

FIRAT UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
TÜRKİYE



**IDENTIFICATION AND ISOLATION OF BIOACTIVE
COMPOUNDS FROM MEDICINAL PLANTS**

Aryan Fathulla QADER

Doctoral Thesis

DEPARTMENT OF CHEMISTRY

Division of Analytical Chemistry

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GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
T Ü R K İ Y E

Department of Chemistry

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THESIS APPROVAL

This thesis, which was prepared according to the thesis writing rules of the Graduate School of Natural and Applied Sciences, Firat University, was evaluated by the committee members who have signed the following signatures and was unanimously approved after the defense exam made open to the academic audience.

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DECLARATION

I hereby declare that I wrote this Doctoral Thesis titled “Identification and Isolation of Bioactive Compounds from Medicinal Plants ” in consistent with the thesis writing guide of the Graduate School of Natural and Applied Sciences, Firat University. I also declare that all information in it is correct, that I acted according to scientific ethics in producing and presenting the findings, cited all the references I used, express all institutions or organizations or persons who supported the thesis financially. I have never used the data and information I provide here in order to get a degree in any way.

10 July 2023

Aryan Fathulla QADER



PREFACE

The study, entitled "Identification and Isolation of Bioactive Compounds from Medicinal Plants," was presented as a PhD thesis at the Chemistry Department of the Graduate School of Natural and Applied Sciences at Firat University.

Foremost, I would like to offer my sincere gratitude to God for His continual mercy and grace, and for providing me with the strength and capability to finish this research even during challenging times.

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ELAIZIG, 2023

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ABSTRACT

Identification and Isolation of Bioactive Compounds from Medicinal Plants

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Plants have been demonstrated to be a valuable source of medication, and most plant parts contain bioactive components used in traditional therapies. Medicinal plants are rich in naturally occurring antioxidants and display potent antioxidant action. Several North Iraqi medicinal plants that have been used to treat different illnesses for a long time were tested for their natural phytochemicals and antioxidants.

Four medicinal plants that grow naturally in the northern Iraqi town of Koysinjaq/Erbil, including sumac, purslane, blackberry, and fenugreek, were collected in October 2020, extracted, and investigated. Characteristics such as phenolic, flavonoid, flavonol, anthocyanin, reducing chemicals, metal-chelating, and α -amylase inhibitory were determined for the methanolic plant extracts. Phytochemical analysis revealed that sumac had the highest amount of phenolics (138.46 mg GAE/g), fenugreek had the highest amount of flavonoids (16.11 mg QE/g) and flavonols (21.81 mg RE/g), and blackberry had the highest amount of anthocyanins (7.73 mg cy-3-glu/kg). Meanwhile, the antioxidant analysis showed that sumac had the highest ferric-reducing antioxidant power (92.31 mg QE/g) and metal-chelating (638.46 mg Fe^{2+} /g), while purslane extract most inhibited α -amylase (89.09%).

Different chromatography techniques started with thin-layer (TLC), column chromatography and high-resolution chromatographic techniques like HPLC were used in the investigation to isolate plants extracts, separate the distinct fractions of it and measure the amount of seven polyphenolic compounds in the medicinal plants. As this study proves, selected medicinal plants can be an excellent source of phytochemicals, and natural antioxidants can be used as a potential source of natural antioxidants in the food or pharmaceutical industries.

Keywords: Plants, Antioxidants, Phenolics, Isolation, HPLC-DAD, Chromatography

ÖZET

Tıbbi Bitkilerden Biyoaktif Bileşiklerin Tanımlanması ve İzolasyonu

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Bitkilerin değerli bir ilaç kaynağı olduğu kanıtlanmıştır ve çoğu bitki parçası, geleneksel terapilerde kullanılan biyoaktif bileşenler içerir. Şifalı bitkiler, doğal olarak oluşan antioksidanlar açısından zengindir ve güçlü antioksidan etki gösterir. Uzun süredir farklı hastalıkları tedavi etmek için kullanılan birkaç Kuzey Irak şifalı bitkisi, doğal fitokimyasalları ve antioksidanları açısından test edildi.

Kuzey Irak'ın Koysinjq/Erbil kasabasında doğal olarak yetişen sumak, semizotu, böğürtlén ve çemen dahil dört şifalı bitki Ekim 2020'de toplandı, ekstrakte edildi ve araştırıldı. Metanolik bitki ekstraktlarının fenolik, flavonoid, flavonol, antosiyanin, indirgeyici kimyasallar, metal şelatlayıcı ve α -amilaz inhibitörü gibi özellikleri belirlendi. Fitokimyasal analizler, en yüksek fenolik miktarının sumakta (138.46 mg GAE/g), en yüksek flavonoid (16.11 mg QE/g) ve flavonol (21.81 mg RE/g) çemen otunda ve en yüksek miktarda antosiyaninlerin (7.73 mg cy-3-glu/kg) böğürtlénde bulunduğunu ortaya koydu. Bu arada, antioksidan analizi, sumakın en yüksek ferrik indirgeyici antioksidan güce (92.31 mg QE/g) ve metal şelatlamaya (638.46 mg Fe²⁺/g) sahip olduğunu, semizotu ekstresinin ise α -amilazını (%89.09) en çok inhibe ettiğini gösterdi.

Araştırmada ince tabaka (TLC) ile başlayan farklı kromatografi teknikleri, kolon kromatografisi ve HPLC gibi yüksek çözünürlüklü kromatografik teknikler kullanılarak bitki özlerinin izole edilmesi, farklı fraksiyonlarının ayrılması ve tıbbi bitkilerde bulunan yedi polifenolik bileşik miktarının ölçülmesi sağlandı. Bu çalışmanın kanıtladığı gibi, seçilmiş şifalı bitkiler mükemmel bir fitokimyasal kaynağı olabilir ve doğal antioksidanlar, gıda veya ilaç endüstrilerinde potansiyel bir doğal antioksidan kaynağı olarak kullanılabilir.

Anahtar Kelimeler: Bitkiler, Antioksidanlar, Fenolikler, İzolasyon, HPLC-DAD, Kromatografi

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SYMBOLS AND ABBREVIATIONS

Symbols

- : Radical
- λ : Wave length

Abbreviations

- μg : Microgram
- AAI : α -amylase inhibition
- $^{\circ}\text{C}$: Degree of Celsius
- DAD : Diode array detection
- DCM : Dichloromethane
- EtOAc : Ethyl acetate
- EtOH : Ethanol
- FA : Formic acid
- FRAP : Ferric-reducing antioxidant power
- FT-IR : Fourier transform infrared
- g : Gram
- GAE : Gallic acid equivalent
- HPLC : High-performance liquid chromatography
- Kg : Kilogram
- L : Liter
- M : Molarity
- MCA : Metal chelating ability
- MeOH : Methanol
- mg : Milligram
- mL : Milliliter
- Mol : Mole
- MW : Molecular weight
- nm : Nanometer
- QE : Quercetin equivalent
- RE : Rutin equivalent
- R_f : Retention factor
- RNS : Reactive nitrogen species
- ROS : Reactive Oxygen Species
- SD : Standard deviations
- TAC : Total anthocyanin content
- TF : Total flavonol
- TFC : Total Flavonoid Content
- TLC : Thin Layer Chromatography
- TPC : Total Phenolic Content
- UV-Vis : Ultraviolet visible
- V : Volume

1. INTRODUCTION

Scientists, food suppliers, and consumers know that certain foods have ingredients that assistance the body work better and make people healthier. Hippocrates, 2500 years, said, "Let food be the medicine and medicine be the food," which is still true today [1]. History has shown that spices have played a significant part in the lives of humans throughout much of the world's prehistoric history. For millennia, spices have been used to taste and colour food and preserve it, as well as for their therapeutic effects, which have been well-documented in traditional medicine [2].

To begin, one might say that plants are the embodiment of life. All other life relies on them as a source of food and energy. Ecosystems and the climate are shaped and controlled by the actions of these organisms. A wide range of items is created from their raw resources, including food, clothing, and shelter. These advantages are well-known, but they are not well understood. The variety of life on Earth depends on plants, which are also significant economic resources for human survival [3].

Plants are the most crucial aspect of the Earth's life, and all other living species rely on them for survival. Food comes from plants, which use sunlight to transform it into chemical energy and produce a primary source. Every living entity uses this energy for its life cycle, internal metabolism, mobility, and defence against external circumstances and other living species. Green plants are the only connection between human populations and solar energy. This energy is used indirectly in animal milk, meat, and leather. In addition to producing food, plants help humans and other creatures in various ways. They offer an environment for wild creatures to live and breed [4].

Plants produce various types of organic compounds but do not seem to contribute directly to their ability to grow and develop. These substances are referred to as natural products, secondary products, or secondary metabolites. Secondary products differ from primary products (proteins, carbohydrates, nucleotides, and lipids) because their distribution within the plant kingdom is restricted. Specialized natural products are typically limited to one specific plant species or a few closely related species, while primary products can be found in many different plant species [5].

People with complex medical issues must seek medical professionals in traditional and contemporary medicine. Many traditional healers have a particular area of focus. Almost every community has a traditional birth attendant. Bone setters and experts in mental and chronic disorders are two more types of traditional healers that practice their craft. The activities of healers are not always restricted to addressing body ailments. Treating a sickness is sometimes seen as a religious or social practice because of the belief that social and religious conflicts are to blame for the illness. When it comes to the healers' treatments, they keep their methods secret and refuse to share them. Only the healer's family members may pass them on, and the healer must be

compensated for his services. The patient's socioeconomic standing often determines the cost of therapy, and as payment, animals such as chickens and goats are commonly accepted [6].

"Medicinal plants" refers to various parts of plants, such as leaves, roots, flowers, fruits, and seeds, which can be used in fresh or processed forms, including extracts and compounds, for herbal remedies, pharmaceuticals, veterinary medicine, and human medicine. Stimulants, condiments, spices, essential oils, and other high-level plant life generate distinct effects on cell metabolism and are intimately connected to these plants. Plants with medicinal properties were among the first to be employed by humans. According to legend, eastern nations were believed to be exceptionally rich in plant variety and understanding of their use in medicine, even though most countries had national formularies [7].



2. MEDICINAL PLANTS

The phrase "traditional medicine" encompasses scientific knowledge and beliefs documented in written sources concerning the origins of illness. Examples of traditional medical systems include Ayurveda, which started in India; Unani, which developed in the Arab lands; and traditional Chinese medicine [6]. Herbal medicine has a long history in China, India, and many African and South American nations, and it is still widely used today. Medicinal plants are often found at village markets with other goods like vegetables [8].

Traditionally based, medicine has been acknowledged by the World Health Organization (WHO) for many years as a vital part of health care as a preventative, curative, and rehabilitation modality that is typically well-established. To what extent traditional medicine may be incorporated into or supplemented by an allopathic health care system depends on the country's history and development. For traditional and allopathic medicine to function together in a healthcare system, it must also be founded on research; the WHO acknowledges the evidence-based approach [9].

Over the past few decades, there has been an increase in the worldwide use of medicinal herbs. The World Health Organization states that 80% of the global population utilizes herbal medicines in forms such as teas, decoctions, or extracts with common liquids such as water, milk, or alcohol. Despite the widespread use of modern synthetic pharmaceuticals in industrialized countries, herbal medicines are widely accepted in the West. There is a persistently strong demand for plant materials and extracted natural products. It is essential to highlight that those plants represent only a tiny portion of the emphasis on biotechnology and genetic modification. Today's approaches to therapeutic plant biotechnology focus more on specific natural compounds and biochemical pathways. To a large extent, this is since patients and customers are unwilling to use drugs made from genetically modified plants. Many patients believe that items derived from nature are safe; hence, genetically engineered plants are not included in their view of phytotherapy. Thus, therapeutic plant biotechnology focuses more on natural compounds and biosynthetic processes [10].

Typically, medicinal plants are gathered from the wild, resulting in habitat damage and decreased genetic diversity that cannot be replaced. Cultivating medicinal plants systematically has been promoted and carried out as an alternative to harvesting them from their natural habitats. Systematic cultivation offers these benefits: (i) increased yield and quality, (ii) reduced risk of misidentification, (iii) reduced genetic and phenotypic variability, (iv) elimination of contaminants, (v) reduced variability and instability of extracts, and (vi) ample availability in large quantities while protecting the environment. Compound spectra from medicinal and aromatic plants (MAPs) grown in a lab may occasionally diverge significantly from those in wild populations. Molecular marker-aided breeding has been widely utilized to increase agronomic and medicinal features with

significant investment returns to achieve top germplasm with improved attributes. On the other hand, biotechnological treatments have been seen as the only way to fulfil the ever-increasing need for MAPs in the future [11].

Residents, village grocers, and practitioners harvest medicinal herbs in the area. Traditional wisdom and not scientific understanding are used to harvest therapeutic herbs. To maximize active ingredients and ensure the long-term sustainability and viability of resources, most people lack the understanding of when and how to harvest the best quality specimens. Sunlight and shade are used to dry the plants after they are harvested. Drying flowers and leaves usually take 4–8 days in the sun. The plants are dried by laying them out on a cloth or plastic sheet on the ground, allowing them to decompose. Some plants may also be eaten raw. The specimen being dried is often contaminated throughout the drying process, which results in a simple drying procedure. Plant material is often not kept. However, some plants with significant therapeutic potential are kept in cotton bags and plastic or glass bottles for future use [4].

Many medications are derived from plants. The foundation of these medicines is based on rigorous or straightforward observation of people, culture, or animals or an ethnobotanical study. From the *Cinchona officinalis* L. tree in South America, Cinchona bark was one of the earliest plant-based remedies used in the Western world. Only when the Spanish explorers recorded the native Andean people's usage of this bark did it become immensely prized in Spain as a potent fever remedy. Artemisinin from the *Artemisia* genus is a frequent therapy for cardiac problems, and *Catharanthus roseus* (L.) G. Don for leukaemia is another modern remedy derived from plants [12]. There are countless cases of indigenous peoples using plants that piqued the curiosity of naturalists or scientists, resulting in the "discovery" of a new medication. The diversity of plants shows the breadth of possible chemical compounds and the possibilities of human-beneficial bioactivity [13]. Herbal medicines are often found in countries with a long history of culture and an extensive range of flora. "Biodiversity prospecting may help considerably with the ecologically sound development and possibly disseminate beneficial medical care to people globally," provided research on medicinal plants is conducted ethically and scientifically correctly [14].

The pharmaceutical sector is interested in many natural goods, not only plants. The fact that 25% of all medications prescribed in the United States throughout the 1970s included chemicals derived from higher plants supports this claim [15]. Furthermore, 11% of the 252 WHO-designated "basic and essential" medications are extracted and utilized from plant sources. As a result, over 40% of the pharmacological compounds employed in synthesized medicines today come from natural sources [16]. Only 10% of the medical plant species are grown in labs, with the vast majority coming from natural sources [10].

Concerning plant species' active components, the place, period, season, and harvest method all impact the quality and quantity. Nevertheless, it is required to standardize a medicine before it

may be marketed. The standardization techniques are tested by phytomedicines that include complex mixes of chemicals, some of which are at low concentrations. Currently, phytomedicine quality control focuses on the chemical characterization and identification of a particular constituent responsible for the pharmacological activity [17]. Identifying the compounds in a specific phytomedicine is essential to guarantee accurate medication identification. Most of today's pharmaceuticals may be traced back to plants, at least in theory. Drugs made from plant compounds are very harmless. There are no unlicensed or unprotected pharmaceuticals in the current world. The plant ingredient employed is referred to as a drug for ease of reference. When the plant is harvested, the medication can be extracted from it. Separating the plant's active ingredient from the rest is combined with additional compounds to create pharmaceutical products for the intended use [9].

2.1. Bioactivity of medicinal plants

The term "bioactive" comprises two parts: "bio" and "active". The word "bios" in Greek originates from "life", while the Latin word "activus" means "capable of action", "dynamic", or "lively". According to the medical lexicon, a bioactive component is a material that impacts, causes a reaction, or triggers a response in living tissue [18]. Secondary metabolites generated by plants, known as bioactive chemicals, may directly or negatively impact people and animals, depending on the dosage and bioavailability. These secondary metabolites result from primary metabolism and help plants adapt to environmental stress by enabling interaction with their surroundings [19]. Plants are unaffected by their absence since these bio-compounds act as defence molecules. In humans, these chemicals may have a positive impact on health. Studies on plant-based diets have demonstrated a preventive impact [20] on cardiovascular disease, cancer [21], osteoporosis, breast cancer [22], and other chronic conditions [23].

Every portion of the plant cell produces bioactive chemicals concentrated in a single secretory tissue. Biocomponents of varied chemical characteristics can be produced in higher quantities by combining cells from different organisms. They can only be generated under certain circumstances (during the development phase) and seasons, making extraction and purification challenging [24]. The primary phases of using plant-derived bioactive chemicals are extraction, screening, isolation, characterization, toxicology, and clinical assessment [25]. There are three primary subgroups of secondary metabolites in plants, each with its unique classification system [26]: alkaloids, phenolics, and terpenoids. Subgroups have been created within these primary groups (see Figure 2.1) It has also been suggested that these secondary metabolites have been used as food additives to enhance food functioning [27].

Interest in the creation of novel plant-based medicines has increased recently. Despite remarkable improvements in synthetic chemistry, natural products play a significant role in

developing novel pharmaceuticals in many nations and for various biological activities. Biological screening of plants is conducted as the first stage in searching for a plant. There are three essential steps in creating a new drug from plants; the first is to uncover new pharmaceuticals, the second is looking for lead compounds that can be converted into new drugs and finally is to give a justification for the clinical usage of the conventional drug [7].

