol, and Water) on Extraction yield.

Extracts	**Extraction Yield (%)**
Verbena tenuisecta Ethyl acetate	1.25
Verbena tenuisecta Methanol	5.2
Verbena tenuisecta Water	7.15

Results from Table. No. 4.1 demonstrated a significant variation in the extraction yield obtained using different solvents, highlighting the impact of solvent polarity on the extraction process. Among the three solvents tested, ethyl acetate exhibited the lowest extraction yield, measuring only 1.25%. On the other hand, methanol, a moderately polar solvent, showed a considerably higher extraction yield of 5.2%, indicating its ability to dissolve a broader spectrum of phytochemicals, particularly phenolics and flavonoids. Water, a highly polar solvent, produced the highest ext-

raction yield, reaching 7.15%.

Results suggested that ethyl acetate being a non-polar solvent is less effective in extracting a wide range of bioactive compounds while water is the most efficient solvent for extracting bioactive compounds from *Verbena tenuisecta*, as it effectively dissolves polar phytochemicals. The extraction yield results indicate that solvent efficiency is directly influenced by polarity, with polar solvents demonstrating superior extraction capabilities. The high yield obtained with water recommended that many of the bioactive compounds in *Verbena tenuisecta* are more soluble in polar solvents, reinforcing the importance of solvent selection in optimizing extraction processes for medicinal plant research.

4.2 Total Phenolic Content Determination

The Total Phenolic Content (TPC) of extracts from three solvents (Methanol, Ethyl acetate, and Water) expressed in milligrams of Gallic Acid Equivalent (GAE) per gram of dry extract. Table No. 4.2 represents the significant difference in The Total Phenolic Content of *Verbena tenuisecta* extracts. Among the three solvents tested, methanol demonstrated the highest efficacy in extracting phenolics, yielding 192.72±11.76 (GAE/g) of extract. Ethyl acetate, a less polar solvent compared to methanol, exhibited a lower extraction efficiency, yielding 168.34±22.86 GAE/g of phenolics. Water, being the most polar solvent among the three, extracted the lowest amount of phenolics, with a yield of 161.69±1.39 GAE/g.

Results suggests that methanol is highly effective in dissolving and extracting phenolic compounds, likely due to its intermediate polarity, which allows it to solubilize both polar and moderately non-polar phenolics. While ethyl acetate is capable of extracting some phenolic compounds, it is less effective than methanol, and its higher variability suggests inconsistencies in its extraction ability. Water with the lowest phenolics extraction efficiency is likely due to the limited solubility of certain phenolics in highly polar solvents, as well as the potential loss of some compounds during the extraction process. These findings highlight the critical

role of solvent selection in optimizing the extraction efficiency and consistency of bioactive phytochemicals for medicinal plant research.

TABLE 4.2: The Total Phenolic Content (TPC) of extracts.

Extracts	**Total Phenolic Content (mg GAE/g of dry Extract)**
Verbena tenuisecta Ethyl acetate	168.34 ± 22.86
Verbena tenuisecta Methanol	192.72 ± 11.76
Verbena tenuisecta Water	161.69 ± 1.39

4.3 Total Flavonoid Content Determination

The Total Flavonoid Content (TFC) of extracts from three solvents (Methanol, Ethyl acetate, and Water) expressed in milligrams of Quercetin Equivalent (QE) per gram of dry extract (Table 4.3).

TABLE 4.3: The Total Flavonoid Content (TFC) of extracts.

Extracts	**Total Flavonoid Content (mg QE/g of dry Extract)**
Verbena tenuisecta Ethyl acetate	72.53±7.84
Verbena tenuisecta Methanol	32.74±5.78
Verbena tenuisecta Water	11.17±1.68

The data presented in Table No. 4.3 illustrated a significant variation in the total flavonoid content (TFC) of *Verbena tenuisecta* extracts, depending on the solvent used for extraction. Among the three solvents tested, ethyl acetate demonstrated the highest efficiency in extracting flavonoids, yielding 72.53±7.84 mg quercetin equivalent per gram (QE/g) of extract. Methanol, a polar solvent, extracted a moderate amount of flavonoids, with a yield of 32.74±5.78 QE/g. Water, being the most polar solvent, exhibited the lowest efficiency in flavonoid extraction, yie-

lding only 11.17±1.68 QE/g.

These results suggested that ethyl acetate, due to its intermediate polarity, is particularly effective in dissolving and extracting flavonoid compounds, many of which are moderately polar and more soluble in organic solvents. While methanol is known for its effectiveness in extracting phenolic compounds, its lower efficiency in flavonoid extraction compared to ethyl acetate suggests that some flavonoids may have better solubility in less polar environments. Despite its moderate performance, methanol still extracted a considerably higher flavonoid content than water. Water, being the most polar solvent indicated that most flavonoids in *Verbena tenuisecta* are either weakly polar or non-polar, making them less soluble in water. The low extraction yield suggests that water is not an ideal solvent for flavonoid extraction.

4.4 DPPH Radical Scavenging Assay

The results of DPPH Radical Scavenging assay are presented in the Table No. 4.4. The table shows the DPPH Radical scavenging percentage of extracts such as Ethyl acetate, Methanol and Water expressed at 1µg/ml, 5µg/ml and 50µg/ml concentrations. At different concentrations varying DPPH Radical Scavenging percentage is shown.

Table 4.4: DPPH Radical Scavenging Percentage of Ethyl acetate, Methanol and Water solvent extracts at 1µg/ml, 5µg/ml and 50µg/ml concentrations.