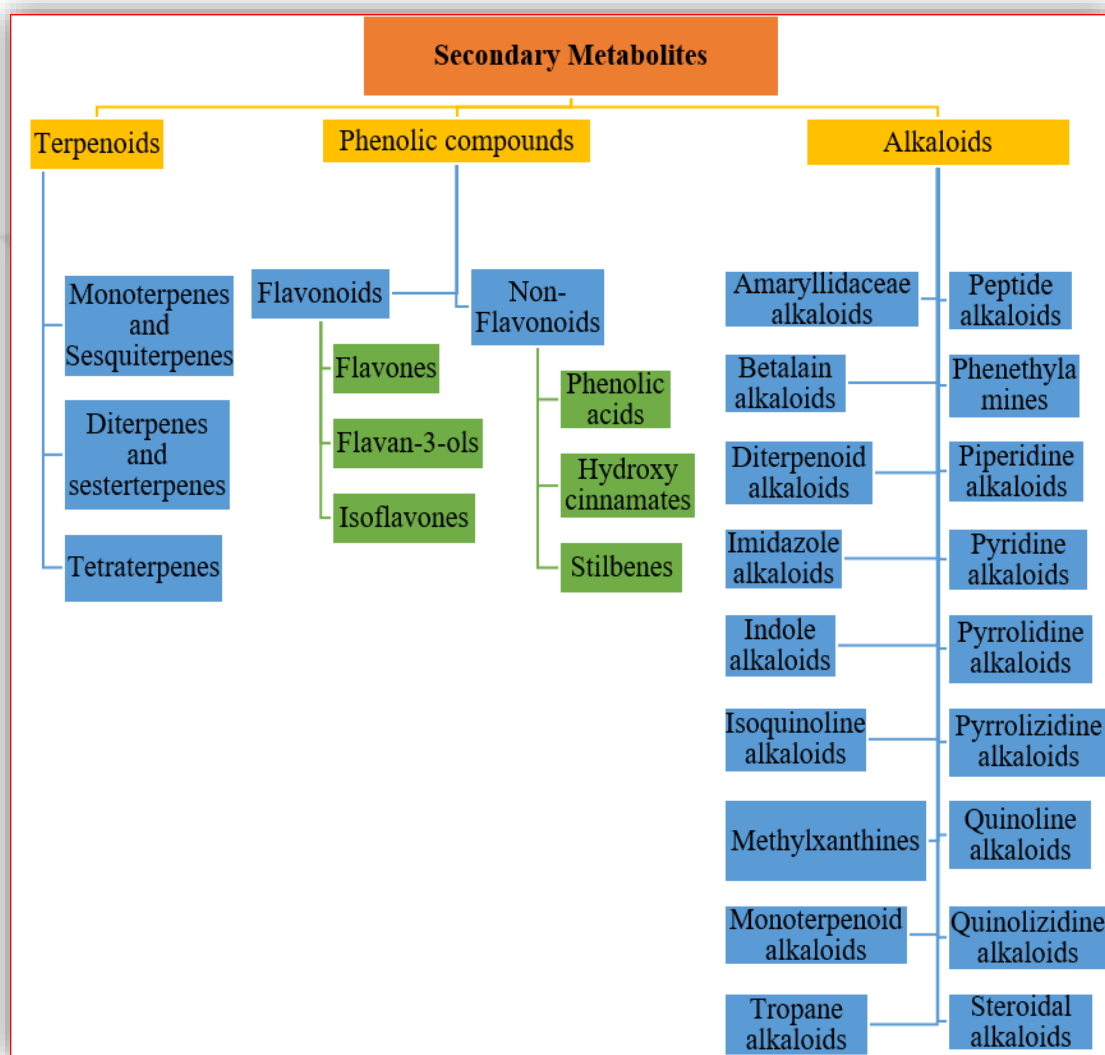


Figure 2.1. Plant-based secondary metabolite classification [18]

Plants produce a wide variety of bioactive compounds, giving them a valuable source of potential medications. Human health has relied heavily on the healing properties of higher plants since the beginning of time [28]. The pharmaceutical industry relies heavily on natural ingredients in its medication development efforts [29]. Manufacturing synthetic pharmaceutical goods is inadequate for preventing and treating the most common illnesses of this century. This constraint

necessitates discovering novel chemical structures, which naturally leads every researcher to focus on the kingdom of plants. An ethnobotanical analysis is a popular strategy for selecting plants for pharmacological investigation [30]. There is still a need for rigorous scientific research of traditional medicinal practices to conserve important therapeutic information and enhance scientific proof of efficacy [31].

Secondary metabolites are the primary source of bioactivity. Many different microorganisms and microbes are susceptible to secondary metabolites because of their broad spectrum of antimicrobial activity. These secondary metabolites are what give the plants their therapeutic qualities. These metabolites stop harmful organisms from growing by directly interacting with them. The active principles may not directly harm the infection but may encourage the synthesis of the body's antimicrobial agents. The drug's mechanism of action is a multistep process in the body. Thus, the plant extract's pharmacological effects may differ *in vivo*. Personal history, environmental factors, and the resistance of other symbiotic microorganisms may all play a role in a patient's ability to be cured [9]. Novel antimicrobial molecules may be derived from plant extracts, particularly those effective against bacterial infections. Screening procedures could be more effective if crude plant extracts are used instead of purified chemicals extracted from natural goods [32].

2.2. Antioxidants

A commonly accepted definition of antioxidants is that stable and harmless chemicals strongly inhibit oxidants, particularly at low concentrations [38]. Antioxidants play a crucial role in our lives by neutralizing "reactive oxygen species" (ROS) or free radicals, thus protecting cells from harm. They are substances that slow down lipid oxidation reactions in food. Antioxidants are defined as substances that prevent or inhibit oxidation or oxygen-driven processes and many preservatives used in products are antioxidants. They can be natural or artificial compounds added to items to prevent degradation caused by air oxygen, such as enzymes, vitamin E, or beta-carotene. Antioxidants are chemical substances that attach to free oxygen radicals, preventing them from causing damage to healthy cells [33].

The body continually creates free radicals due to regular oxygen usage [34]. Oxygen is a vital component of all living organisms. The mitochondria generate free radicals when cells utilize oxygen to generate energy. Cellular redox primarily generates ROS and RNS. Free radicals attack the four types of molecules: lipids, proteins, carbohydrates, and nucleic acids [35].

Scientists in nutrition, biomedicine, and food production are increasingly interested in antioxidant-rich phytochemicals and agronomic food crops. Natural plant compounds known as antioxidants and phytochemicals are widely distributed worldwide. Allelochemicals are mostly phenolic derivatives with polyhydric or monohydric phenols in their structure [36]. Antioxidant

phytochemicals have been shown to prevent chronic and cholesterol-related disorders caused by oxidation. In recent years, phytochemicals found in various farm-based nutrition plants have been extensively examined for their antioxidant and fitness benefits. The antioxidant action of phytochemicals is believed to be mediated by the presence of phenolic rings or hydroxylated aromatic rings. In plants, they are widely spread and produced through chorismite biosynthesis and shikimate from the crucial precursor L-phenylalanine [37].

The body established an antioxidant defence system to manage increased levels of reactive species (see Figure 2.2) [38]. To put it another way, sustaining homeostasis requires the presence of an internal mechanism capable of scavenging free radicals and ensuring that the generation and elimination of reactive species are effective in equilibrium. Oxidative stress may be prevented by antioxidants, which control the redox state of biological systems. Antioxidants are vital to keep in mind because they prevent reactive species from forming uncontrollably. Also, the low concentration of antioxidant exposure to oxidized substances is delayed, controlled, or eliminated, counteracting the oxidative degradation of biological structures and biomacromolecules. This inhibitory function may counter internally damaging reactive species to avoid damage and minimize illness symptoms [39]. The antioxidants fall into the following categories: direct antioxidants with a radical-scavenging effect (SOD/O₂^{•-}) or non-radicals (Catalase/H₂O₂) [40]. The primary antioxidant enzymes are directly involved in ROS neutralization [41]. Other indirect antioxidants that influence the cellular sources of free radicals (chelators/metals, apocynin/Nox), or the cellular antioxidants (Sulforaphane/Nrf2 targets-GSH), are other kinds of antioxidants [38].

Reduced ROS production may be achieved using metal-chelating proteins like metallothionein to bind to metals. Other dietary sources of antioxidants (vitamins and carotenoids) exist. Low molecular weight indirect antioxidants (vitamins C and E, β -carotene, uric acid, and GSH) scavenge ROS. Inhibition of lipid peroxidation is an essential purpose for vitamin E [42]. In normal circumstances, the actions of intracellular antioxidants are in equilibrium. This equilibrium is critical to the survival and well-being of creatures [43].



Figure 2.2. Role of antioxidants [33]

2.3. Polyphenolic compounds

"Phenolics" is often used when discussing a medicinal plant's active ingredients. As a result of their significance in atherosclerosis, stroke, cerebral thrombosis, diabetes, cancer, Alzheimer's, and Parkinson's, these antioxidants have been elevated to pharmacologically active components [44]. Natural and synthetic antioxidants are both subcategories of antioxidants. The vast majority of synthetic antioxidants are phenolic. Because of their chemical structures, many antioxidants have different physical characteristics, such as volatility, solubility and thermal stability. Natural phenolic chemicals, found in abundance in plants, play a significant role in food's antioxidant properties [45].

Phenolics are the most abundant and diverse secondary chemicals. Many structures are classified as phenolic because of a benzene ring with a single hydroxyl group. The majority of the over twenty-five thousand known varieties of phenolics are effective antioxidants and are often

employed as preservatives; in plants. They have inhibited membrane oxidation and other forms of oxidative damage. Some phenolics are disagreeable, poisonous, and hinder digestion [46].

In nature, polyphenolic chemicals constitute a substantial category of secondary plant metabolites found in abundance and have different physiological and morphological activities. Phytoalexins, repellents, attractants, ligands, and UV radiation protection factors are all chemicals that function in plant physiology. In addition, this group of chemicals has a considerable impact on the flavour and aroma of a specific plant species. Human health and nutrition benefit from polyphenolic compounds because of their antibacterial, anti-inflammatory, and antiviral activities and their high antioxidant capacity, which help reduce the risk of many illnesses [47].

One or more phenolic nuclei (polyphenols) and various functional groups connected to the phenolic nucleus make up this category of chemicals. Polyphenolic compounds are not confined to free and monomeric forms. Glycosides are the most common polyphenolic chemical in nature and are found in various forms, such as tannins [48]. Many polyphenolics can be found in fruits, vegetables, nuts, seeds, and alcoholic beverages. Polyphenolic content is inconsistent throughout the plant and varies at different points in the plant's life cycle [49]. Bees obtain nectar from various melliferous plants, and the chemical makeup of this nectar is very dependent on the climate. However, polyphenols found in honey may serve as possible identifiers for tracing honey's botanical and geographic origin. Polyphenolic substances may be linked to the establishment of stress-inducing situations in plants through their composition fluctuations [47].

Foods derived from plants include phenolic acids, a broad chemical category. "Phenolic acids" refers to phenols with a carboxylic acid functional group. Phenolic acids can have either hydroxycinnamic or hydroxybenzoic carbon frameworks in nature. Benzoic and cinnamic acid derivatives make up most of them. Figure 2.3 illustrates the unique polyphenols. Protein synthesis, enzyme activity, and photosynthesis are among the many roles in plants. According to recent research, many age-related illnesses and cancers of the circulatory system and other organs could be prevented or slowed by the protective effects of phenolic acids [50].

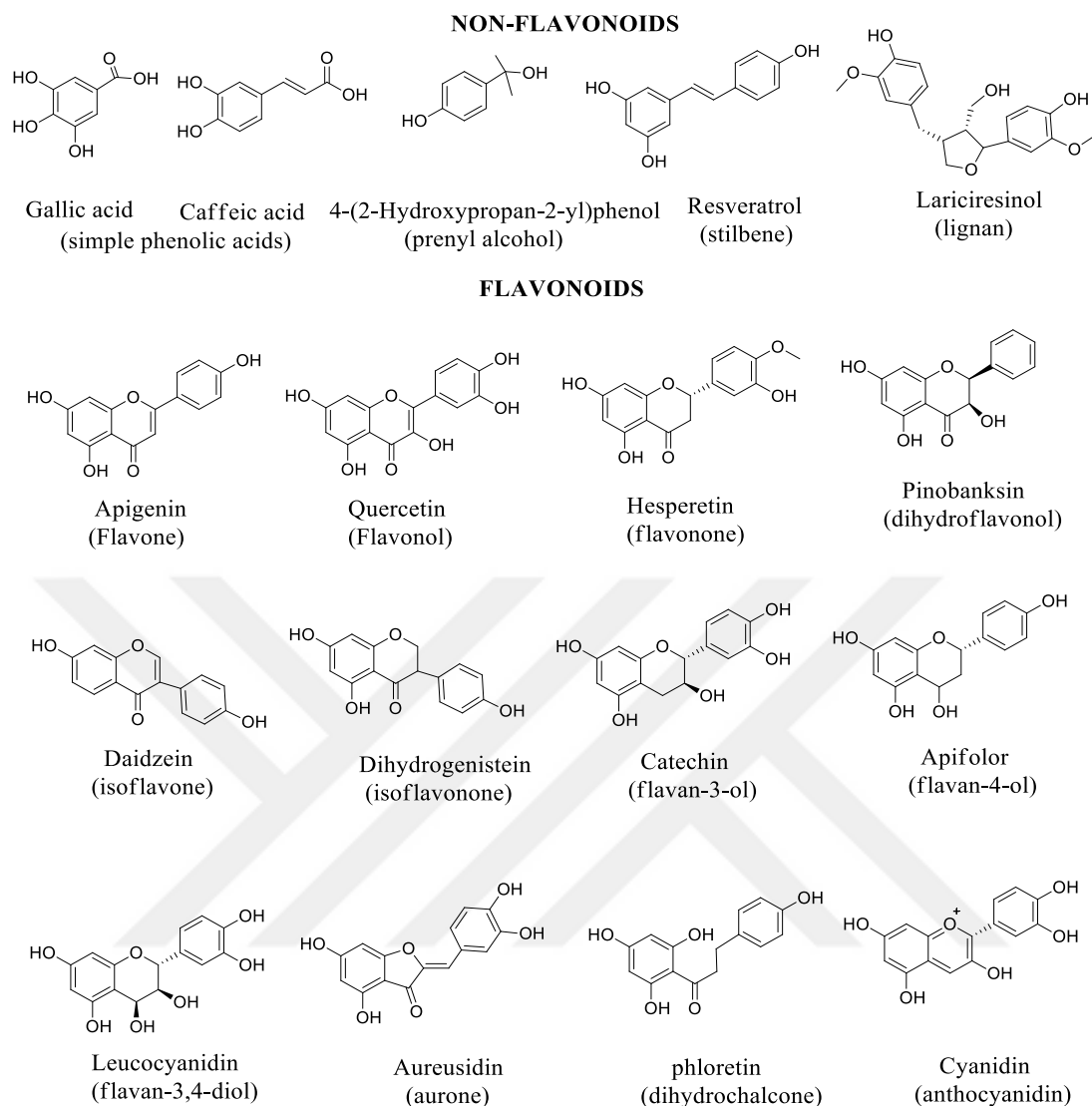


Figure 2.3. Some of the most significant polyphenols, as well as their chemical structures

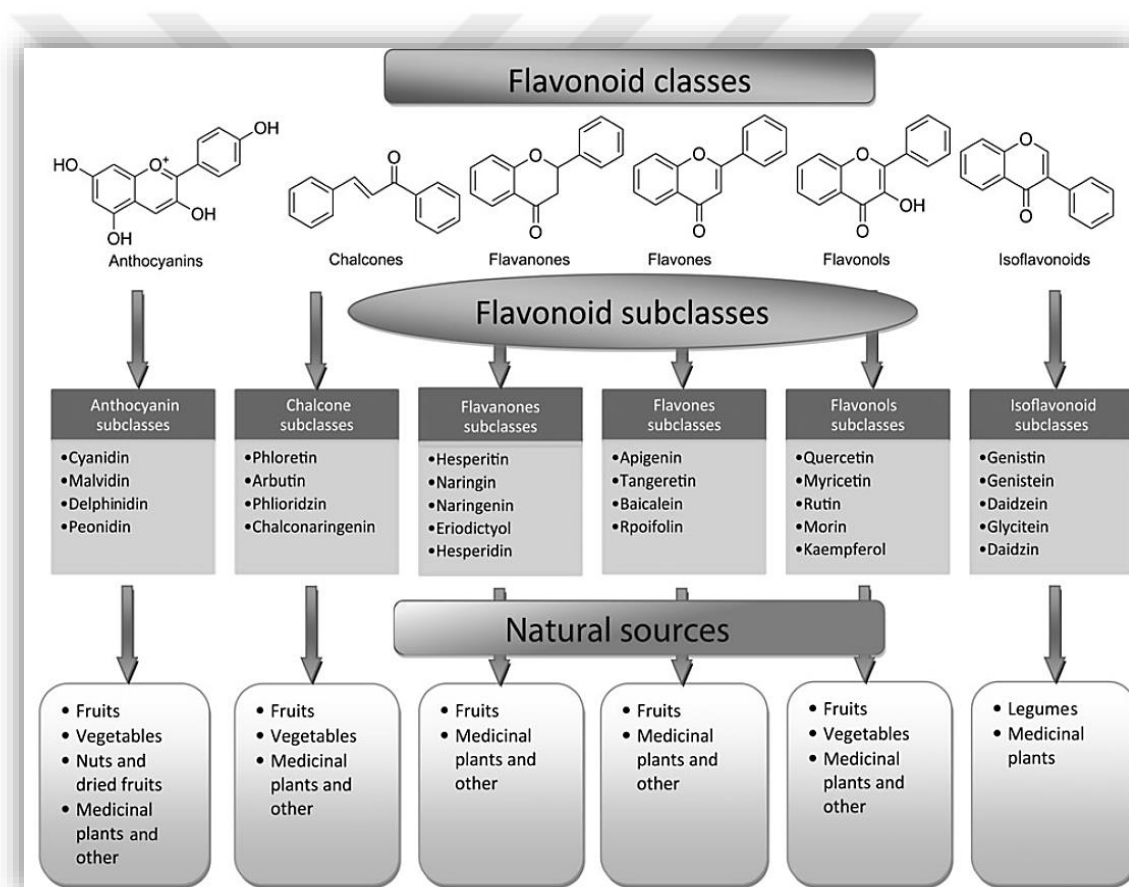
2.4. Flavonoid

This group of polyphenolic secondary plant metabolites, called flavonoids, is abundant in fruits, vegetables, and certain beverages. Flavonoids have been linked to various health benefits and are essential in nutraceutical, pharmaceutical, therapeutic, and cosmetic uses [51]. Flavonoid molecules, which have antioxidative, antibacterial, could be anticarcinogenic, and cardioprotective qualities, are of interest to scientists because of their wide range of biological features [52]. Their capacity to donate electrons is responsible for their antioxidant properties [53].

Flavonoids, found exclusively in higher plants, are plant-derived chemicals present in various parts of plants. They play a crucial role in the growth and protection of vegetables against disease [54]. Most angiosperm families contain many flavonoids that function as floral pigments. It is not only in flowers where they can be found but in any plant section [55]. Plants, animals, and

microorganisms benefit from flavonoids, which have many biological effects. Flavonoids in flowers and fruits give them colour, and fragrance, and attract pollinators for seed and spore dispersal, aiding in seedling growth and germination [56]. Flavonoids also serve as unique UV filters and protect plants from biotic and abiotic challenges [57]. Flavonoids could have a role in a plant's ability to withstand cold temperatures and drought and acclimate to heat [58].

Flavonoids are grouped into subtypes based on the linkage of the B ring to the C ring, and the unsaturation/oxidation of the C ring. Isoflavone has the B ring attached to position 3 of the C ring, neo-flavonoids to position 4, and flavones, flavonols, flavanones, flavanonols, flavanols/catechins, anthocyanins, and chalcones based on C ring structure when the B ring links to position 2. (see Figure 2.4) [51].



fruits and vegetables. Compared to flavones, the 3rd position of the C-ring in flavonols can also be glycosylated. Flavonols, similar to flavones, exhibit diverse patterns of methylation and hydroxylation and are considered a prevalent and important class of flavonoids in fruits and vegetables [59].

2.6. TLC solvent selection for the mobile phase

TLC is a type of separation method that was developed in the 1950s and is used by analysts and researchers all over the world. It is helpful for organic and inorganic chemicals in concentrations ranging from nanograms to micrograms, whether produced from natural sources or created in labs [60].

Silica-based sorbents are the most prevalent for TLC. Silica gel (SiO_2) is a white, porous substance produced by precipitating silicate solutions with acid. It is composed of silicon and oxygen linked together with leftover hydroxyl groups. Plates made of silica gel can separate all types of chemicals and can be treated with additional substances to boost their selectivity [61].

The mobile phase used for silica gel and modified silica gel plates in TLC consists of a single solvent or a single solvent to which a polar solvent has been added to boost the mobile phase's polarity. The sample should be spotted on a suitable plate and examined in beakers or small jars containing 1 cm of a single solvent to create an effective solvent system. The solvent concentration was increased following the elutropic sequence until the sample was raised halfway up the plate. Lloyd Snyder created the elutropic series and stated that the strength of the solvent is based on the physical characteristics of the liquid and the sorbent, with ϵ_0 representing the amount of adsorption energy per unit of a standard solvent for a solvent/sorbent combination. Another solvent of comparable strength but from a different selectivity group should be introduced to increase resolution. Typically, the most remarkable improvement is shown when the additional solvent belongs to a substantially distinct selectivity group. The resolutions and selectivities of solvent systems of the same strength but comprised of various solvents will vary significantly [62].

TLC solvents are selected based on the polarity of the element being separated and the type of method being used, such as adsorption or partition chromatography. Different solvents have different elutive powers, these solvents may be ranked in a hierarchy known as the elutropic series (Table 2.1). This hierarchy determines how quickly different substances migrate over a particular adsorbent [60].

Table 2.1. A series of eluotropic solvents for oxygen-containing adsorbents with increasing elutive power

No.	Series- A	Series- B
1	Petroleum Ether	n-Pentane
2	Carbon Tetrachloride	Petroleum Ether (b.p 30-50 °C)
3	Trichloro Ethylene	Petroleum Ether (b.p 80-100 °C)
4	Benzene	n-Hexane
5	Dichloromethane	n-Heptane
6	Chloroform	Cyclohexane
7	Diethyl Ether	Carbon Tetrachloride
8	Dimethyl	Trichloroethylene
9	Ethyl Acetate	Benzene
10	Pyridine	Dichloromethane
11	Acetone	Chloroform (Alcohol-Free)
12	n-Propanol	Diethyl Ether (Absolute)
13	Ethyl Alcohol	Ethyl Acetate
14	Methyl Alcohol	Pyridine
15	Formamide	Acetone
16	Water	n-Propanol
17	Glycol	Ethyl Alcohol
18	Glycerine	Methyl Alcohol; Water

Note that (i) these series are not always applicable in the same sequence for all substances; (ii) these series may be considered valuable suggestions for choosing a specific solvent alone; and (iii) these series are only applicable to hydrophilic adsorbents and not hydrophobic ones, such as charcoal.

After developing TLC plates, the separated components are detected by calculating their R_f (Retention factor) values. For example, coloured substances, such as xanthophylls, chlorophylls, and carotenes, may be found visually. Colorless Substances, such as alkaloids, steroids, and amino acids, may be identified using either short-wave or long-wave UV light. When sealed in a desiccator with I_2 -vapours, these compounds may also be recognized as brown or dark brown patches. Unique Detecting Reagents: For a particular class of substances, only a few specific detecting reagents are typically utilized [60].

3. INVESTIGATED PLANTS

Four plants were selected for the investigation: sumac (*Rhus Coriaria* L.), purslane (*Portulaca oleracea* L.), blackberry (*Rubus fruticosus* L.) and fenugreek (*Trigonella foenum-graecum* L.). These plants were selected based on their popularity as medicinal plants in the northern Iraqi villages of Koysinjaq /Erbil. However, based on the review, phytochemicals and antioxidant studies were done on selected plants in other places but not in this study area. As a result, the content of phytochemicals and antioxidants in the research region could be compared to other locations. The reviewed literature shows that column chromatography has not been used to fractionate and separate the four medicinal plants under consideration. This work used column chromatography to fractionate and isolate bioactive components from four medicinal plants. After that, polyphenolic compounds were determined in each extract and isolated fractions using HPLC.

3.1. Sumac (*Rhus coriaria* L.)

The term "Sumac" refers to the *Rhus* genus belonging to the Anacardiaceae family, which contains 91 recognized species names. In Iraq, the species *Rhus coriaria* L. is found either growing naturally or farmed near communities in northern Iraq [63]. Sumac can thrive in lands unsuitable for agriculture, and various species have been used by indigenous communities for medicinal and other purposes, showing the potential to commercialize the bioactivity of these plants without conflicting with agricultural land use [64]. The common name "Sumac" for the plant originates from the Syriac word 'sumaga,' meaning "red." [65].

The term "Sumac" is currently used generically to refer to the spice *Rhus coriaria*, which has been included in spice blends in traditional Asian remedies since ancient times [66]. Sumac powder is commonly used to add a lemon-like flavour to meat or salads. It is also an ingredient in zaatar, a blend of spices and herbs, and a substitute for red pepper and capers in regions such as Syria and Lebanon [67].

The flowers are tiny and exhibit a greenish-yellow hue. The fruits are lenticular drupes, measuring 4-6 mm in diameter, slightly fleshy, and reddish-brown. Short glandular hairs encircle them, and inside is a single seed (see Figure 3.1).

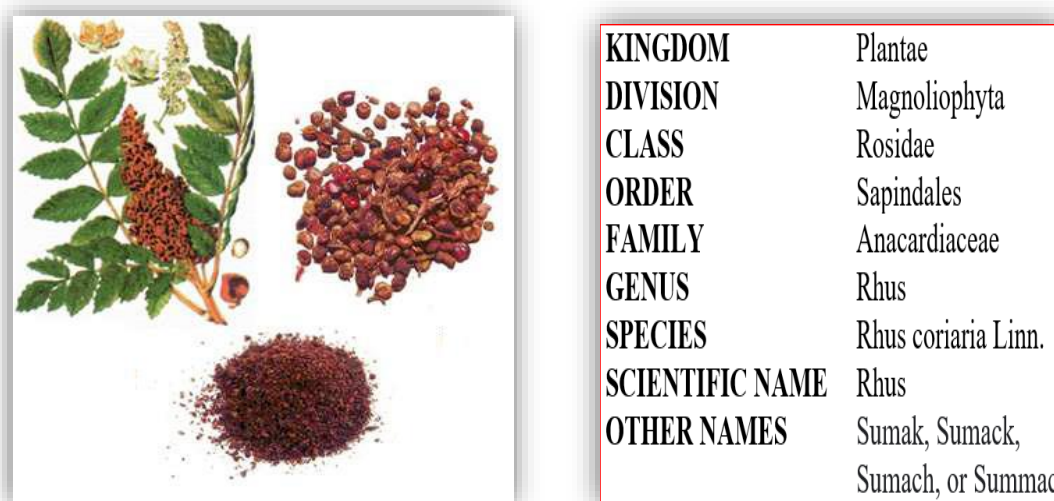


Figure 3.1. Sumac plant organs; fruit, stem, leaf and flowers, as well as sumac fruit powder with Classification [66]

The *Rhus coriaria* L. fruit has minerals like calcium (Ca), phosphorus (P), magnesium (Mg), and iron, as well as fatty acids, organic acids, essential and non-essential amino acids, carbohydrates, and vitamins [68]. Studies have mostly revealed that phytochemicals exhibit anti-cancer effects by suppressing inflammation, changing the immune response, exhibiting antioxidant activity, and altering various cellular processes, particularly cancer cell proliferation, apoptosis, and angiogenesis. Because of this, it is very important to use phytochemicals from plants as a source for making new and effective cancer drugs [69]. Sumac extracts have been used to treat various human diseases [70], including cancer [71]. *Rhus coriaria* bark powder is suitable for cleaning teeth. At the same time, its infusion is beneficial for treating viral eye infections, and its bruised water is applied to the forehead to cure epistaxis. Powdered fruits are sprinkled over hard-boiled eggs and consumed to alleviate diarrhoea. A fruit decoction is prepared and administered orally thrice daily to treat liver illnesses, urinary system abnormalities, and diarrhoea till improvement [72]. Sumac's active ingredients, which include flavonoids, xanthones, and tannins [69], have been linked to hypoglycemic, anti-microbial, antioxidant, antifungal, and radical-scavenging properties [73].

Ten polyphenolic compounds, including gallic acid, quercetin-3-O-rhamnoside, ellagic acid, m-gluco caffeic acid, and quercetin, as well as five significant anthocyanins, were found. These phenolics were the primary contributors to the high antioxidant activity [74]. *Rhus coriaria* has a lot of uses in the food and cosmetics industries, as well as in veterinary medicine, which makes it precious [75]. Several studies have been done to find out what the main parts of *Rhus coriaria* are [76] and what makes it work [77].

3.2. Purslane (*Portulaca oleracea* L.)

Portulaca oleracea L. is the botanical name for this plant, which belongs to the Portulacaceae family [78]. The word "Portulaca" comes from the Latin words "portare," which means "to carry," and "lac," which means "milk," and refers to the plant's milky sap. The word "oleracea" comes from the phrase "pertaining to kitchen gardens," so the plant is used for cooking [79]. It is considered a weed in the United States, whereas purslane (*Portulaca oleracea*) grows in many parts of Europe, Africa, the Mediterranean, and Asia (see Figure 3.2).



KINGDOM	Plantae
DIVISION	Tracheophyta
CLASS	Magnoliopsida
ORDER	Caryophyllales
FAMILY	Portulacaceae
GENUS	Portulaca
SPECIES	<i>P. oleracea</i>
SCIENTIFIC NAME	<i>Portulaca oleracea</i>
OTHER NAMES	Garden purslane, beldroega, children's spinach, jump up and kiss me, little hogweed, money plant, pot purslane, ma chi xian

Figure 3.2. Purslane (*Portulaca oleracea* L.) plant with Classification

Foods like salads, sides, soups, and stews can all benefit from the addition of purslane. The purslane plant can be eaten by roasting the stems and leaves or preserving them [80]. It is a very invasive plant, formerly classified as the eighth-most prevalent weed. It is a synanthropic species since it has always been a part of urban flora, dating back to prehistoric times [81]. One of the most significant sources of omega-3 fatty acids is *Portulaca oleracea* L., which is five times more abundant than spinach. Antioxidants, such as α -tocopherol and melatonin, are found in purslane. Besides that, it is an excellent source of vitamin A, vitamin C, calcium, phosphorus, and iron.

Purslane holds a vast array of potential for pharmaceutical applications. It is widely used in Chinese anti-bacterial and anti-viral products and has shown wound healing and muscle relaxation properties in mouse muscles [82].

Purslane contains several phytochemicals with pharmacological properties, including immunomodulatory, [83] anticancer, [84] antioxidant, [85] hypoglycemic, [86] anti-inflammatory, [87] antibacterial, [88] and anticholinesterase properties [89].

Several new studies have shown that *P. oleracea* has potent virucidal effects against influenza A virus infection in vitro, [90] and it reduces heavy menstrual bleeding (menorrhagia), [91] which its polysaccharides protect rats from Pb-induced memory and learning impairments [92]. Patients with metabolic syndrome may benefit from purslane's ability to lower their blood glucose and cholesterol levels [93]. Purslane's flavonoids are the source of its antioxidant activity, anti-germ, anti-virus, anti-ulcer, and anti-inflammatory effects, as well as its ability to suppress coughing and eliminate phlegm. The five main flavonoids in purslane are kaempferol, apigenin, myricetin, quercetin, and luteolin [94].

3.3. Fenugreek (*Trigonella foenum-graecum* L.)

Fenugreek is a centuries-old medicinal plant that has been used globally for both dietary and therapeutic purposes. The scientific name for this small plant, belonging to the Papilionaceae family, is *Trigonella foenum-graecum* Linn. It is named "three-angled" in ancient Greek, referring to the triangular shape of its seeds (see Figure 3.3) [95]. Its natural habitats are the Mediterranean, India, China, Northern Africa, and Ukraine. Many countries worldwide grow fenugreek as a food and medicine crop because of its wide range of uses [96]. Food-based uses for this plant include stew with rice in Iran and flavouring cheese in Switzerland, bitter rum and syrup in Germany, and Egyptian flatbread. Coffee substitutes made from the roasted grain of young seedlings are widespread in Africa [97].

Trigonella foenum-graecum L., Fabaceae, is native to the Near East and Mediterranean areas and is grown mainly in India, northern Africa, Germany, France, and Russia. Despite the lack of breeding or assessment in the southern hemisphere, at least two cultivars have been issued in Canada. Several accessions have been studied in the mid-western United States [98].

Asian, African, and Mediterranean people eat the seeds of this plant as part of their everyday diet. It is employed in various areas, including medicine, nutrition, drinks, perfumes, cosmetics, and more [99]. An excellent source of protein, the seeds also include inaccessible carbohydrates, mucilage, and saponins. Compared to other legumes, the seed is richer in minerals (Ca, P, Fe, Zn, and Mn) [100]. The seeds include flavonoids, carotenoids, and coumarins, among other compounds [101]. Bioactive phytochemicals such as flavonoids are linked to fenugreek's antioxidant and anti-inflammatory properties. Flavonoid antioxidant and anti-inflammatory properties are the most well-recognized benefits of this class of compounds [102].

For medicinal purposes, the seeds may be employed in cooking or as a flavour enhancer for other treatments [103]. Fenugreek seeds are widely used in folk medicine to cure various ailments, including paralysis, diabetes, epilepsy, and fever [104]. Traditionally, fenugreek was suggested to breastfeeding mothers to boost milk production and, curiously, to Moroccan Saharawi women to enhance their physical beauty by increasing their appetite [105].



KINGDOM	Plantae
DIVISION	Eudicots
CLASS	Rosids
ORDER	Fabales
FAMILY	Fabaceae
GENUS	Trigonella
SPECIES	Foenum
SCIENTIFIC NAME	Trigonella foenum-graecum
OTHER NAMES	Alholva, Bird's Foot, Bockshornklee, Bockshornsame, Chandrika, Fenogreco, Foenugraeci Semen, Greek Clover, Greek Hay, Greek Hay Seed, Hu Lu Ba, Medhika, Methi, Sénégrain, Trigonella, Woo Lu Bar

Figure 3.3. *Trigonella foenum-graecum* seeds, leaves, and classification

The fenugreek seeds include proteins high in lysine and L-tryptophan, mucilaginous fibre, and other uncommon chemical elements such as saponins, coumarin, fenugreekine, nicotinic acid, sapogenins, phytic acid, scopoletin, and trigonelline [106]. These ingredients possess sugar-lowering, cholesterol-lowering, fever-reducing, anti-inflammatory, cytotoxic, apoptosis-activating, antifertility properties [107], antifungal, antibacterial, and anticarcinogenic [105].