Extracts	**DPPH Radical Scavenging Percentage Concentrations**		
	1µg/ml	**5µg/ml**	**50µg/ml**
V. tenuisecta Ethyl acetate	7.76±1.82	9.40±1.22	13.90±0.90
V. tenuisecta Methanol	5.31±2.28	8.56±0.82	25.50±1.82
V. tenuisecta Water	5.01±0.09	6.25±1.26	7.14±1.55
Ascorbic acid	7.01±1.1	11.92±1.6	76.34±0.01

The results presented in Table No. 4.4 represented a significant variation in the antioxidant activity of *Verbena tenuisecta* extracts, with the scavenging percentage increasing progressively as the concentration of the extracts was elevated. At the lowest concentration of 1µg/ml, all three solvent extracts exhibited the lowest scavenging percentages, with ethyl acetate showing 7.76%, methanol at 5.31%, and water at 5.01%. This indicates that at lower concentrations, the antioxidant activity of all three extracts was relatively modest, with water exhibiting the lowest activity, followed by methanol, and ethyl acetate showing the highest scavenging percentage.

At the concentration of 5µg/ml, the scavenging percentages increased for all extracts, demonstrated a moderate improvement in antioxidant activity. The ethyl acetate extract exhibited 9.40%, methanol showed 8.56%, and water had 6.25% scavenging. Although all three extracts showed a noticeable increase in scavenging activity at this concentration, the pattern of their relative effectiveness remained consistent, with ethyl acetate still showing the highest scavenging percentage, followed by methanol, and water exhibiting the lowest.

At the highest concentration of 50µg/ml, a more distinct difference in scavenging activity was observed. The ethyl acetate extract reached 13.90%, methanol exhibited 25.50%, and water had 7.14%. This significant increase in scavenging activity, particularly for methanol, highlights the increased efficacy of the extracts at higher concentrations. Methanol, at this concentration, demonstrated the highest scavenging activity, followed by ethyl acetate, and again, water showed the lowest scavenging percentage.

Results suggested that the scavenging activity of all three extracts increased as the concentration of the extract was elevated, with methanol demonstrating the most pronounced antioxidant activity at higher concentrations. At 1µg/ml, ethyl acetate was the most effective, followed by methanol and water. However, at the 50µg/ml concentration, methanol showed the highest scavenging potential, followed by ethyl acetate, and water continued to exhibit the lowest scavenging percentage. These findings indicate that solvent choice and concentration both play a critical role in determining the antioxidant efficacy of *Verbena tenuisecta*

extracts.

4.5 Protein Denaturation Assay

Protein denaturation inhibition percentage of all three solvents extracts along with standard drug Quercetin was represented below at the concentrations of 0.5 mg/mL, 1 mg/mL and 1.5 mg/mL.

The protein denaturation inhibitory effects of the ethyl acetate extract increase steadily with rising concentrations (Fig 4.1). The extract shows increasing inhibition activity starting from 0.5 mg/mL and reaching its peak at 1.5 mg/mL. The anti-inflammatory potential of phytochemicals in the ethyl acetate extract grows stronger through time although the inhibitory capacity remains lower than the standard. The extract demonstrates promising but moderate effectiveness for protein denaturation prevention primarily through increasing concentration levels.

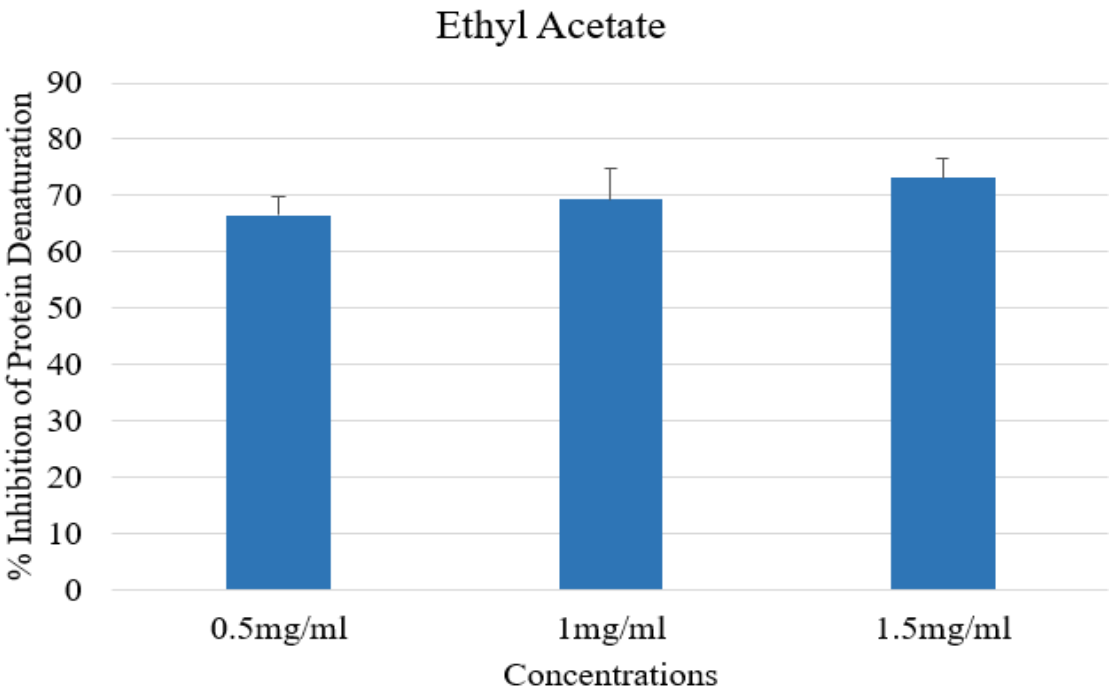

FIGURE 4.1: Protein Denaturation Inhibition by Ethyl Acetate Extract at 0.5 mg/mL, 1 mg/mL and 5 mg/mL.

Among the tested extracts the methanol extract demonstrates the highest capabil-

ity to prevent protein denaturation (Fig 4.2). The inhibitory activity of the extract increases steadily from 0.5 mg/mL to 1.5 mg/mL which demonstrates its strong concentration-dependent response. The methanol extract displays strong anti-inflammatory potential because it contains multiple potent bioactive compounds which become evident through its high inhibition percentages across all tested concentrations. The methanol extract demonstrates performance levels comparable to standard Quercetin at elevated concentrations which positions it as an excellent candidate for pharmacological research.

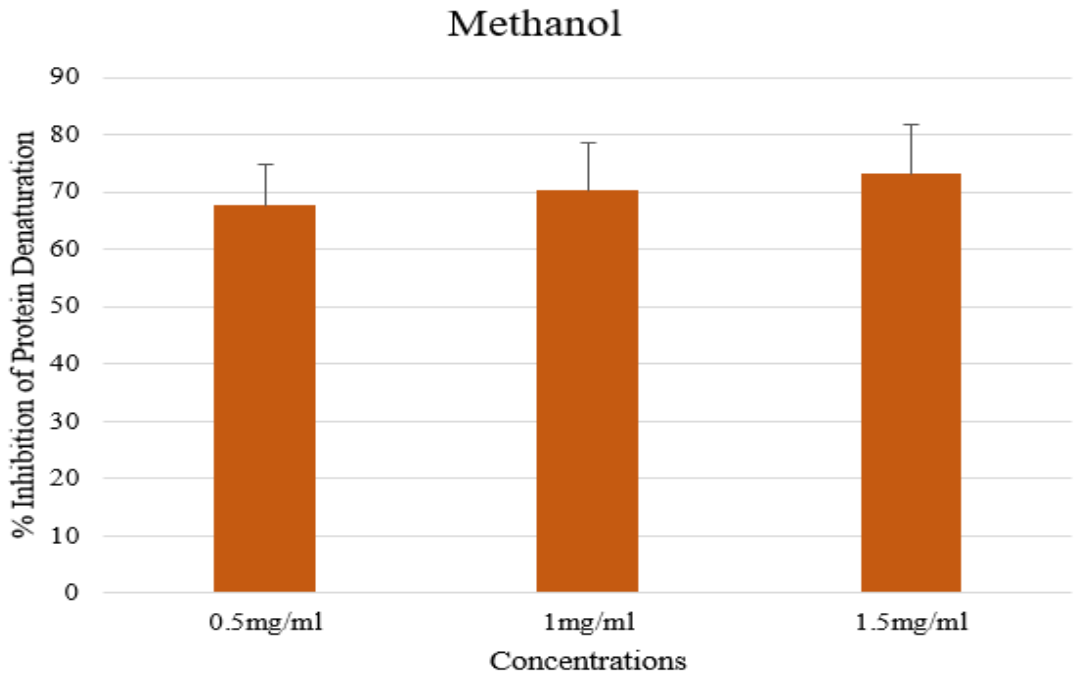

FIGURE 4.2: Protein Denaturation Inhibition by Methanol Extract at 0.5 mg/mL, 1 mg/mL and 5 mg/mL.

The inhibition percentages from water extract remain the lowest among the three different solvent extracts (Fig 4.3). The inhibition level of the extract at 0.5 mg/mL remains low but increases moderately at 1 mg/mL and further at 1.5 mg/mL. The inhibitory effect of the sample shows traditional dose-dependent characteristics yet maintains a consistently low inhibitory response. The water-soluble phytoconstituents in the extract show restricted capability to stop protein denaturation thus producing weaker anti-inflammatory effects than methanol and ethyl acetate extracts.

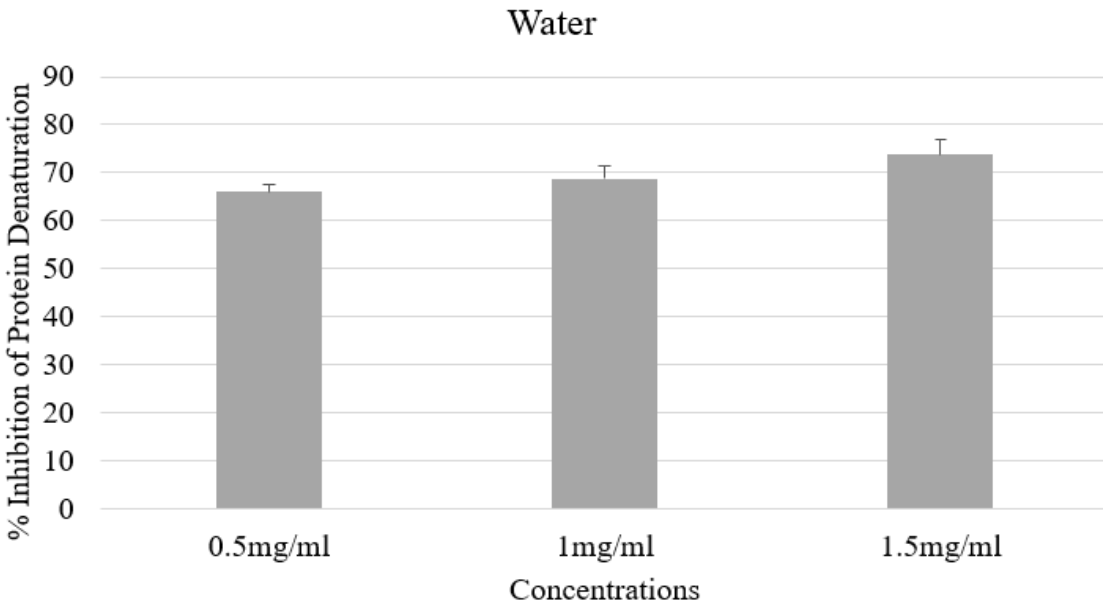

FIGURE 4.3: Protein Denaturation Inhibition by Water Extract at 0.5 mg/mL, 1 mg/mL and 5 mg/mL.