3.4. Blackberry (*Rubus fruticosus* L.):

Berries are a vital part of the fruit kingdom because of their distinctive colour, flavour, aroma, and high vitamin and mineral content [108]. Blackberry is a fruit belonging to the genus *Rubus* (family Rosaceae, genus *Rubus*) extensively spread worldwide. This fruit is endemic to tropical America, primarily Colombia and Ecuador [109]. This fruit comprises several tiny drupes on a 1–2.5 cm long receptacle and is recognized by its dark crimson colour and bitter flavour (see Figure 3.4) [109]. A ripening fruit that goes from green to crimson to black [110].



KINGDOM	Plantae
DIVISION	Magnoliophyta
CLASS	Magnoliopsida
ORDER	Rosales
FAMILY	Rosaceae
GENUS	Rubus
SPECIES	<i>R. fruticosus</i>
SCIENTIFIC NAME	<i>Rubus fruticosus</i>
OTHER NAMES	Bramble, European blackberry, scaldhead, shrubby blackberry, wild blackberry, wild blackberry complex

Figure 3.4. Blackberry (*Rubus fruticosus* L.) fruit with classification [111]

Wild fruits like the blackberry (*Rubus fruticosus*) are popular because of their appealing colour and flavour and their potential health advantages to humans [112]. The blackberry fruit is packed with vitamins C, K, and E. The fruit has a low sugar content and is high in nutritional fibre. The high anthocyanin and ellagitannin (ETs) content of blackberries and other phenolic components that support their high antioxidant activity make them a fruit of interest. In reality, blackberries' healing properties have been well-known in Europe since the 16th century, when they were utilized to cure eye and mouth ailments [113]. Blackberries' most significant bioactive substances include flavonoids, phenolic acids, tannins, vitamins, and antioxidants [114]. The blackberry fruit is included in the phenolic substances of anthocyanins, flavonols, chlorogenic acid, and procyanidins that positively impact human health [115].

Blackberries have been used medicinally in Europe since the 16th century when they were utilized to cure eye and mouth diseases [116]. Several studies show that regular blackberry consumption benefits human health by preventing and treating certain chronic degenerative disorders [117]. Through laboratory tests and studies using animals as models, scientists discovered that the phenolic chemicals in blackberries could prevent numerous malignancies, inflammatory illnesses, and age-related cognitive problems [113].

4. MATERIAL AND METHOD

4.1. Apparatus

The laboratory apparatus and equipment used in all experiments are listed in Table 4.1.

Table 4.1. The list of laboratory apparatus

No.	Apparatus	Company	Country
1	Shimadzu HPLC-DAD	Shimadzu	Germany
2	Vacuum drying oven	LabTech	India
3	Water bath	Memmert	Germany
4	Centrifuge	Hermle	Germany
5	Magnetic stirrer	LabTech	India
6	Sensitive Balance	Radwag	Germany
7	pH meter	CyberScan	Singapore
8	Rotary evaporator	IKA	United States
9	UV-VIS spectrophotometer	Thermo Scientific GENESYS	United States
10	Advanced vortex mixer	VELP Scientific	Italy
11	Grinding Machine	Mill Powder Tech	Taiwan
12	UV-Lamp	Fisher Scientific	Austria
13	TLC Silica gel	Merck	Germany

4.2. Chemicals

The solid chemicals and solvents used in all experiments are listed in Table 4.2.

Table 4.2. Chemicals and solvents

No.	Chemicals	Company	Country
1	Aluminium chloride	Merck	Germany
2	Sodium acetate trihydrate	Merck	Germany
3	Potassium ferricyanide	Merck	Germany
4	1, 10-Phenanthroline monohydrate	Merck	Germany
5	Iodine	Merck	Germany
6	Ferrous sulfate heptahydrate	Merck	Germany
7	Potassium dihydrogen phosphate	Merck	Germany
8	Dipotassium hydrogen phosphate	Merck	Germany
9	Sodium carbonate	Merck	Germany

10	Folin-phenol ciocalteu's reagent	Merck	Germany
11	Sodium hydroxide	Merck	Germany
12	Trichloroacetic acid	Merck	Germany
13	Ferric chloride trihydrate	Merck	Germany
14	Potassium hydroxide	Merck	Germany
15	potassium chloride	Merck	Germany
16	37% Hydrochloric acid	Merck	Germany
17	85% Formic acid	Merck	Germany
18	99.8% Methanol	Carlo Erba	Sabadell (Spain)
19	99.9% Ethanol	Carlo Erba	Sabadell (Spain)
20	99% Hexane	Carlo Erba	Sabadell (Spain)
21	Sodium dihydrogen phosphate	Carlo Erba	Sabadell (Spain)
22	Quercetin	Sigma-Aldrich	Germany
23	Ethyl acetate	Sigma-Aldrich	Germany
24	Anhydrous gallic acid	ISOLAB GmbH	Germany
25	99.9% Dichloromethane	Scharlau	Spain
26	99.5% Diethyl ether	Scharlau	Spain
27	99.0% Chloroform	Scharlau	Spain
28	99.8% Acetone	Scharlau	Spain
29	99.8% Tetrahydrofuran,	Scharlau	Spain
30	99.9% Acetonitrile	Scharlau	Spain
31	Petroleum ether	Scharlau	Spain
32	99.5% Toluene	J.T.Baker	India
33	Rutin trihydrate	Dr EhrenstorferTM	

4.3. Reagents preparation

To make the following solutions:

- 10% (w/v) sodium carbonate: dissolve 10 g of sodium carbonate in 50 ml of distilled water and bring the volume up to 100 ml with more distilled water.
- 5% (w/v) AlCl_3 : dissolve 5 g of AlCl_3 in 50 ml of distilled water, mix until dissolved, then complete the volume to 100 ml with the same solvent.
- 1.0 M $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$: dissolve 13.608 g of sodium carbonate (MW = 136.08 g/mol) in distilled water and dilute to 100 ml.
- Aluminium chloride 2% (w/v): dissolve 2 g of AlCl_3 in 50 ml of MeOH, mix until dissolved, then add more MeOH to reach 100 ml.
- $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ 5% (w/v): dissolve 5 g of $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ in 50 ml of MeOH, mix until dissolved, then add more MeOH to reach 100 ml.

- 0.025 M potassium chloride: dissolve 0.1864 g of potassium chloride (MW = 74.55 g/mol) in distilled water and dilute to 100 ml.
- 0.40 M $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$: dissolve 5.44 g of $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ (MW = 136.08 g/mol) in distilled water and dilute to 100 ml.
- 1.0 M HCl: dilute 8.28 ml of concentrated hydrochloric acid (Sp. gr. = 1.19, purity 37% and MW = 36.46 g/mol) to 100 ml.
- 1% (w/v) $\text{K}_3\text{Fe}(\text{CN})_6$: dissolve 1 g of $\text{K}_3\text{Fe}(\text{CN})_6$ in distilled water and dilute to 100 ml.
- 10% trichloroacetic acid: dissolves 10 g of trichloroacetic acid in distilled water and dilutes to 100 ml.
- 0.1% (w/v) fresh FeCl_3 : dissolve 0.1 g of FeCl_3 in 100 ml of distilled water and fill the rest of the bottle with more of the same solvent.
- 0.2% (w/v) FeCl_3 : dissolve 0.2 g of FeCl_3 in distilled water and dilute to 100 ml.
- 0.1% phenanthroline: dissolve 0.1 g of phenanthroline in 50 ml of distilled water, and bring the volume up to 100 ml with the same solvent.
- pH: 6.6 phosphate buffer: dissolves 1 g of potassium dihydrogen phosphate and 0.7 g of dipotassium hydrogen phosphate in 50 ml of distilled water.

4.4. Plant sampling and preparation

This part of our research was conducted in Koysinjaq/Erbil in northern Iraq. It involves surveying, collecting, identifying, cleaning, chopping, and powdering plants.

4.4.1. Plant surveys

The samples of plants sumac, purslane and blackberry were collected from the north of Iraq, the villages near Koysenjaq, about one hour from Erbil, in October 2020. Dried fenugreek seeds are the only plant purchased from the local Bazar of Koysenjaq. Figure 4.1 displays a GIS (Geographic Information System) map of the research area's location. Prof. Dr Ikbal Tahir, a distinguished taxonomist from Koya University, assisted in the identification of plants.

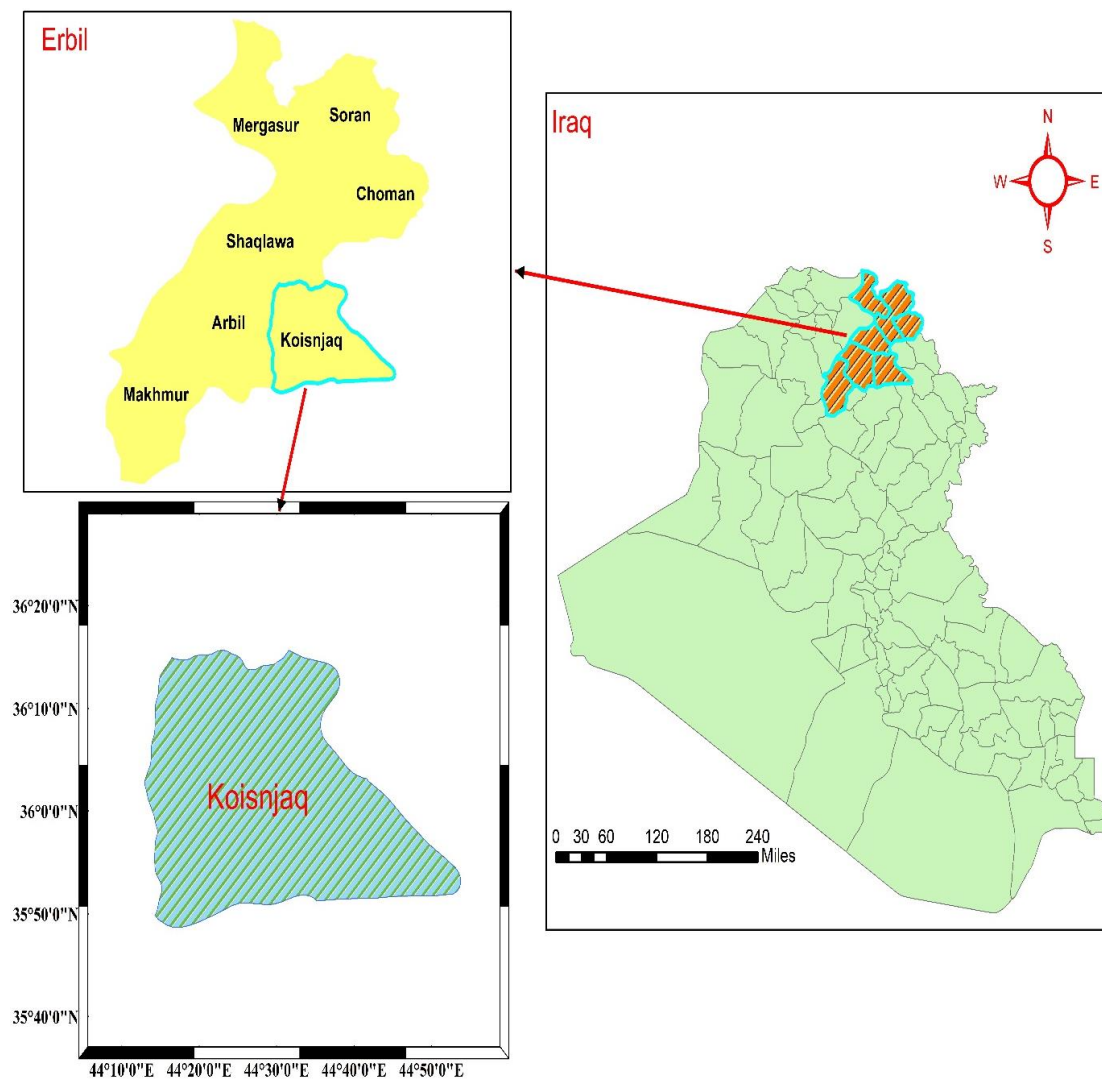


Figure 4.1. GIS Map: Iraqi map, Erbil governorate location and Koisinjq study area location

4.4.2. Cleaning plant samples

As stated in Table 4.3, the plants were first collected for the identification procedure and taxonomic identification. The samples were rinsed with tap water to remove any dirt particles and later washed one more time with distilled water.

Table 4.3. Classification of plants taxonomically

Scientific classification	Plants			
	Sumac	Purslane	Fenugreek	Blackberry
Scientific name	Rhus	Portulaca oleracea	Trigonella foenum-graecum	Rubus fruticosus
Kingdom	Plantae	Plantae	Plantae	Plantae
Division	Magnoliophyta	Tracheophyta	Eudicots	Magnoliophyta
Class	Rosidae	Magnoliopsida	Rosids	Magnoliopsida
Order	Sapindales	Caryophyllales	Fabales	Rosales
Family	Anacardiaceae	Portulacaceae	Fabaceae	Rosaceae
Genus	Rhus	Portulaca	Trigonella	Rubus
Species	Rhus coriaria Linn. [72]	P. oleracea [118]	Foenum [119]	R. fruticosus [120]

4.4.3. Plants drying and powdering

The plants were shade-dried before being pulverized into a fine powder using an electric grinder (see Figure 4.2). This action was performed at the appropriate time and place. The powder was then placed in airtight vials for future examination and stored.

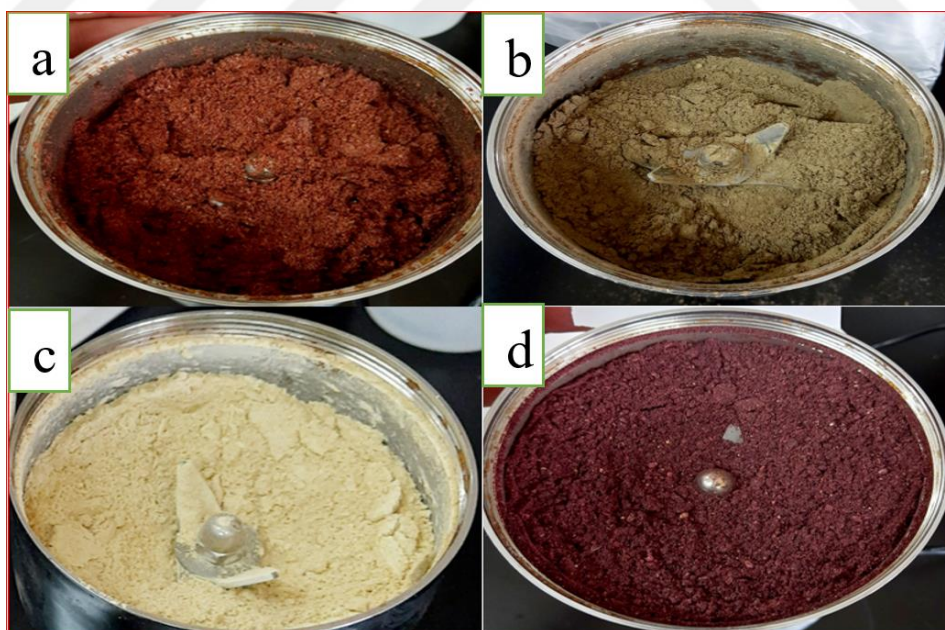


Figure 4.2. Crushed and dried plant samples (a) Sumac (b) Purslane (c) Fenugreek (d) Blackberry

4.4.4. Plants extraction

Important chemical components of medicinal plants must be extracted from plant materials before they can be separated and characterized, making extraction the first and most crucial stage in researching medicinal plants [25]. The extraction methods and solvents are responsible for dissolving the plant's endogenous components. In addition, plant components might be polar or non-polar. Due to the presence of a hydroxyl group, phenolic compounds are more soluble in polar organic solvents; consequently [121], MeOH was chosen as the extraction solvent [122].

Plant components were successfully extracted using maceration. The powder was extracted for 24 hours at room temperature three times using 75 ml of MeOH each time (see Figure 4.3a). The extract was filtered using Whatman filter paper No. 1 (see Figure 4.3b), and the filtrate was concentrated in a rotary evaporator under a temperature of 40 °C and pressure of 337 mbar. Hexane solvent was utilized to eliminate the fat remaining after the extract evaporated. Once air-dried, the extract was finished in a vacuum oven at 40 °C. All dry methanolic extracts were stored at 4–6°C until usage.

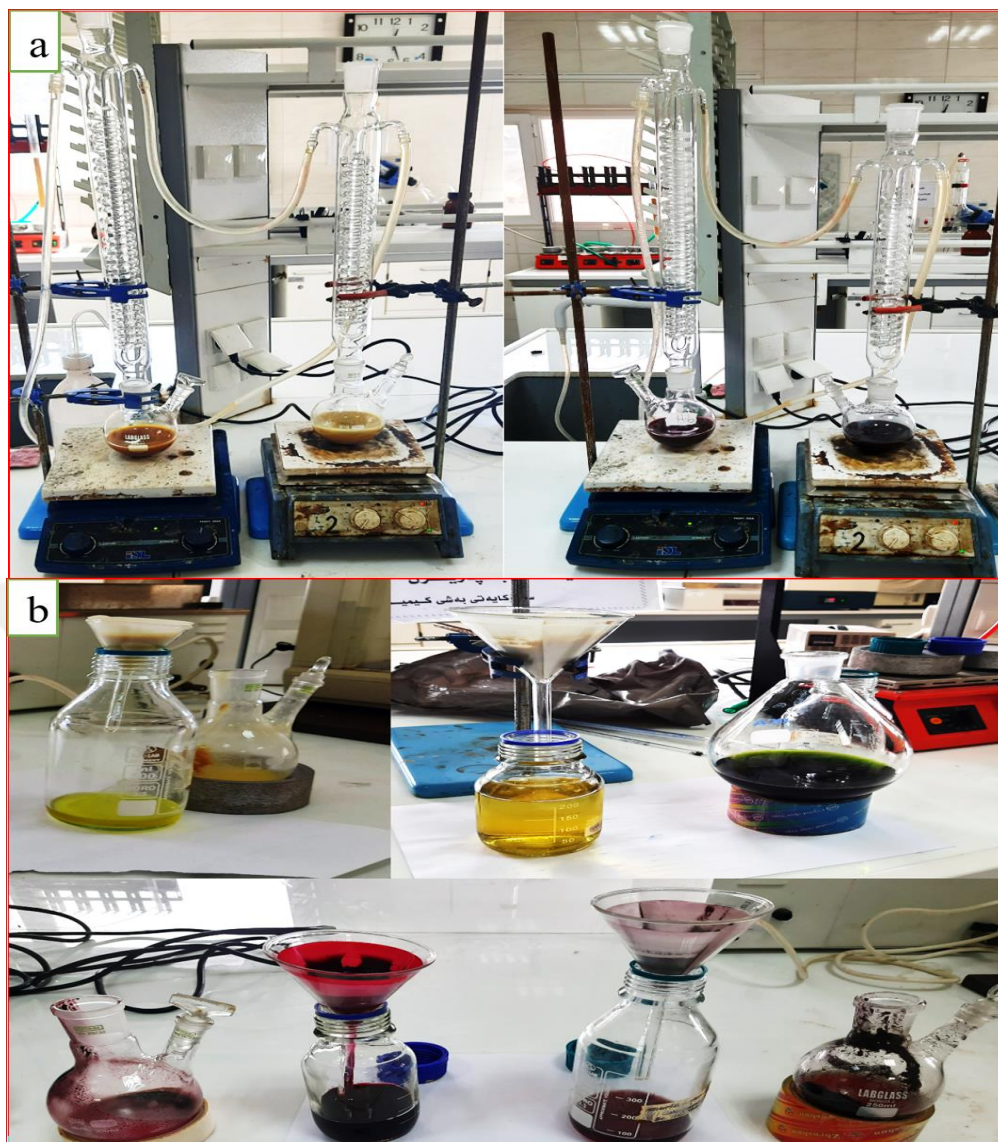


Figure 4.3. (a) Extracting plant material using MeOH at an ambient temperature, (b) Extracted sample plants filtered into sterile bottles with filter paper

Most modern chemical labs cannot operate without an electronic rotary evaporator (see Figure 4.4). Many labs let a volatile solvent evaporate to separate it from a non-volatile substance. A rotary evaporator also called a "rotovap," is a machine that removes the solvent from a flask. The rotovap technique involves:

- lowering the pressure within the flask with a vacuum,
- rotating the sample to increase its surface area, and
- heating the solution to evaporate the solvent.

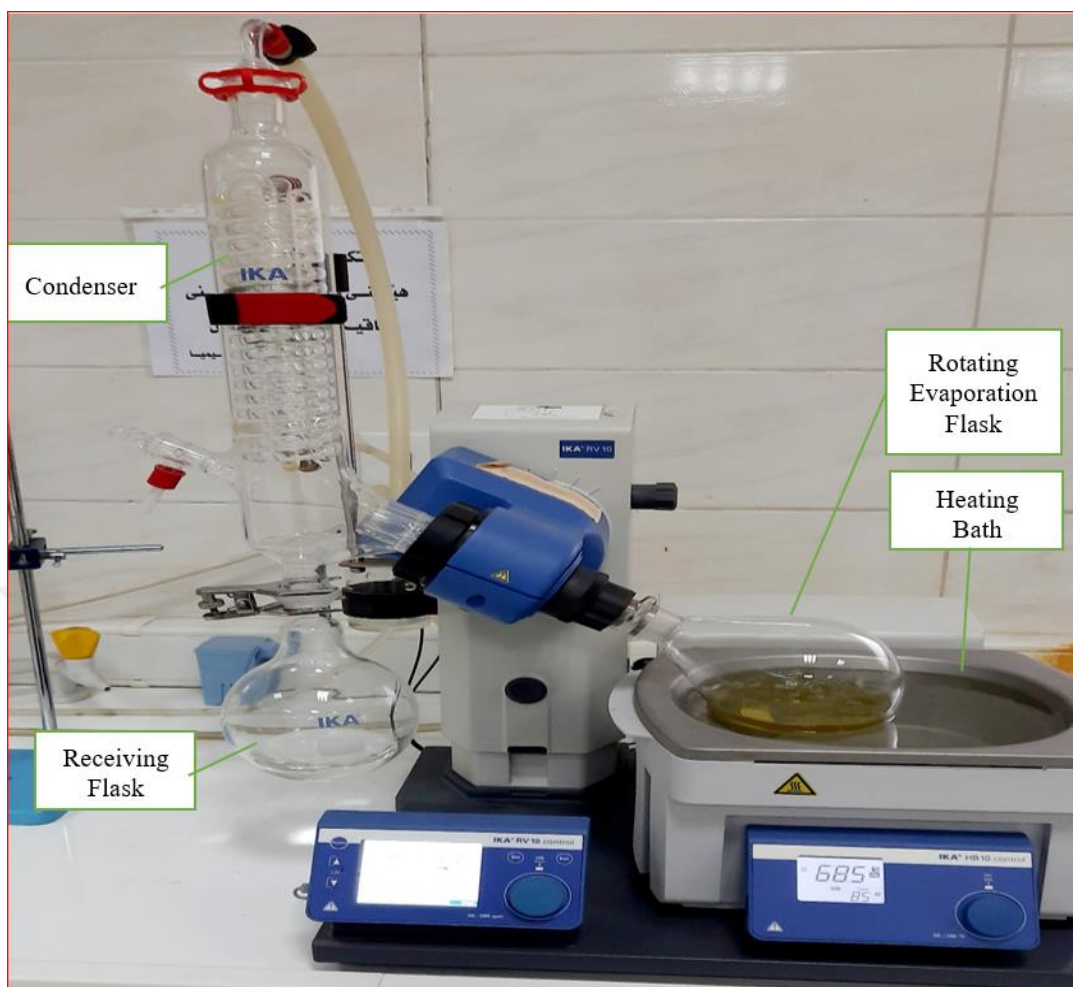


Figure 4.4. Concentrating of extract plants using a rotary evaporator at 40 °C

Treatment for fat removal could be roughly classified as either chemical or mechanical. As part of the chemical process, organic solvents are used chiefly for fat extraction. Pure organic solvents or organic solvent combinations are suitable for this method [123].

A separating funnel is a particular funnel designed to separate immiscible liquids. A 20 mL of each extracted plant was placed individually in a 100 mL volume size separating funnel and held by an iron ring (see Figure 4.5). Then, 5.0 mL of hexane solvent was added, and the stopper was closed. The separatory funnel was removed from the ring and held tightly at the stopper and stopcock. The funnel turned over slowly and was vent (open the stopcock) towards the back of the hood. A sound like a whistle was heard when the pressure took off. The stopcock was closed, and the funnel was shaken for emulsion production. The stopcock was repeatedly vented until no more gas came out. After putting the separatory funnel back into the iron ring, the layers were allowed to split. Then, the stopper was removed, and the bottom layer was poured into a clean container (glass petri dish). The bottom layer is MeOH, which pulls out the investigated products, and the top is hexane.

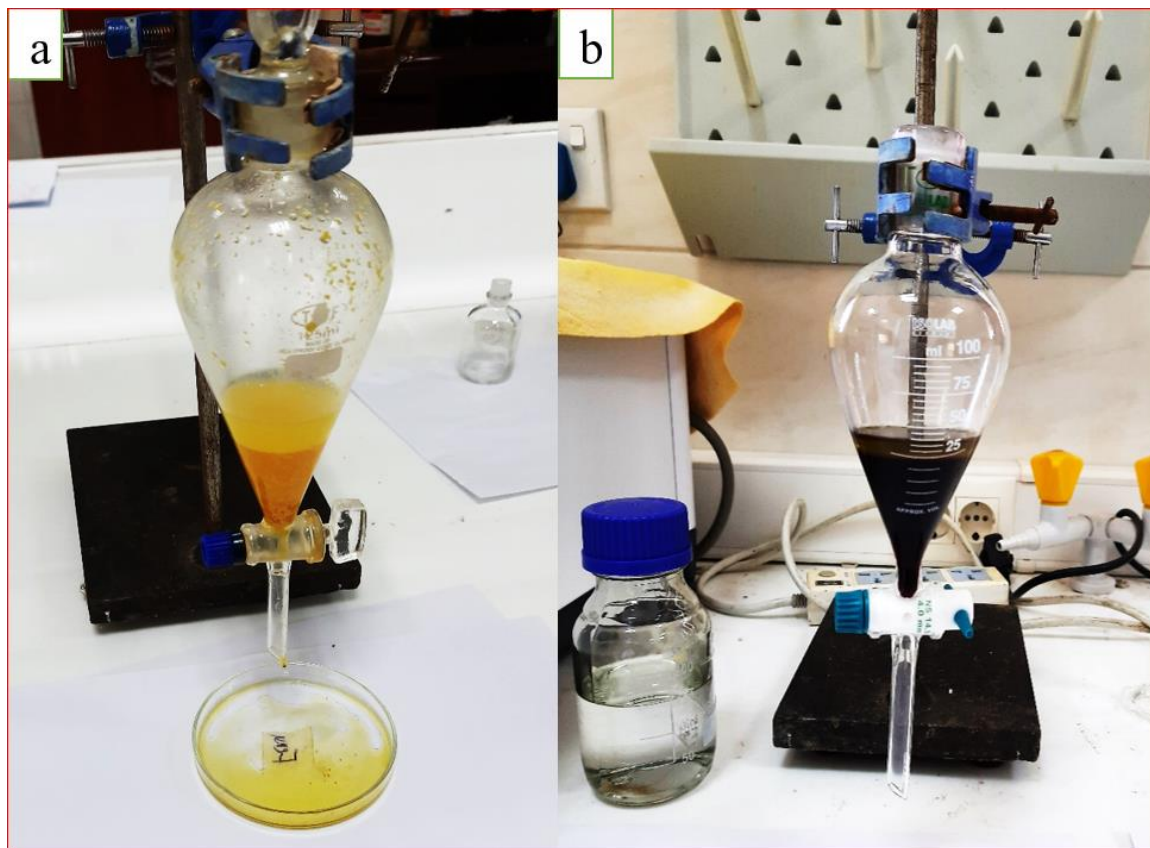


Figure 4.5. Hexane-defatted (a) fenugreek and (b) blackberry extracts in a separating funnel

A low-pressure (vacuum) atmosphere has a lower boiling point; therefore, using a vacuum oven, samples that cannot be dried at average high temperatures are dried at a lower temperature. The drying tests were done in a vacuum oven at 40 °C. The samples were convectively dried in a glass petri dish over 24 hours (see Figure 4.6).

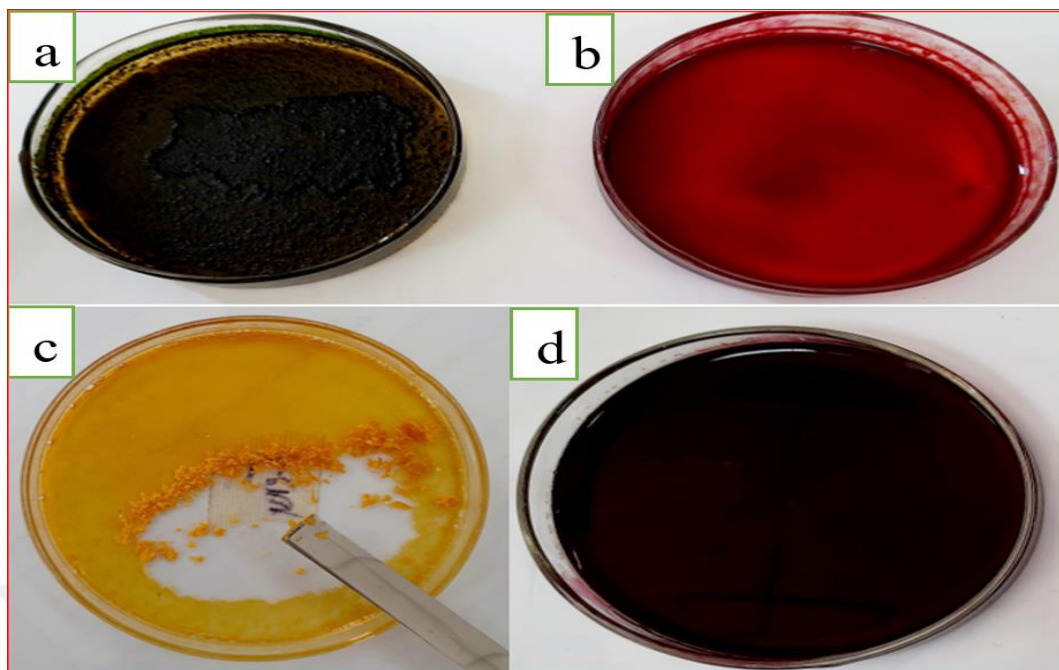


Figure 4.6. Dried plant extracts of (a) purslane, (b) sumac, (c) fenugreek, (d) blackberry

4.5. Extracts' percentage yield

The proportion of extracts was obtained by comparing the weight of the extracts to the weight of the original dried sample. The yield is estimated as a percentage using the following formula:

$$\% \text{ Yield} = \frac{W_1}{W_2} \times 100 \quad (4.1)$$

Where W1 is the weight of the extract residue after the removal of the solvent and W2 is the weight of the dried plant powder [124].

4.6. Phytochemical Analysis

This study investigated the phytochemical profiles of four medicinal methanolic plant extracts.

4.6.1. Total phenolic content (TPC)

The TPC of each plant extract was determined by altering the Folin-Ciocalteu procedure [125]. A 1.5 mL of distilled water was added to 0.1 mL of extract (3 mg/mL MeOH) and vigorously agitated for 5 minutes with 0.1 mL of Folin–Ciocalteu reagents, followed by 1.5 mL of 10% (w/v) sodium carbonate. After 60 minutes in the dark, the mixture's absorbance was determined at 765 nm using a UV-VIS spectrophotometer. A Folin- Ciocalteu reagent was used to oxidize the dried plant extracts, and sodium carbonate neutralized the reaction. A stock solution concentration (1000

µg/ml) of gallic acid was prepared by dissolving 50 mg of dry gallic acid in 10 ml of MeOH and then diluting it to 50 ml with the same solvent in a volumetric flask. Serial dilutions of the stock gallic acid solution in MeOH were made to achieve concentrations ranging from 5 to 200 µg/ml. A regression equation derived from the gallic acid standard calibration curve was used to calculate the extract's TPC equivalents (mg GAE/g extract).

4.6.2. Total flavonoid content (TFC)

The TFC of each extract was determined using a colourimetric method by adding aluminium chloride [126]. A 0.2 mL of extract (3 mg/mL) was combined with 1 mL of 5% (w/v) AlCl₃ solution, followed by 0.1 mL of 1.0 M CH₃COONa.3H₂O solution and 2.7 mL of MeOH, then allowed to stand for 60 minutes. The absorbance was measured at 420 nm. A quercetin stock solution of (1000 µg/ml) concentration was made by dissolving 10 mg of dry quercetin in 5.0 ml of MeOH and then diluted to 10 ml with the same solvent in a volumetric flask. The stock solution of quercetin in MeOH was diluted serially to produce working solutions in the range of 5 to 200 µg/ml concentrations. A calibration curve was used to compute the TFC, expressed as mg QE/g of dry weight.

4.6.3. Total flavonol (TF) content

A colourimetric method is used to determine the TF content of plant extracts by using aluminium chloride [127]. A standard calibration curve of the method was created using rutin. A 0.5 mL extract (3 mg/mL) was added to a test tube, followed by 0.5 mL of 2% (w/v) aluminium chloride and 5% (w/v) sodium acetate (6 mL). After constant stirring, all tubes were incubated for 2.5 hours at room temperature in a dark place. The absorbance was measured for reference and sample solutions at 440 nm to get the plant's results in mg RE/g dry weight. A concentration of (1000 µg/ml) stock solution of rutin was produced by dissolving 10 mg of dried rutin in 5.0 ml of MeOH and then diluting it to 10 ml with the same solvent in a volumetric flask. To prepare concentrations ranging from 5 to 200 g/ml, the stock rutin solution in MeOH was serially diluted. The TF was calculated using a calibration curve in mg RE/g dry weight.

4.6.4. Total anthocyanin content (TAC)

The TAC was determined using a differential pH method [128]. Anthocyanin concentration of plant extract was measured by diluting it with potassium chloride (0.025 M) and sodium acetate (0.40 M) solutions, then adjusting the pH with 1.0 M HCl to 1.0 or 4.5 as appropriate. The cyanidin-3-glucoside standard was used for absorbance measurement against distilled water as a blank. They recorded the absorbance of the sample diluted with buffers of (a) pH 1.0 and (b) pH 4.5 at 520 and

700 nm. The diluted test samples were compared to a blank cell containing distilled water. The reason absorbance is measured at 700 nm is to account for haze. If the diluted sample is too cloudy, clear it by centrifuging or filtering before measurement [129].

Absorbance at different wavelengths is related to pH levels in the following way:

$$A = (A_{520} - A_{700})_{pH1.0} - (A_{520} - A_{700})_{pH4.5} \quad (4.2)$$

The concentration of anthocyanin in the extract samples was calculated as follows:

$$\text{Anthocyanin extraction yield (mg/g)} = \frac{A \times MW \times DF \times V \times 1000}{\epsilon \times L \times wt} \quad (4.3)$$

Where; DF= dilution factor, MW = Molecular weight of cyanidin-3-glucoside (449.2 g/mol), ϵ = molar extinction coefficient of cyanidin-3-glucoside (26900 L/mol.cm), V = extract volume (L), L = sample cell Length (cm), 1000 = conversion factor from g to mg, wt. = sample weight (g). All data represented in mg of cyanidin-3-glucoside/g.