The reference standard compound quercetin shows very strong inhibition of protein denaturation across its entire concentration range (Fig 4.4). The anti-denaturation effects of the extract become more pronounced as the concentration increases from 0.5 mg/mL to 1.5 mg/mL where the peak activity is observed. The experimental findings strengthen Quercetin's potency as an anti-inflammatory drug and validate it as a standard measure to evaluate different plant extracts. The assay validity becomes stronger due to this substance's stable performance which demonstrates its potential therapeutic applications.

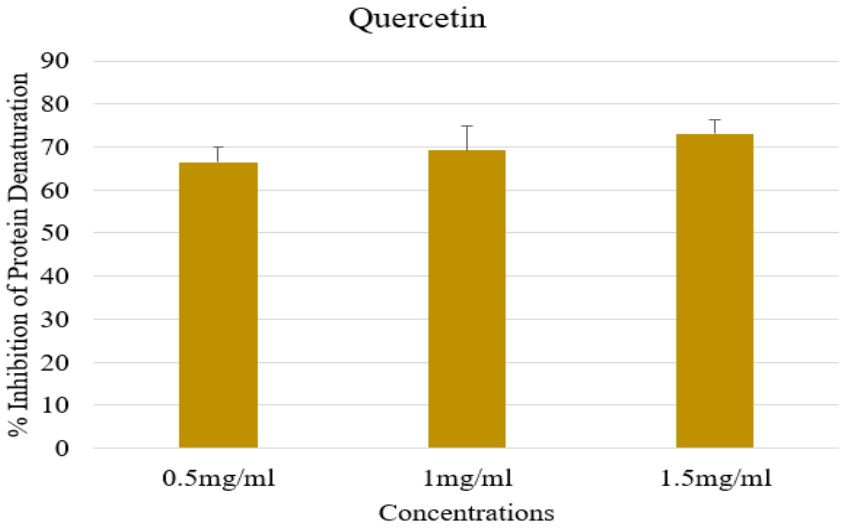

FIGURE 4.4: Protein Denaturation Inhibition by Quercetin (Standard Drug).

The graph illustrated the anti-inflammatory potential of *Verbena tenuisecta* extracts by measuring their ability to inhibit protein denaturation at a concentration of 1 mg/mL. All three extracts demonstrated appreciable activity, with variation in effectiveness depending on the extraction solvent used (Fig 4.5).

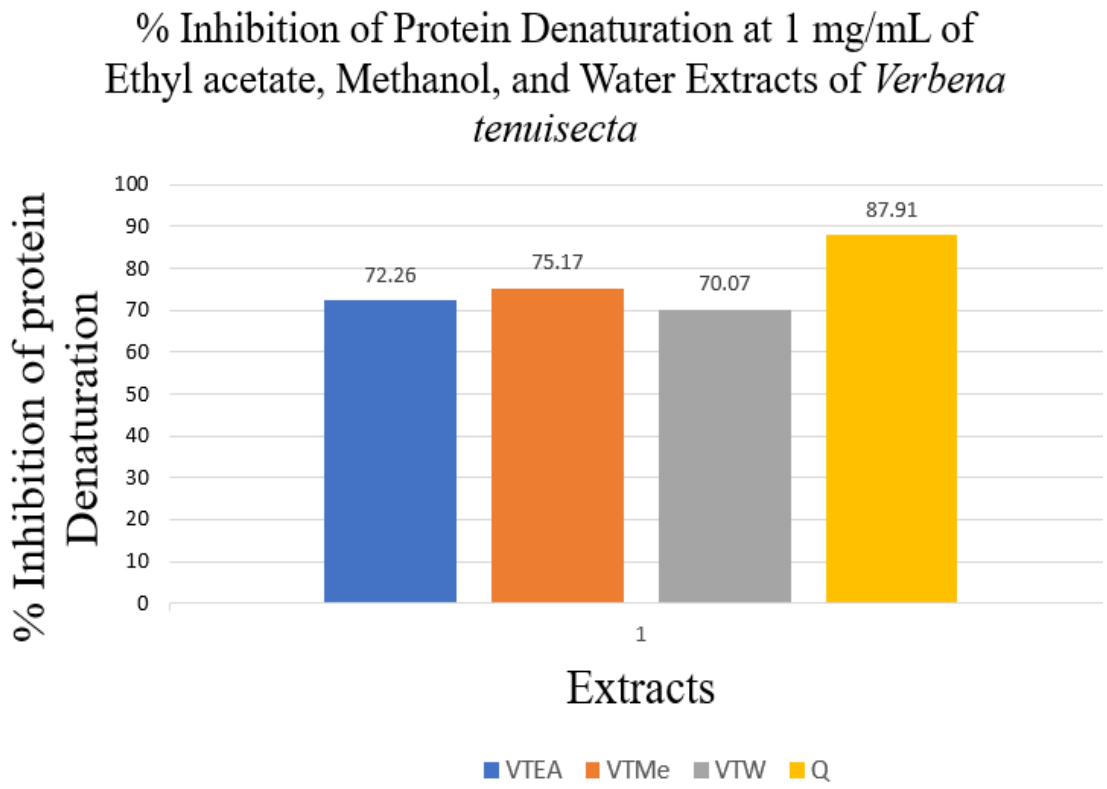

FIGURE 4.5: Inhibition (%) of protein denaturation at a concentration of 1 mg/mL for Ethyl acetate (VTEA), Methanol (VTMe), and Water (VTW) extracts of *Verbena tenuisecta*, compared to standard Quercetin.

The illustrated bar graph provides a well defined comparision of the protein denaturation inhibition activity of *Verbena tenuisecta* extracts in relation to the standard drug, Quercetin, which demonstrated an inhibition of 87.91%. In comparison, the methanol extract exhibited the highest level of inhibition at 75.17%, indicating that methanol was particularly effective in preventing protein denaturation among the three solvents tested. Ethyl acetate, while effective, showed a slightly lower inhibition at 72.26%, indicating that it also contributes to protein denaturation inhibition but to a lesser extent than methanol. Water, as expected, exhibited the lowest inhibition of protein denaturation at 70.07%.

This suggests that methanol has a strong potential for inhibiting the denaturation of proteins, possibly due to its ability to extract a broader range of bioactive compounds with anti-denaturation properties. The moderate inhibition from ethyl acetate suggests that it is somewhat less efficient than methanol in extracting compounds that prevent protein denaturation, although it still demonstrates significant activity compared to water. While water exhibited the relatively lower percentage suggests that water is less effective at extracting the bioactive compounds responsible for this activity. This finding is in line with the previous results indicating that polar solvents like water may not be as efficient as methanol and ethyl acetate in extracting compounds with strong protein-denaturing inhibition potential.

Results from the bar graph suggested that the varying degrees of protein denaturation inhibition among the different *Verbena tenuisecta* extracts, with methanol being the most effective solvent in this regard, followed by ethyl acetate, and water showing the least inhibition. These results emphasize the importance of solvent selection in extracting bioactive compounds with specific therapeutic properties, such as protein denaturation inhibition.

4.6 α-amylase Inhibition Assay

In the amylase inhibition activity, all the extracts of *Verbena tenuisecta* demonstrated positive results, indicating that each solvent used for extraction confined the proficiency of inhibiting the activity of the α-amylase enzyme.

Table 4.5 showed that *Verbena tenuisecta* extract solutions exhibited different levels of α-amylase inhibitory activity depending on the solvent used and concentration tested. All extracts demonstrated an increase in inhibition percentage as the concentration level increased which indicated concentration-dependent enzyme inhibition.

The inhibition activity remained low when using 10 μg/ml of all extracts as the primary substance. The methanolic extract achieved 17.47 $\pm$ 2.99% inhibition at

this concentration while ethyl acetate extract produced 10.095 ± 2.01% inhibition and the water extract reached 8.78 ± 2.71% inhibition. The standard inhibitor Berberine achieved the least enzyme inhibition at 3.4 ± 2.58% when tested at this specific concentration. The α-amylase inhibitory activity results demonstrated that methanol provided the strongest inhibition effects at lower concentrations among all the extracts.

TABLE 4.5: % Inhibition of α-amylase Assay of Ethyl acetate, Methanol and Water solvent extracts at 10µg/ml, 50µg/ml and 200µg/ml concentrations.

Extracts	**% Inhibition of α-amylase Concentrations**		
	10µg/ml	**50µg/ml**	**200µg/ml**
Verbena tenuisecta Ethyl acetate	10.095 ± 2.01	34.62 ± 1.26	40.80 ± 0.29
Verbena tenuisecta Methanol	17.47 ± 2.99	22.83 ± 0.58	36.67 ± 7.57
Verbena tenuisecta Water	8.78 ± 2.71	21.92 ± 4.71	36.46 ± 1.28
Berberine	3.4 ± 2.58	12.83 ± 3.33	34.55 ± 0.44

The inhibitory activity of all extracts increased when tested them at the medium concentration of 50 μg/ml. The ethyl acetate extract achieved the maximum inhibition of 34.62 ± 1.26% at this concentration followed by methanol extract inhibition of 22.83 ± 0.58% and the water extract inhibition of 21.92 ± 4.71%. Berberine showed 12.83 ± 3.33% inhibition. The inhibitory strength of ethyl acetate extract surpasses both methanolic and water extracts when the concentration rises thus achieving better inhibitory outcomes at moderate concentration.

The trend of increasing inhibition persisted up to the highest concentration level of 200 μg/ml. The ethyl acetate extract achieved maximum inhibition at 40.80 ± 0.2895% while the aqueous extract followed at 36.46 ± 1.28% and the methanol extract at 36.665 ± 7.57%. A single 200 μg/ml concentration of berberine produced 34.55 ± 0.44% inhibition against α-amylase activity. The results demonstrate that ethyl acetate extracted the most effective inhibition at high concentration but all three extracts achieved similar α-amylase inhibition at this level approaching standard compound activity.

These findings suggest that *Verbena tenuisecta* exhibits promising α-amylase inhibitory activity, which is solvent-dependent and concentration-dependent. Ethyl acetate extract was found to be the most effective overall, especially at moderate and high concentrations, making it a strong candidate for further investigation in the context of antidiabetic or carbohydrate metabolism-modulating therapies.

Chapter 5

Discussion

The present study was conducted to evaluated *Verbena tenuisecta* extraction with three different solvents and measured phenolic and flavonoid content delivered essential findings about suitable extraction conditions for these compounds while evaluating their antioxidant antidiabetic activity, protein denaturation assay and activity.

5.1 Extraction Yield (%)

The extraction efficiency of phytochemicals depends on their chemical nature in addition to the extraction procedure along with sample uniformity and solvent selection and potential interferences [87]. The extraction yield quantifies how well a solvent extracts particular constituent from the source material. It will provide insight into the plant's extractability under various circumstances [88].

The extraction yield varied greatly among the solvents. Water produced the highest extraction yield (7.15%), followed by methanol (5.2%) and ethyl acetate (1.25%) as shown in the Table 4.1. These results were consistent with earlier study by Ngo et al. [89] about polar solvents yield better results. The study demonstrated that methanol and water extraction yielded optimal phytochemical extraction results as opposed to low polar extraction systems [90].

A research [91] evaluated multiple solvents such as water, methanol, ethanol, and acetone to determine their effectiveness as bioactive compound extractors from Sudanese medicinal plants. Most plant samples showed better extraction results for Total Phenolic Content and Total Flavonoid Content through the use of methanol as the solvent [91].