4.7. Antioxidant Analysis

This study investigated the antioxidant properties of four medicinal methanolic plant extracts.

4.7.1. Ferric-reducing antioxidant power (FRAP)

Potassium ferricyanide made a coloured complex containing antioxidant properties to test the reducing power [130]. A 0.1 mL extract sample of 3 mg/mL concentration was added to a tube containing 2.5 mL of 1% (w/v) $K_3Fe(CN)_6$ and 2.5 mL of 0.2 M phosphate buffer (pH: 6.6). After 30 minutes at a temperature of 50°C, a 2.5 mL of 10% (w/v) Trichloroacetic acid was added to the mixture and then centrifuged at 3000 rpm for 10 minutes. The supernatant was pipetted into a second tube containing 2.5 mL water and 0.5 mL of freshly prepared 0.1% (w/v) $FeCl_3$ concentration. To prepare a quercetin stock solution (1000 µg/ml), 10 mg of dry quercetin was dissolved in 5.0 ml of MeOH and diluted to 10 ml with the same solvent in a volumetric flask. The stock quercetin solution in MeOH was serially diluted to obtain working solutions ranging from 20 to 200 g/ml. The absorbances were recorded at 700 nm. The FRAP was calculated in mg QE/g dry weight using the quercetin standard calibration curve and regression equation.

4.7.2. Metal chelating ability (MCA)

The MCA of plant extracts was studied using the 1,10-phenanthroline technique [131] by filling the tube with 0.1 mL of sample solution (3 mg/mL) and then 1.5 mL of water, 1 mL of 0.2%

(w/v) FeCl₃, 1 mL of 0.1% (w/v) phenanthroline, and 1.4 mL of water were added. The tubes were vigorously shaken before incubation for 20 minutes in a dark place. The absorbance was determined at 510 nm. The identical method established a standard calibration curve for iron II sulfate. To prepare a stock solution of (1000 µg/ml) concentration of FeSO₄, 50.0 mg of the salt was dissolved in 10 ml of distilled water and diluted with the same solvent to 50 ml in a volumetric flask. The stock FeSO₄ solution in water was serially diluted to obtain working solutions with concentrations ranging from 100 to 800 mg/ml. The equivalent iron II sulfate concentrations were given in mg Fe²⁺/g dry weight.

Absorbance at 510 nm was plotted versus concentration to generate standard calibration curves. Using the FeSO₄ standard calibration curve and regression equation, the MCA was calculated as the FeSO₄ equivalent.

4.7.3. α -amylase inhibition (AAI)

The AAI of plant extracts was determined using the starch-iodine method [132]. To make an iodine solution, 0.1 g I₂ and 1.0 g KI were dissolved in 50 mL of distilled water. The starch solution was made by dissolving 1.0 g of starch in 50 mL of distilled water, with slow heating, then cooling it, and completing to 100 mL with distilled water in a volumetric flask. The amylase solution was made by mixing 15 mg of α -amylase suspension with 30 mL of phosphate buffer (pH 6.9). A 0.25 mL of α -amylase was incubated with 0.25 mL (3 mg/mL) of plant extract for 15 minutes at 37 °C. After adding 0.25 mL of starch solution, the mixture was re-incubated for 30 minutes. A 0.1 mL of HCl (1.0 M) was added to halt the reaction. An iodine reagent of 1.0 mL was added to 3.0 mL of distilled water with continuous vigorous stirring. A spectrophotometer was used to quantify the absorbance at 580 nm. Blank was prepared to modify the background absorbance of the measurements. The controls were done the same way as the trials, where plant extract for 0.25 ml of distilled water was substituted. The commonly used medicine for antidiabetic is acarbose, which was used as a positive control. The % inhibition was determined using the following formula:

$$\% \text{ Inhibition} = \frac{(A_c - A_s)}{A_c} \times 100 \quad (4.4)$$

Ac-absorbance for control; As-absorbance for standard.

4.8. Select a solvent for isolating plant extracts

TLC is often used to separate substances since its creation and operation require minimum effort and expense [133]. The solute and the mobile phase compete for binding sites on the stationary phase to separate molecules. UV radiation at 350-365 nm and 250-260 nm is primarily used for detection [134].

TLC could be seen as a micro-version of column chromatography, which it closely resembles. Quickly assess the purity of a mixture, the number of compounds present, and the combination of eluents to separate the compounds. TLC could be used to choose a solvent system for column chromatography. TLC, on the other hand, involves little sample preparation [135].

The adsorbent used in TLC is a thin, homogeneous layer (usually 0.24 mm thick) of a dry, finely ground substance placed on a suitable substrate, such as a glass plate, an aluminium sheet, or plastic foil. The mobile phase is then allowed to travel over the surface of the plate (often by capillary action), and the chromatographic phenomena may rely simply on adsorption, partition, or a mixture of the two, depending on the adsorbent, its treatment, and the nature of the solvents used. [60].

TLC confirmed the bioactive component separation from the methanolic extract. Numerous solvent systems were tested to choose the optimum one. Each extract was dissolved in MeOH to produce a 5% solution of crude extract. An appropriate solvent for TLC was chosen to use this solution (5% plant extract) for the fractionation and isolation of bioactive compounds. The application locations for the crude extract were indicated on the cut-to-size aluminium TLC plates. Capillaries were used to administer a crude extract solution to the indicated spots. The capillary with the crude extract solution was held vertically on the plate and then dropped by contact. After anticipating the solvent evaporating, place a second drop on it and allow it to dry thoroughly.

A 2.0 mL of the running solvent combination was added to the tank. The solvent combination in the tank was allowed some time to reach equilibrium with its vapour. The TLC plate containing the sample spot was positioned at a 45° angle in the development chamber, with the solvent covering the bottom of the plate up to roughly 1 cm. The solvent was let go around the plate without moving the tank. When the required solvent level was attained, the plates were removed from the tank using forceps. Before the solvent evaporated from the plate and dried, the solvent level was marked with a pencil. The plates were placed in an airtight container with a few iodine crystals. The chromatogram was examined under UV light (254 nm and 365 nm) to detect spots in an iodine chamber. According to TLC, each plant discovered an appropriate mobile phase. The selected mobile phase was used for biochemical separation in column chromatography due to its higher R_f (Retention factor) value.

4.9. Column chromatographic separation of bioactive compounds

TLC and column chromatography operate on the same basic principle. However, column chromatography is carried out differently. Unlike TLC, column chromatography uses a cylindrical tube (column) to hold the stationary phase (adsorbent) in place, while TLC uses flat solid support to hold the stationary phase (adsorbent) [136]. In the column chromatography experiment, a

cylindrical tube was used to hold the stationary phase, which consisted of 70-230 mesh silica gel (see Figure 4.7).

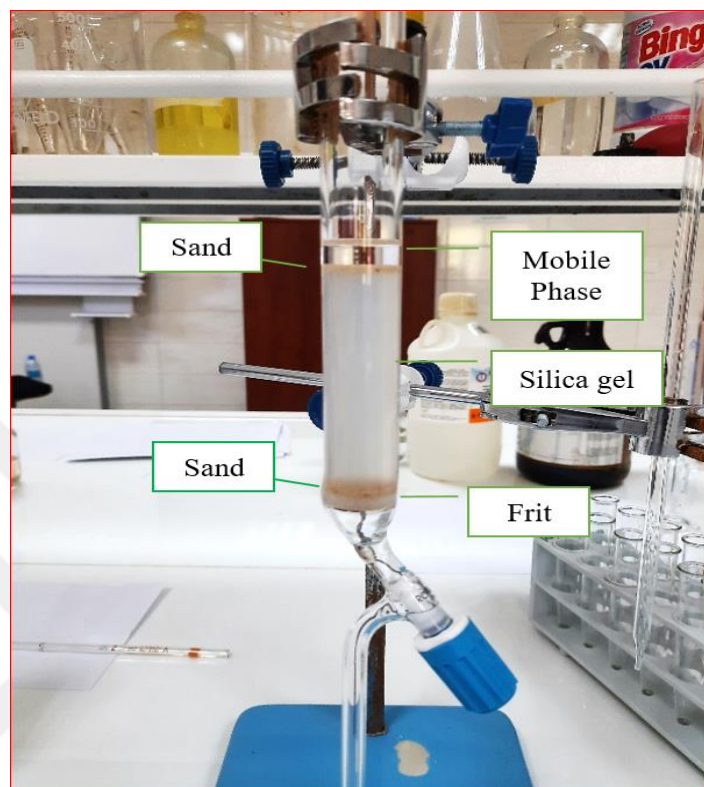


Figure 4.7. Column chromatography was applied to the separation of bioactive compounds in plant extracts

Before adding the adsorbent, a tiny piece of frit was put into the bottom of the column and then fill it halfway with the solvent (mobile phase) used for separation. Enough sand of about (0.5–1 cm) was added to cover the column's curved part completely. Then the required quantity of adsorbent was added to avoid any air bubbles that may enter the tightly packed column (the column was tapped during the addition of the adsorbent). A slurry suspension was formed by combining 10 grams of silica gel with five times the amount of mobile phase. The suspension was put into the column piecemeal and stirred frequently. The column was repeatedly struck with a plastic pipe to prevent air bubbles from forming during the pouring process. After filling the column, 0.5 to 1 cm of sand was added to the silica gel to protect its surface during sample injection. It was time to open the column tap and raise the solvent level to match the sand level.

The concentrated sample solution was applied to the adsorbent in the column's top bed after the column had been packed. During this step, a sufficient quantity of crude extract was mixed in the mobile phase solvent mixture to produce a concentrated solution. When the solution level reached the sand level, the continuous mobile phase was added to the column.

As soon as the stopcock was opened, the solvent (mobile phase) was added from the top of the column. Several bands were formed when the mobile phase went down the column under gravitational pressure. The topmost band was formed by the most heavily adsorbed component, whereas the least highly adsorbed component formed the bottommost band. Eventually, the separate components of the mixture began to emerge from the bottom of the column while the solvent continued to flow from the top. Fractions of the eluent exiting the column were collected in tubes (1-2 mL each) and analyzed. Separate tubes were used to collect the samples. TLC was performed on each fraction collected in the tubes using the mobile phase solvent combination. After that, we combined the fractions with identical R_f values and TLC chromatograms.

4.10. HPLC technique for analysis of phenolic components

The HPLC instrument equipped with a Shimadzu DGU-20A5 vacuum degasser and a Shimadzu 20 ADXR solvent pump was used to determine the polyphenol concentrations (see Figure 4.8a). Separations were performed using a reversed-phase Cliepus C18 5 m column (250 mm x 4.6 mm). Detection was carried out using a Shimadzu SPD-M20A photodiode array detector. Stock solutions for analytically pure polyphenol standards were prepared by dissolving 0.01 g of polyphenol in deionized clean water and completing to 10 mL volumetric flask with a 1:1 ratio of MeOH and water. This allowed us to produce 1000 mg/mL stock solutions to prepare analytically pure polyphenol standards. Prepared stock solutions were used as a starting point, and solution dilutions of each polyphenol were prepared. HPLC-DAD method was used to identify the phenolic compounds [137]. Likewise, MeOH was used to prepare plant extracts and fractions (see Figure 4.8b). Gradient elution using solvent A: 4.5% acetic acid solution and solvent B: acetonitrile as mobile phases at a flow rate of 1.0 mL/min and an injection volume of 20 μ L was used to ascertain the concentration. The diluted extract and the fractions were injected straight into the HPLC apparatus and screened using a photodiode array detector.



Figure 4.8. (a) Shimadzu HPLC-DAD instrument, (b) Preparation of the sample extracts and fractions for HPLC analysis

4.11. Statistical analysis

All parameters were measured in triplicate, and Microsoft Excel 2016 was used for statistical analysis. The results are reported as the average $\pm$ standard deviations (S.D.).

5. RESULTS AND DISCUSSION

5.1. Yield percentages of extracts

The extraction yield %(w/w) is defined as the mass of solid extract recovered relative to the initial dry extract quantity, which measures the solvent's effectiveness in extracting the target components from the starting material [138]. Table 5.1 shows the highest percentage yield obtained when sumac was extracted using the maceration technique with MeOH (39.3%), followed by blackberry sample percentage yield (34.4%) and fenugreek (11.6%). The lowest percentage yield (10.1%) was for purslane. (see Figure 5.1).

Table 5.1. Four medicinal plants' maceration extraction yielded a total weight

No.	Plants	Plant parts	Pre-extraction plant dry weight (g)	Plants' post-extraction dry weight (g)
1	Sumac	Fruit	10	3.93
2	Purslane	plant	10	1.01
3	Fenugreek	Seed	10	1.16
4	Blackberry	Fruit	10	3.44

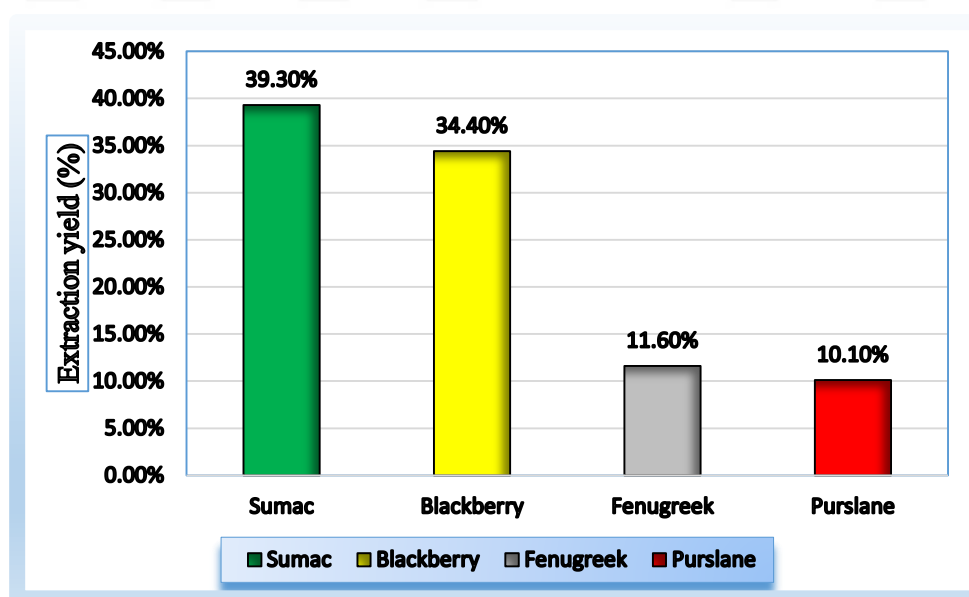


Figure 5.1. The percentage yield of each plant extract

5.2. Phytochemical quantification

Phytochemical analysis employs both qualitative and quantitative methods, with qualitative methods examining the presence or absence of particular compounds. This study focuses on quantitative analysis to determine the concentration or amount of phytochemicals in the four medicinal plant extracts obtained using methanol as a solvent.

5.2.1. Total phenolic content (TPC) analysis of dried extracts

Phenolic chemicals are significant plant ingredients that have antioxidant action due to their redox characteristics. Plant extracts' hydroxyl groups enhance free radical scavenging [139]. The Folin–Ciocalteu technique is a standard method that was used to determine the TPC of the dried plant extracts. This technique depends on transmitting electrons from phenolic compounds to the Folin–Ciocalteu reagent under alkaline conditions. The visible-light spectrophotometric analysis of polyphenols in plant extracts is based on their reaction with particular redox reagents (Folin–Ciocalteu reagent). The reaction produces a phosphotungstic-phosphomolybdenum complex, which acts as a blue chromophore, with maximal absorption varying with the solution's pH and the phenolic chemicals' content [140]. However, this reagent decomposes fast in alkaline solutions, necessitating a massive excess of the reagent for a complete reaction. This excess may cause precipitates and severe turbidity, preventing spectrophotometric analysis. Folin and Ciocalteu resolved this issue by including lithium salts in the reagent, which avoided turbidity [141]. The reaction generally yields reliable and specific data for multiple classes of phenolic compounds since several compounds change colour differentially owing to unit mass changes [140].

Folin—Ciocalteu reagent was used to quantify phenolic content as a standard for all extracts. The findings were calculated as the gallic acid equivalents (GAE) per gram of dry extract weight using a calibration curve ($y = 4.4519x + 15.747$, $R^2 = 0.9946$) for gallic acid concentrations between 5 and 200 $\mu\text{g/mL}$ (see Figure 5.2).

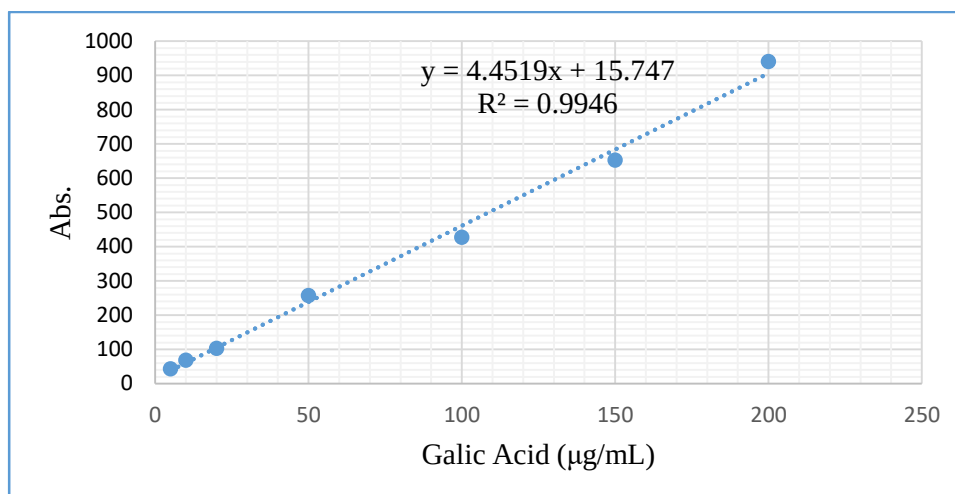


Figure 5.2. Gallic acid calibration curve for the TPC analysis of plant samples

Approximately an eight-fold difference was observed in the phenolic component concentration of MeOH extracts, ranging from 138.46 to 16.13 mg GAE/g. The lowest phenolic content was identified in purslane (16.13 ± 1.20 mg GAE/g), while the highest was found in sumac (138.46 ± 1.44 mg GAE/g), followed by blackberry (38.08 ± 0.19 mg GAE/g), and fenugreek (33.58 ± 1.39 mg GAE/g) (Table 5.2).