5.2 Total Phenolic Content (TPC)

The majority of medicinal plants include phenolic compounds, which are vital to human nutrition because of their numerous health benefits, including antioxidants [91]. The phenolic components demonstrate antibacterial along with antioxidant and anti-inflammatory and antidepressant properties. The active plant compounds contain good reducing potential enabling them to function as effective antioxidants [92].

Quantification of phenolic compounds is dependent on a number of factors, including the chemical makeup of the compounds, the extraction technique, particle size, standard selection, and impurities and interfering chemicals. As analytical science has advanced, many techniques, including spectrophotometry, HPLC, GC, and their combinations, have been employed to quantify phenolic chemicals from plant sources [93]. Phenolic compounds are essential in the treatment of medical conditions like diabetes, cancer, and obesity and are also important for their inherent anti-inflammatory, antibacterial, and antioxidant properties [93].

The extraction of phenolic compounds yields better results when alcohol mixtures with variable water concentrations replace single solvents. The presence of a minor water amount in organic solvent creates a more polar extraction environment that enables polyphenol extraction [94].

The concentration of total phenolic contents value is expressed as mg of GAE/g of extracts as shown in the Table 4.2. The extracted materials that were analyzed had total phenolic levels ranging from 161.69 to 192.72 mg GAE/g. Because phenols are very soluble in polar solvents, there is a large concentration of these chemicals

in the extracts made with polar solvents [95]. Methanol was the best solvent when assessing the Total Phenolic Content (TPC), producing 192.72 mg GAE/gas opposed to 168.34 mg GAE/g for ethyl acetate and 161.69 mg GAE/g for water. The fact that methanol exhibits less variability implies that it consistently removes more phenolic compounds from the sample. Methanol serves as an ideal extracting solvent because it effectively dissolves molecules that range from polar to non-polar in nature. The known antioxidant properties of phenolics make these compounds extremely essential since they boost the overall health benefits of plant extracts. The high level of TPC that was achieved using methanol may be explained by the solvent's ability to make flavonoids' hydroxyl groups more soluble [94].

These results were confirmed by earlier research that also demonstrated that the extraction yields of TPC were greatly impacted by various extraction solvents [87, 89, 96]. Methanol is one of the best solvents for extracting TPC from plants, and numerous studies have shown that TPC depends on the polarity of organic solvents [94]. In a study, Methanol extracts produced greater TPC values compared to alternative solvents which demonstrates its superior capability to extract phenolic compounds [91].

The extraction using ethanol solution produced the highest total phenolic content for *C. carthagenensis* [97]. Ethanol serves as one of the main extraction solvents for plant polyphenolic compounds because it allows extensive solubility for various phenolic plant substances. According to Grujic et al., *I. paraguariensis* yielded higher phenolic compound yields with the utilization of polar organic solvents as extraction method [98]. The literature shows ethyl acetate functions weakly for extracting polar phenolic compounds [97].

5.3 Total Flavonoid Content (TFC)

Flavonoids are class of secondary plant metabolites with profound biological activities. The specific biological effects of flavonoids function based on their chemical composition and hydroxyl group variation [91]. Phenolic substances with free hydroxyl groups are responsible for the extracts' antioxidant, effects since they have

potent free radical scavenging properties [95]. The TFC of the extracts are reported in a Table 4.3. The ethyl acetate extract possessed the maximum TFC content amounting to 72.53 ± 7.84 mg QE/g whereas methanol extract contained 32.74 ± 5.78 mg QE/g and water extract had 11.17 ± 1.68 mg QE/g. Ethyl acetate shows exceptional performance in flavonoid extractions because it dissolves non-polar flavonoids better than both polar solvents methanol and water. The diverse flavonoid composition present in *Verbena tenuisecta* might explain the observed differences among ethyl acetate extracted products.

Previous investigations on *S. chinensis* fruit pulp together with *Limnophila aromatica* and Macadamia tetraphylla skin waste validated that extraction solvents substantially influence flavonoids [87, 99]. Compounds display choice-based solubility patterns due to their distinctive polarities in solution [89]. The determination of flavonoids in *M. peregrinum* plant extracts occurred through spectrophotometric assessment with aluminum chloride [95]. Each of the methanolic extract along with acetone extract and ethyl acetate extract showed maximum flavonoid levels. The petroleum ether solution in combination with water showed the least number of detected flavonoids. Plant extracts show varying levels of flavonoids depending on the polarity of solvents applied during their extraction process [95].

5.4 DPPH Radical Scavenging Assay

The scientific interest in antioxidant activity of plant species utilized in Phytotherapeutic medicine products has dramatically increased over the recent years. Phenolic compounds are known to have potential antioxidant qualities. Their effectiveness is closely correlated with the type of solvent used for extraction, as well as with the plant's origin, growth environment, harvest date, and storage circumstances [93]. The primary sources of the antioxidant potential are the phenolic compounds that are extracted from the *Lippia sidoides*, *Buchenavia tetraphylla*, and *Buchenavia tomentosa* species of medicinal plants [100].

The mechanism through which antioxidants secure DPPH free radicals represents an established pathway for lipid peroxidation inhibition. The widely applied analy-

tical method uses DPPH to determine antioxidant activities because it requires minimal time for testing. The antioxidant capacity of the extract increased proportionally with rising concentration levels [101].

Scavenging activity tests were applied to measure the extracts' antioxidant potentials of the extracts. The DPPH radical scavenging activity measurements of extracts from ethyl acetate (VTEA), methanol (VTMeOH), and water (VTW) solutions help determine their respective antioxidant potential [102].

The highest antioxidant scavenging effect among the extracts was observed in methanol solutions over 25% at 50 μg/mL; methanol proved itself as an efficient solvent for taking out antioxidant compounds. The antioxidant effects observed from ethyl acetate extracts were moderate (15% scavenging at 50 μg/mL) but lower activities appeared in water (10-12%) extracts at all concentration levels. The outcome demonstrates why solvent selection for antioxidant potential extraction matters because methanol proves to be the most effective solvent. The antioxidant activities between the used solvents demonstrated these results: Methanol > Ethyl acetate > Water.

Data demonstrated that higher extract concentrations caused better DPPH scavenging percentages because of dose-dependent antioxidant action. The observational pattern follows standard antioxidant principles which demonstrate that increased extract volume contains additional active compounds which effectively neutralize free radicals. Antioxidant compound yields together with their efficiency in solution depends heavily on the solvent's polarity according to analysis results. Altemimi et al. evaluated the antioxidant and antibacterial properties of *Punica granatum* peel extracts obtained through different solvents (ethyl acetate, acetone, methanol, and water) by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging technique [103].

Research data demonstrated higher decreasing properties in methanol extracts coupled with higher antibacterial effects in acetone extracts. Altemimi et al. examined *Oxalis corniculata* L. by using methanol solvent to evaluate antioxidant and anti-inflammatory effects [103].

5.5 Protein Denaturation Assay

Protein denaturation follows an unpredictable process that affects electrostatic hydrogen bonds and disulfide bonding and hydrophobic interactions [104]. Protein denaturation results in the elimination of biologically active characteristics from protein molecules [105]. The denaturation of proteins happens as an inflammatory response in rheumatoid arthritis and diabetes while also occurring in cancer which leads to inflammatory diseases [92]. Preventing protein denaturation can serve as an additional measure to stop inflammatory conditions from developing [105].

In vitro protein denaturation was used to evaluate anti-inflammatory activities [106]. Methanol extracts exhibited the most effective inhibition rate while ethyl acetate extracts achieved moderate and the water extracts obtained lowest. Both methanol and ethyl acetate extracts showed significant inhibitory effects, indicating that both solvents are effective at extracting bioactive substances that can stabilize proteins against denaturation as shown in Fig. 4.5.

Honari et al. [107] published research showing *Achillea wilhelmsii C.* has a potent anti-inflammatory effect. Sing et al. [106] revealed that methanolic extracts contained phenolic substances with both saponin and tannin components. 4-Hydroxy-3-methylacetophenone combined with 2-isopropyl-5-methylphenol in the methanolic extract inhibit protein denaturation thus preventing autoantigen synthesis. Multiple anti-inflammatory effects have been discovered within the roots of *M. siamensis* during the study [108]. Research suggests that the plant effectively inhibits protein denaturation like the well-known plant *C. adansonii* bark indicating its potential as an anti-inflammatory herb substitute for *C. adansonii* bark.

The findings of study [109] demonstrate that the extracts of *Adansonia digitata* and *Flueggea leucopyrus* leaves and *Solanum xanthocarum* aerial, *Adansonia digitata* leaf extract and *Flueggea leucopyrus* extract and *Punica granatum epicarp* extract exhibited anti-inflammatory properties against protein denaturation. Flavonoids and related polyphenols appear to be responsible for the observed activity. The

research [110] showed *M. siamensis* roots contain anti-inflammatory substances. The plant showed remarkable inhibition against protein denaturation similar to what *C. adansonii* bark does which suggests *M. siamensis* could be valuable as a plant-based anti-inflammatory alternative.

5.6 α-Amylase Inhibition Assay

Scientists have identified numerous plants as fundamental drug sources for effective antidiabetic treatment. The therapeutic use of medicinal plants for disease treatment including diabetes is now encouraged because these plants include antidiabetic phytoconstituents such as flavonoids, terpenoids, saponins, carotenoids, alkaloids and glycosides [111]. Some antidiabetic plants activate the βcells to increase their numbers or improve their function which results in elevated insulin release. Studies show that specific plants reduce the synthesis of blood glucose at the same time they work to decrease the production of blood glucose. Some plants lower blood sugar through decreased enzyme activity of glucose6-phosphatase, fructose 1,6-bisphosphatase and more which enables carbohydrate absorption to slow down while blocking glucose transport [112]. Multiple medicinal plants demonstrate antidiabetic properties that are utilized throughout various regions worldwide [113]. Scientific analyses indicate three major plant inhibitors that function in antidiabetic plants are terpenes, saponins, and polyphenols [114, 115].

Starch-digesting enzyme inhibition inside the intestine delays carbohydrate breakdown and minimized glucose absorption to lower blood glucose after eating represents a widely accepted method. α-amylase performs vital functions in starch breakdown making its inhibition crucial for controlling post-meal high blood glucose. Recent research investigates α-amylase inhibitor properties found in natural plant extracts because plants present abundant potential for therapeutic antidiabetic compounds [116].

The α-amylase inhibitory response exhibited positive results for all *Verbena tenuisecta* plant extracts while the inhibition level increased proportionally with extract concentration (Table 4.5). The extracts indicated ethyl acetate exhibited maxim-

um inhibition at 200 μg/ml (40.80 $\pm$ 0.29%) while methanol and water exhibited 36.67 $\pm$ 7.57% and 36.46 $\pm$ 1.28% inhibition rate respectively. The band of inhibition measured 17.47 $\pm$ 2.99% at the lowest test concentration of 10 μg/ml for methanol extract while water extract produced the weakest inhibition at 8.78 $\pm$ 2.71%.

The results demonstrate that extraction solvents at different polarities affect the quantity and efficacy of α-amylase inhibitory compounds extracted from *Verbena tenuisecta* where ethyl acetate and methanol provide better access to diverse active phytochemicals compared to water. The ethyl acetate extract demonstrated an enhanced anti-diabetic ability at greater concentrations which signifies its potential value as a therapeutic option. The *Verbena tenuisecta* extracts demonstrate great potential to act as inhibitors based on these results. This suggest that active compounds present in the extracts demonstrate potent inhibition of carbohydrates breakdown into oligosaccharides which supports the reduction of postprandial hyperglycemia effects. Additional research needs to focus on separating key compounds from the tested extracts that lead to alpha-amylase inhibition.