Table 5.2. The TPC of four medicinal plants

No.	Plants	TPC (mg GAE/g dry extract weight)
1	Sumac	138.46 ± 1.44
2	Purslane	16.13 ± 1.20
3	Fenugreek	33.58 ± 1.39
4	Blackberry	38.08 ± 0.19

GAE = gallic acid equivalent, Results are reported as mean $\pm$ SD of three determinations

In a literature review, Abdel-Mawgoud et al. found a TPC of 30.31 mg GAE/g dry weight of sumac in an 80.0% MeOH extract [142]. Santiago-Saenz et al. and Saffaryazdi et al. determined a TPC of 10.06 and 6.986 mg GAE/g dry weight of purslane, respectively [143,144]. Bhanger et al., Priya et al., and Al-Maamari et al., respectively, observed a TPC of 5.75, 0.876, and 2.16 mg GAE/g dry weight of fenugreek seed extract [145–147]. The TPCs for blackberry extract were determined to be 40.16, 4.61, and 3.26 mg GAE/g dry weight by Zafra-Rojas et al., Diaconeasa et al., and Koca and Karadeniz., respectively [115,148,149]. There was some discrepancy between the phenolic content results in this investigation and those previously reported in the literature. Differences in extraction techniques or geographical location may account for these differences in phenolic content [150].

5.2.2. Total flavonoid content (TFC) analysis of dried extracts

Among polyphenols, flavonoids are particularly beneficial since they aid the human body in fighting disease [151]. Flavonoids are hydroxylated phenolic chemicals often produced by plants in response to microbial diseases [152]. Flavonoids can scavenge hydroxyl radicals, superoxide anion radicals, and lipid peroxy radicals, and their pharmacological impact correlates with their antioxidant properties [153]. The potential of flavonoids to operate as powerful antioxidants is determined by their molecular structures, the location of the hydroxyl group, and other structural properties. They are found in abundance in plants as glycosides [151]. Quercetin is the most prevalent flavonol with antioxidant activity, and it possesses all of the necessary structural characteristics for free radical scavenging activity [154]. Flavonoid concentrations were significantly influenced by genetic diversity and biological, environmental, seasonal, and year-to-year changes [155].

The flavonoid content of selected plant extracts was measured using a colourimetric technique with aluminium chloride as a quantitative basis. Aluminium chloride forms acid-stable compounds with the C-4 keto group and the C-3 or C-5 hydroxyl groups of flavones and flavonols. Moreover, aluminium chloride forms acid-labile complexes with the orthodihydroxyl groups in flavonoids' A or B rings. These flavonol-formed complexes (including kaempferol, rutin, quercetin, and quercitrin) exhibit the highest absorbance between 415 and 440 nm [156].

Each extract's flavonoid concentration was calculated using a quercetin standard calibration curve (see Figure 5.3), and the findings were reported in terms of milligrams of quercetin equivalent per gram of dry extract. The extract's flavonoid concentration was calculated using three measurements. The results were based on a calibration curve for quercetin ($y = 4.2398x - 11.326$, $R^2 = 0.9977$) that was built from concentrations ranging from 5 to 200 $\mu\text{g/mL}$.

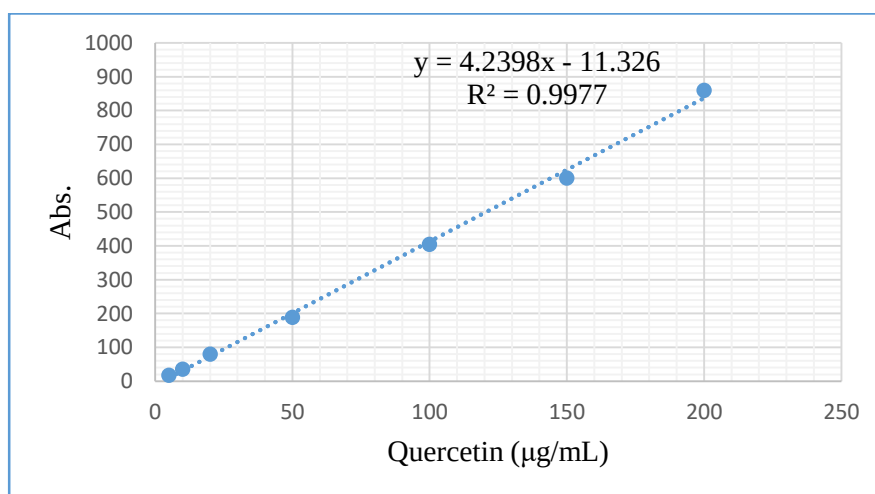


Figure 5.3. Quercetin calibration curve for the TFC analysis of plant samples

The flavonoid concentration of MeOH extracts varied from 16.11 to 5.54 mg QE/g, or by around three times as much as expected. Sumac was found to have the lowest phenolic content (5.54 ± 0.47 mg QE/g), while fenugreek had the highest (16.11 ± 0.71 mg QE/g), followed by blackberry (14.58 ± 0.83 mg QE/g) and purslane (12.91 ± 0.88 mg QE/g) (Table 5.3).

Table 5.3. The TFC of four medicinal plants

No.	Plants	TFC (mg QE/g dry extract weight)
1	Sumac	5.54 ± 0.47
2	Purslane	12.91 ± 0.88
3	Fenugreek	16.11 ± 0.71
4	Blackberry	14.58 ± 0.83

QE = Quercetin equivalent, Results are reported as mean $\pm$ SD of three determinations

According to a literature search by Abdel-Mawgoud et al., 80% MeOH extract of sumac has a TFC of 20.93 mg QE/g dry weight [142]. The TFC values for purslane were 8.97 and 0.469 mg QE/g dry weight by Santiago-Saenz et al. and Saffaryazdi et al., respectively [143,144]. The TFC values of 0.607, 0.489, and 0.328 mg QE/g dry weight of fenugreek seed extract were recorded by Bhanger et al., Priya et al., and Al-Maamari et al., respectively [145–147]. Diaconeasa et al. reported that the TFC for blackberry extract was 0.07 mg QE/g dry weight [148]. There was some inconsistency between our investigation's flavonoid content findings and those found in the literature.

5.2.3. Total flavonol (TF) content analysis of dried extracts

One of the most investigated groups of polyphenolic phytochemicals, flavonols are of great interest due to their powerful antioxidant properties and other biological activities [157]. Flavonols, also known as 3-hydroxyflavones, are a single subgroup of flavonoids. Vegetables, fruits, and their liquid counterparts are the most common sources [158]. Rutin, quercetin, myricetin, and kaempferol are this subclass's most well-known and significant chemicals [159]. The plant compound rutin (3, 3', 4', 5, 7-pentahydroxyflavone-3-rhamnoglucoside) may be found in a wide variety of vegetation. It inhibits the growth of bacteria, viruses, fungi, allergies, diabetes, cancer, the kidneys, the blood vessels, inflammation, and hypertension. Rutin has demonstrated the ability to improve thyroid function by modulating a number of signal transduction pathways [160].

Numerous plant foods include flavonols, a class of flavonoids and polyphenols that critically prevent coronary heart disease and other age-related diseases such as dementia [161]. Because it has the optimal structural characteristics for free radical scavenging action, quercetin is the most prevalent flavonol with excellent antioxidant function [154]. Among flavonols, many names are

rutin, also called quercetin-3-rutinoside, rutoside, and sophorin. It is a powerful plant-based antioxidant often used to treat many human ailments, including diabetes, cancer recurrence, oxidative stress, microbial contamination, and cardiovascular disease [162].

Colourimetric analysis based on aluminium chloride provided a quantitative assessment of the flavonol content of certain plant extracts. The TF content of the MeOH extract of medicinal plants was calculated in milligrams of rutin equivalents (RE) per gram of dry extract using the calibration curve's regression equation ($y = 2.4785x - 17.965$, $R^2 = 0.9963$) (see Figure 5.4).

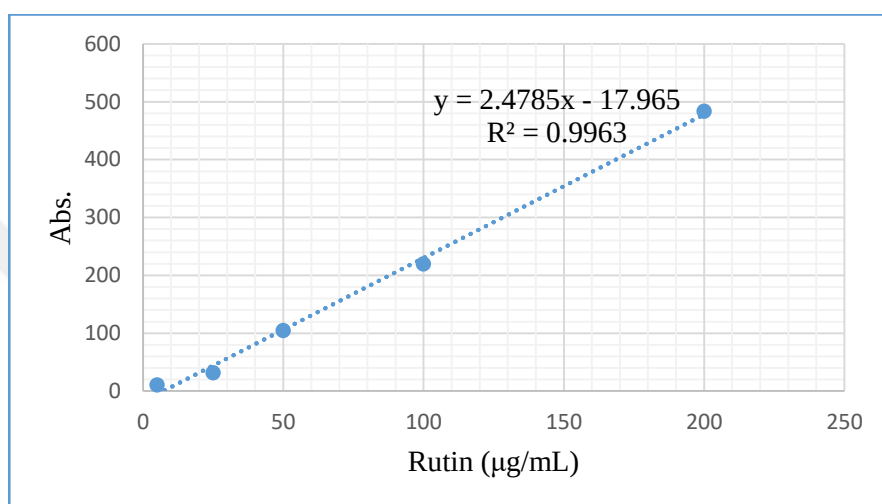


Figure 5.4. Rutin calibration curve for the TF content analysis of plant samples

The flavonol content in MeOH extracts ranged from 21.81 to 6.95 mg RE/g, or around three times the predicted value. The lowest flavonol concentration was identified in blackberry (6.95 ± 0.76 mg RE/g), while the greatest was found in fenugreek (21.81 ± 0.59 mg RE/g), followed by sumac (10.15 ± 0.58 mg RE/g) and purslane (8.07 ± 0.23 mg RE/g) (Table 5.4). Compared to overall flavonoid content, TF values are somewhat more significant for sumac and fenugreek than for other therapeutic plants.

Table 5.4. The TF content of four medicinal plants

No.	Plants	TF (mg RE/g dry extract weight)
1	Sumac	10.15 ± 0.58
2	Purslane	8.07 ± 0.23
3	Fenugreek	21.81 ± 0.59
4	Blackberry	6.95 ± 0.76

RE = Rutin equivalent, Results are reported as mean $\pm$ SD of three determinations

5.2.4. Total anthocyanin content (TAC) analysis of dried extracts

Phytochemicals such as anthocyanins are naturally occurring pigments that colour fruits, vegetables, and plants. Apart from chlorophyll, they are the most prevalent group of plant pigments that may be seen [163]. Antiallergic, anti-inflammatory, antiviral, and antioxidant effects of anthocyanins have been shown in vitro and in vivo, indicating that they also work as reducing agents, hydrogen donors, and singlet oxygen quenchers [164]. To achieve this effect depends on it is crucial to consider how many OH groups are present, how much structural conjugation is present, and if electron-donating and electron-withdrawing substituents exist in the ring structure [165]. cyanidin, delphinidin, pelargonidin, petunidin, peonidin, and malvidin are typical examples of anthocyanidins found in plant foods; their hydroxyl and methoxyl group substituents vary in quantity and location [166].

Because of its speed and simplicity, the pH differential approach is the most often used method for assessing anthocyanins. Colouration in monomeric anthocyanin pigments is pH-dependent, with the colourful oxonium form at pH 1.0 and the colourless hemiketal form dominating at pH 4.5. Degraded anthocyanins in the polymeric form are pH-insensitive and excluded from the assays because they absorb pH 4.5 and 1.0. Absorbance at 520 nm is inversely related to pigment content. Using equation 4.3, the data is reported in cyanidin-3-glucoside equivalents [167].

Approximately a twenty-two-fold difference was observed in the anthocyanin concentration of MeOH extracts, ranging from 0.34 to 7.73 mg cy-3-glu/kg dry extract weight. Blackberry (7.73 ± 1.17 mg cy-3-glu/kg dry weight) had the highest anthocyanin concentration, followed by sumac (5.33 ± 1.67 mg cy-3-glu/kg dry weight) and fenugreek (1.87 ± 1.69 mg cy-3-glu/kg dry weight). Purslane had the lowest anthocyanin concentration (0.34 ± 1.87 mg cy-3-glu/kg dry weight) (Table 5.5).

Table 5.5. The TAC of four medicinal plants

No.	Plants	TAC (mg cy-3-glu/kg dry extract weight)
1	Sumac	5.33 ± 1.67
2	Purslane	0.34 ± 1.87
3	Fenugreek	1.87 ± 1.69
4	Blackberry	7.73 ± 1.17

Results are reported as mean $\pm$ SD of three determinations

5.3. Antioxidant quantification

Due to the chemical variety of natural antioxidants, it is challenging to isolate, identify, and quantify individual antioxidants from a complex food/biological matrix. Furthermore, since individual antioxidant species work together to promote health, measuring the overall antioxidant capacity is frequently more appropriate. Antioxidant tests may be roughly categorized as electron transfer (ET), or hydrogen atom transfer (HAT) based, and because of the absence of standardized measurement methodologies, there is currently no single antioxidant test for food labelling [32].

5.3.1. Ferric reducing antioxidant power (FRAP) analysis of dried extracts

Nutritional scientists have made extensive use of the FRAP test. The FRAP test has been used to study the absorption of antioxidants from foods such as soya milk, cocoa, and tea, as well as the impact of processing and cooking on the antioxidant content of foods. The FRAP test has also been used in the agri-food business as a quality control measure to determine how "total antioxidant content" varies with factors including climate, growing season, and how long food has been stored [168].

The reducing power test is often used to assess an antioxidant's capacity to donate an electron [169]. The FRAP test is a straightforward, colourimetric approach based on the antioxidants' capacity to reduce Fe^{3+} to Fe^{2+} . The FRAP test directly determines a substance's reducing capacity, a critical characteristic for determining if a chemical is an effective antioxidant.[170] In this experiment, extracts were tested for their potential to reduce Fe^{3+} to Fe^{2+} . As a consequence of the antioxidants present in the extracts, the ferric cyanide complex (Fe^{3+}) was reduced to ferrous cyanide (Fe^{2+}). The reducing power of compounds is measured by their ability to induce the reduction of Fe^{3+} to Fe^{2+} in a solution, which results in a colour change from green to blue [171].

The FRAP test is often employed because it is rapid, sensitive, and cheap. The FRAP test is extensively used across the globe, and there is currently an extensive database including the FRAP values of hundreds of foods, drinks, spices, and plants. The antioxidant potential of the plant extracts against ferric oxidation was calculated as quercetin equivalent (mg/g dry extract) using a calibration curve (see Figure 5.5) and equation ($y=1.9869x + 65.366$, $R^2=0.9916$).

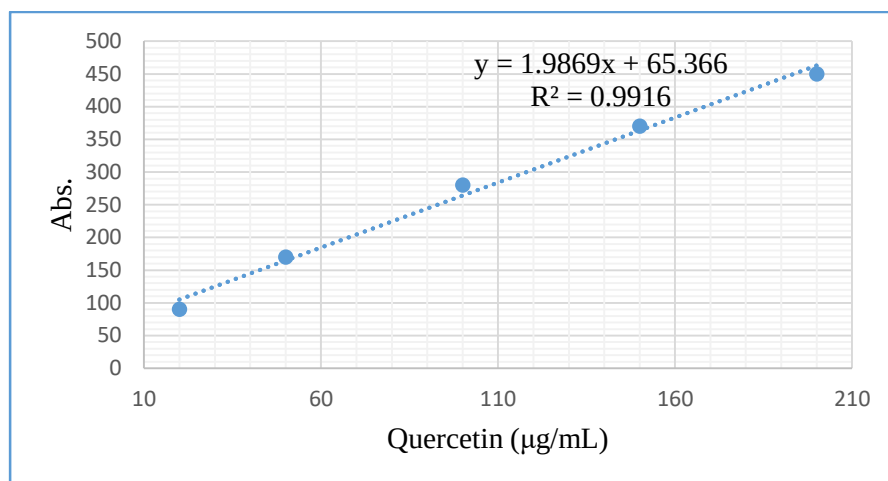


Figure 5.5. Quercetin calibration curve for the FRAP analysis of plant samples

According to the FRAP test findings, sumac has the maximum activity (92.31 mg QE/g dry extract). Blackberries were likewise quite active, with 20.53 mg QE/g dry extract. Fenugreek and purslane, with 11.07 and 6.45 mg QE/g dry extract, respectively, showed the lowest activity (Table 5.6).