In the study [117], ethyl acetate fraction of *Scirpus holoschoenus* demonstrates maximum antioxidant properties as well as bacterial inhibition against *Staphylococcus aureus* and *Bacillus subtilis* which leads to minimal inhibitory. Research from Boussoussa et al introduced the findings on phenolic compounds and antibacterial activities that vary with seasons in *Rhanterium adpressum*. Methanolic extracts of *R. adpressum* harvested in April demonstrated their maximum phenolic content which corresponded to the measured amount of phenolic compounds in active extracts [118]. Research [116] evaluated the inhibition impact that raising plant extract concentrations from *Pelargonium* spp. had on α-amylase activity. The research evaluated the ability of *Pelargonium spp.* extract and *Rhus coriaria* extract to prevent α-amylase activity [116].

Relevant research evaluates the anti-diabetic properties of Sudanese medicinal plants [119]. Similarly, Houacine et al. had reported the anti-diabetic activity for the ethanolic extract of *G. senegalensis* [120]. The research study performed by Salehi et al. identified 703 plants that exhibited inhibition properties against α -

amylaseand/or α-glucosidase [111].

People in India employed raw fruits of *L. cubeba* for both hyperglycemia prevention and monitoring purposes. Bioactive methanol compounds from the extract blocked both α-amylase and α-glucosidase actions [121]. A laboratory study demonstrated that streptozotocin-induced diabetic rats benefited from antidiabetic activity obtained from *M. ferrea* flower methanol extracts. A mechanism was determined as part of these findings [122].

Chapter 6

Conclusion and Future Recommendations

6.1 Conclusion

This study concluded the comprehensive analysis of how different extraction solvents affect the phytochemical composition and biological activities of *Verbena tenuisecta* extracts. Choosing the right solvent was crucial in establishing the extraction yield, efficiency and concentration, of important bioactive substances, such as flavonoids and phenolics. These compounds are known for their significant therapeutic benefits, such as antioxidant, anti-inflammatory, and anti-diabetic properties. The research findings indicate that methanol was the most efficient solvent for extracting phenolic compounds and enhancing antioxidant and antiinflammatory activities. In contrast, ethyl acetate was more suitable for flavonoid extraction and anti-diabetic activity, while water yielded the highest overall extract quantity. These variations in bioactivity among different solvent extracts emphasize the critical importance of solvent selection in maximizing the medicinal potential of *Verbena tenuisecta*.

The results of this study suggested that Methanol is optimal solvent for obtaining phenolic content, antioxidant and anti-inflammatory activities while Ethyle acetate is effective for extracting flavonoids and ant-diabetic activities. By identifying the

most effective extraction medium, this research provides valuable insights into enhancing extraction techniques to enhance the therapeutic properties of *Verbena tenuisecta*. The ability to optimize extraction methods not only improves the bioavailability of beneficial plant compounds but also contributes to the advancement of natural medicine. These findings support the growing interest in plant-based treatments and reinforce the role of traditional medicinal plants in modern healthcare applications.

Furthermore, this study bridges the gap between traditional herbal medicine and scientific validation, reinforcing the importance of evidence-based approaches in natural product research. The insights gained from this research can aid in the development of effective natural remedies, functional foods, and pharmaceutical formulations derived from *Verbena tenuisecta*. However, further studies are necessary to explore additional extraction parameters, optimize processing conditions, and conduct in vivo experiments to confirm the plant's pharmacological potential. Future research should also investigate the synergistic effects of different phytochemicals present in the extracts to better understand their combined therapeutic impact. By expanding knowledge in this field, researchers can contribute to the development of innovative, plant-based treatments for chronic diseases, making traditional herbal remedies more accessible and scientifically validated for broader medical applications.

6.2 Future Recommendations

6.2.1 Implications for Herbal Medicine

These findings have implications for formulation of any herbal medicine or dietary supplement that is developed from *Verbena tenuisecta*. The present work identifies methanol as suitable solvent for determining phenolic content, antioxidant and anti-inflammatory activities and ethyl acetate for extracting flavonoid content and anti-diabetic activity from plant serve as a basis for future research on standardizing extraction procedures to obtain the highest therapeutic effects.

In addition, knowing which phytochemicals are soluble in various solvents may help practitioners determine which extracts provide the necessary health benefits. The data also mean that there is a key area of overlap of indigenous wisdom and modern evidence. Arising from this study is an endorsement of these historical extraction techniques as solvents with better therapeutic claims have been empirically identified.

6.2.2 Limitations of Future Research

It is pertinent, thus, to point out some studies that are on a definite note; nevertheless, research limitations are inherent in this study design. The present study was confined to the chosen set of solvents, but there remains potential to employ other extraction techniques including ultrasound assisted extraction or microwave assisted extraction in the subsequent investigation with the purpose of increasing the yield and the bioactivity.

Thus, future studies should focus on determining the combined influence between different phytochemicals found in the extracts from *Verbena tenuisecta* together with other herbs. There may be synergistic effects of the various phytochemicals in the food that translates to more potent biologic effects worthy of further study.

6.2.3 Integrating Evidence for Future Use in Traditional Medicinal Plant

Overall, this work clearly establishes extraction solvents as essential contributors to the yield and biological activity of *V. tenuisecta* extracts. This study reveals potential of Ethyl Acetate, Methanol and Water as extraction solvent which supports the need for provisions to be made to the selection of the solvent in preparing herbal medicines for treatment of illnesses. Subsequent research contributions must consider examining other unique extraction procedures that may be used for improving health related values of herbs and herbal products.

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