Table 5.6. The FRAP of four medicinal plants

No.	Plants	FRAP (mg QE/g dry extract weight)
1	Sumac	92.31 ± 1.25
2	Purslane	6.45 ± 0.66
3	Fenugreek	11.07 ± 0.81
4	Blackberry	20.53 ± 0.47

Results are reported as mean ±SD of three determinations

A literature review conducted by CEYLAN et al. indicates that 0.31 μmol Fe²⁺/g dry weight was attributed to the FRAP of a sumac MeOH extract [172]. Studies by Habibian et al. found that purslane required 2000 mM FeSO₄/g dry weight to achieve FRAP levels [173]. Al-Maamari et al. found a FRAP of 0.259 mMoles/L dry weight for fenugreek extract [147]. Blackberry extract has FRAP values of 12511.44 μmol Fe (II)/100 g as reported by Zafra-Rojas et al., and 59.36 μmol Fe (II)/g dry weight as recorded by Koca and Karadeniz [115][149].

5.3.2. Metal chelating ability (MCA) analysis of dried extracts

Transition metals are essential for all living organisms to perform their crucial tasks. Different metal ions have various purposes in living organisms. In most situations, metals firmly attach to metalloproteins by establishing coordination bonds, and proteins gain a three-dimensional

structure [174]. Iron is required for efficient cell function, including oxygen transport and respiration, and serves as a cofactor for various iron metallic enzymes. Conversely, an excess of free iron may form reactive oxygen species through Fenton reactions, which are the breakdown of hydrogen or lipid peroxide generating highly reactive and biologically harmful hydroxyl and lipid peroxy radicals catalyzed by transition metals [175]. The beneficial Fe-binding potential of antioxidants owing to the antioxidants' metal-binding functional groups has been documented. Biological effects, such as antioxidant characteristics, may be modified by interacting Fe ions with antioxidant molecules [176,177].

The majority of reactive oxygen species (ROS) are by-products of mitochondrial electron transport and other metabolic activities. As a result of metal-catalyzed oxidation processes, ROS are generated as essential intermediates. The transition metal ion Fe^{2+} can generate free radicals in the solution using electron gain or loss. As a result, chelating metal ions with chelating agents reduces the generation of reactive oxygen species. The chelation ability of the extract was assessed using a chelation power test [178].

The chelation power test is included iron-chelation agent 1,10-phenanthroline is assumed to chelate primarily Fe^{2+} as $[\text{Fe}(\text{phenanthroline})]^{2+}$. The Fe^{2+} -phenanthroline combination absorbs visible wavelength light at 510 nm, and the measurement is directly proportional to the sample's Fe^{2+} level in the pH range of 2.5 to 8.0 [179]. Antioxidants are oxidized when they are exposed to FeCl_3 as they reduce Fe (III) to Fe (II). An excessive amount of FeCl_3 is used to ensure that all antioxidants are oxidized. The Fe-phenanthroline generated matches a sample's antioxidant content [180]. The MCA of plant extracts was estimated as ferrous sulfate equivalent (mg/g dry extract) using a calibration curve and equation ($y = 0.8821x - 52.514$, $R^2 = 0.9909$) (see Figure 5.6).

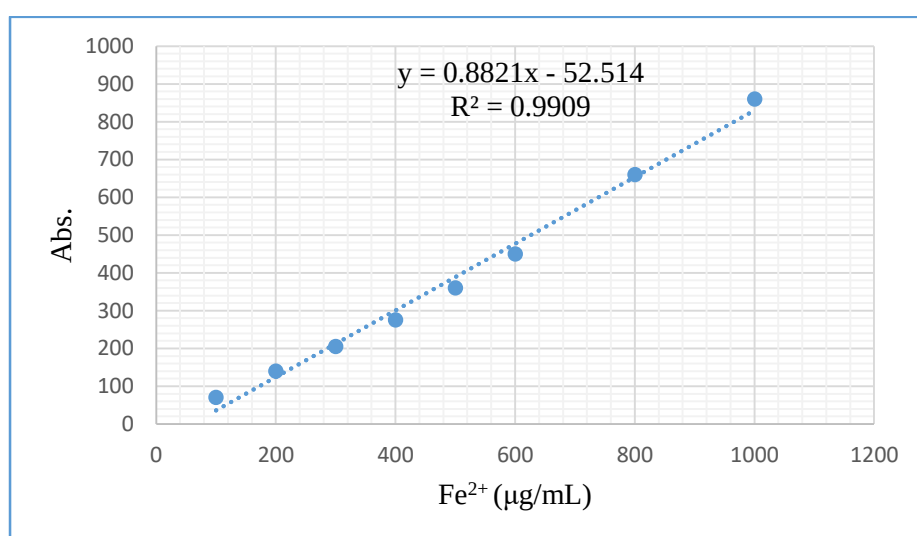


Figure 5.6. Ferrous sulfate calibration curve for the MCA analysis of plant samples

In MCA test found that sumac has the highest activity (638.46 mg Fe²⁺/g dry extract), while blackberries contents were quite dynamic with 182.12 mg Fe²⁺/g dry extract. The two herbs with minor activity were fenugreek (76.35 mg Fe²⁺/g dry extract) and purslane (63.71 mg Fe²⁺/g dry extract) (Table 5.7).

Table 5.7. The MCA of four medicinal plants

No.	Plants	MCA (mg Fe ²⁺ /g dry extract weight)
1	Sumac	638.46 ± 0.89
2	Purslane	63.71 ± 0.12
3	Fenugreek	76.35 ± 0.25
4	Blackberry	182.12 ± 0.26

Results are reported as mean ±SD of three determinations

According to a literature study by Bursal and Köksal, a sumac water extract's MCA was estimated to be 23.4 µg/ml dry weight [181]. Research by Binici et al. showed that 56.30 mg Fe²⁺/g dry weight was necessary for MCA levels in purslane [127]. Extracts of fenugreek were discovered to have an MCA of 1.02 mg/g dry weight by Bhanger et al. [145]. Bhagat and Thusoo found that the MCA content of blackberry extract was 437 µg/mL dry weight [182].

5.3.3. α-Amylase inhibition (AAI) assay of dried extracts

One of the essential enzymes in the human body is α-amylase, which breaks down starch into simpler sugars. Glucose absorption may be slowed by inhibiting this enzyme, causing a delay in carbohydrate breakdown. Consequently, diabetics' postprandial blood glucose levels are reduced, and their glucose tolerance is improved [183]. Amylase inhibitors reduce postprandial blood glucose levels by inhibiting amylase, sometimes called starch blockers. Reduced glycemic index and insulin resistance may be achieved by slowing down the digestion and breakdown of starch in people with diabetes [184].

Inhibitors of α-amylase have been found in many plants, making them potentially effective in treating diabetes. Studying natural extracts from accessible traditional medicinal plants might discover new antidiabetic medications. Several plants have been shown to inhibit α-amylase, making them potentially useful in treating diabetes [185]. By inhibiting α-amylase, carbohydrates (such as starch and maltose) undergo slower hydrolysis of α-1,4-glycosidic linkages, resulting in slower glucose absorption into the bloodstream [186].

The plant extracts were examined using the quantitative starch-iodine method to determine their capacity to inhibit amylase. Control samples containing no plant extract were generated and compared to test samples containing varying plant extracts in all trials. As a positive control,

acarbose was employed. Table 5.8 displays the amount of amylase inhibition enzyme in the plant extracts of four selected sample plants. The AAI test results show that purslane has the highest activity level (89.09%) and in the second level fenugreek (84.21%) came. Blackberry and sumac have the lowest activity levels at 76.01% and 65.71%, respectively.

Table 5.8. The AAI of four medicinal plants

No.	Plants	AAI (%)
1	Sumac	65.71 ± 1.90
2	Purslane	89.09 ± 1.16
3	Fenugreek	84.21 ± 0.99
4	Blackberry	76.01± 1.57

Results are reported as mean ±SD of three determinations

5.4. Sumac extract natural ingredients isolation

The sumac extract was produced according to the protocols detailed in Section 4.4.4. After collecting the sumac extract, TLC was performed to choose appropriate solvents. Open-column chromatography was utilized in the isolation procedure, and HPLC was performed to determine polyphonic compounds in the extract and fractions.

5.4.1. TLC-based solvent system selection for sumac extract isolation

The crude extract was dissolved in MeOH to create a solution containing 5% crude extract. An appropriate solvent for TLC was chosen using this solution. A small quantity of the mixture of extracts was spotted towards the plate's base of the TLC. The plate was then put in a developing chamber inside a shallow pool of a solvent, such that only the very bottom of the plate was submerged. This liquid, or eluent, is the mobile phase, and through capillary action, it progressively ascends the TLC plate.

As the solvent passes past the applied area, an equilibrium is created for each component of the mixture between the adsorbed molecules of that component and the molecules in the solution. In theory, the components will vary in their solubility and adsorption strengths to the adsorbent, and some will be transported higher up the plate than others. When the solvent has reached the top of the plate, the plate is taken out of the developing chamber, dried, and the separated components of the mixture are seen. If the compounds are coloured, their visibility is simplified. Typically, the compounds are colourless; thus, UV light is employed to see the plates. The solvents used in selecting solvent combinations and the resulting mixtures are listed in Table 5.9.

Table 5.9. Mobile phase solvent combinations for isolating sumac extract by TLC

No.	Solvent-1	Solvent-2	Solvent-3	Mixing ratio (v/v)
1	Acetone	MeOH		100:10
2	Chloroform	Acetone	MeOH	50:50:10
3	Chloroform	MeOH	Water	100:10:3
4	Chloroform	MeOH	Water	100:10:5
5	Chloroform	MeOH	Water	100:10:8
6	chloroform	Acetone		100:5
7	Chloroform	Acetone		100:10
8	Chloroform	Acetone		100:15
9	Chloroform	MeOH	Water	90:10:3
10	Chloroform	MeOH	Water	90:10:5
11	Chloroform	MeOH	Water	90:10:8
12	Chloroform	MeOH	Water	80:10:3
13	Chloroform	MeOH	Water	80:10:5
14	Hexane	EtOAc	MeOH	80:20:10
15	Hexane	EtOAc	MeOH	70:20:10
16	Hexane	EtOAc	MeOH	80:20:5
17	Hexane	EtOAc	MeOH	110:20:10
18	chloroform	Acetone	MeOH	90:10
19	Hexane	EtOAc	MeOH	110:20:20
20	Hexane	EtOAc	MeOH	90:20:20
21	Hexane	EtOAc	MeOH	110:20:20
22	Hexane	EtOAc	MeOH	110:20:15
23	Hexane	EtOAc		100:20
24	Hexane	EtOAc		100:30
25	Hexane	EtOAc		90:20
26	Hexane	EtOAc		80:20
27	Hexane	EtOAc		70:20
28	Chloroform	MeOH	Water	100:10:8
29	Chloroform	MeOH	Water	90:10:3
30	Chloroform	MeOH	Water	85:10:3
31	Chloroform	MeOH	Water	90:5:3
32	Chloroform	MeOH	Water	90:7:3
33	Chloroform	MeOH	Water	90:15:3
34	Chloroform	MeOH	Water	90:5:5
35	Chloroform	MeOH	Water	90:5:10
36	Chloroform	MeOH	Water: FA	90:5:3:5
37	Chloroform	MeOH	FA	90:5:5
38	Chloroform	Water	FA	90:5:5
39	Chloroform	MeOH	Water: FA	90:8:5:3
40	Chloroform	MeOH	Water	90:10:5
41	Chloroform	MeOH	Water: FA	100:8:3:3
42	Chloroform	MeOH	FA	90:10:3
43	Chloroform	MeOH	FA	125:15:3

44	Chloroform	MeOH	FA	100:10:3
45	Chloroform	MeOH	Water	95:10:3
46	Chloroform	MeOH	FA	200:20:3
47	Chloroform	MeOH	Water	150:15:3
48	Chloroform	MeOH	Water	150:15:10
49	Chloroform	MeOH	Water	160:15:15
50	Chloroform	MeOH	Water	140:15:15
51	Chloroform	MeOH	Water	130:15:15
52	Chloroform	MeOH	Water	170:15:15
53	Chloroform	MeOH	Water	120:15:15
54	Chloroform	MeOH	Water	100:15:15
55	Chloroform	MeOH	Water	200:15:15
56	Chloroform	MeOH	Water	150:15:15



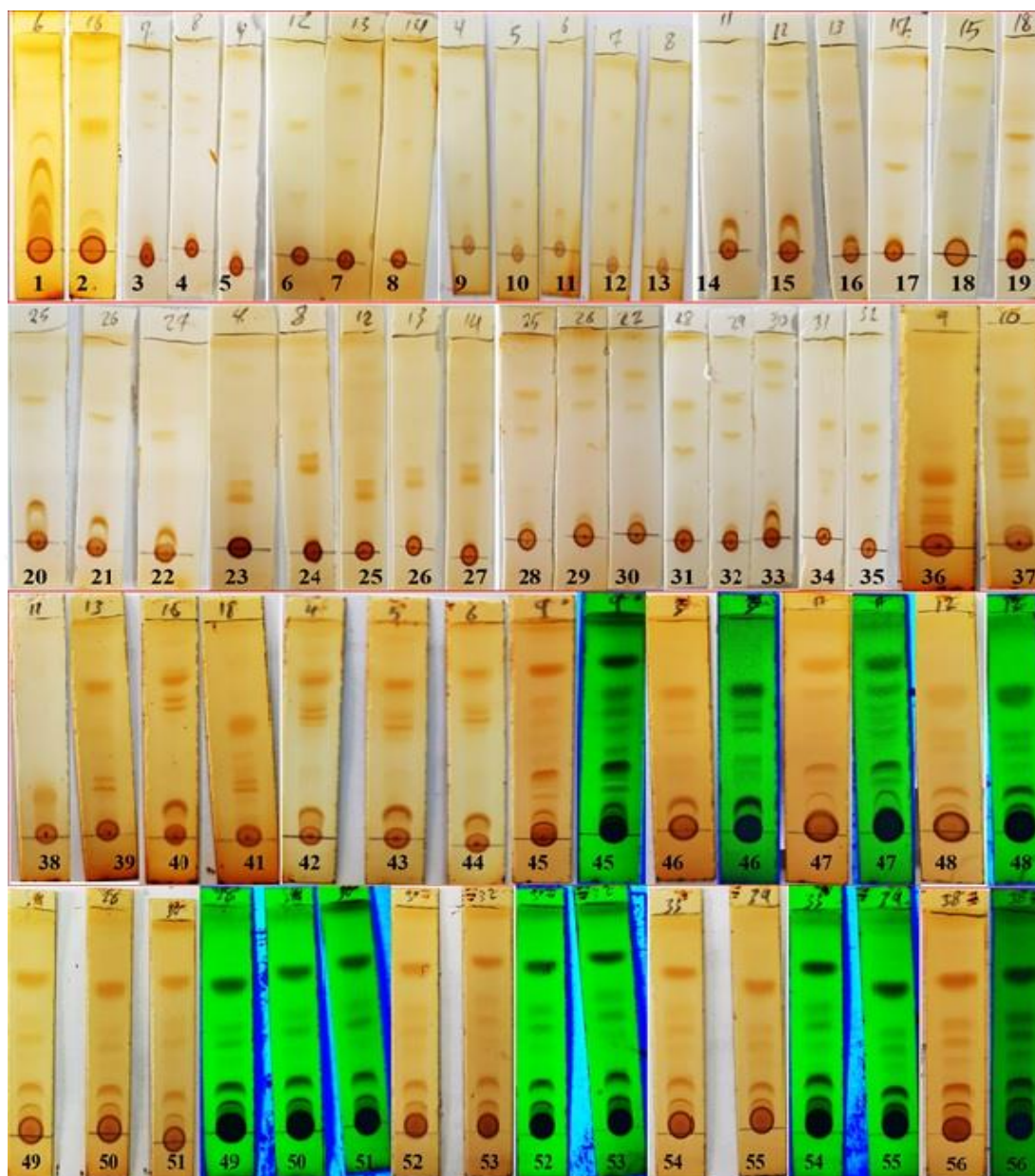


Figure 5.7. Sumac extract TLC chromatograms obtained in various solvent combinations

Analysis of the TLC chromatograms led to identifying the optimal solvent combination. Figure 5.7 shows chromatograms developed from solvent mixtures applied to TLC plates. A majority of solvent combinations exhibited two spots or more on TLC plates. For instance, the mobile phase solvents mixtures of (acetone:MeOH) in the ratio of (100:10) and (chloroform:MeOH:water) in the ratio of (90:10:5) produced four spots. However, the mobile phase solvents mixture of (chloroform:MeOH:water) in the ratio (150:15:15) provided more than six spots and had a high R_f value. Hence, it was chosen for column chromatography. When attempting to do column chromatography using the mobile phase (chloroform:MeOH:water, 150:15:15), the

sumac extract did not dissolve in this mobile phase. Therefore, an attempt was made to perform column chromatography using the dry packing technique.

5.4.2. Dry loading column chromatography for sumac extract isolation

Column chromatography is a technique for purifying specific compounds based on the polarities of the stationary phase, eluents, and compounds. This approach is based on the interaction of polar chemicals with silica, which causes the molecules in the target extract/fraction to separate. The separation is determined by the degree of polarity of the eluent. TLC was used to determine retention variables. A similar eluent method was later utilized in column chromatography. The mixture's quantity and the number of chemicals were considered while selecting column size and silica type [187]. Column chromatography is one of the best ways to separate phytocomponents.

First, a dry loading column was employed for column chromatography, which is dependent on the materials being dried onto silica gel for loading. When this is done, a 2- to 10-fold excess (by mass) of silica is usually adequate. When drying samples on silica, the sample-silica to chromatographic-silica ratio must be correct. While a 1:20 sample-to-silica ratio is adequate for wet loading, a 1:40 ratio may be more suited for dry loading [188]. The mobile phase of chloroform:MeOH:water in the ratio (150:15:15) was chosen for the dry loading column chromatography depending on the highest R_f value obtained from the TLC investigation of the sumac extract.

"Dry loading procedure" First, dilute the sample to be tested with the smallest quantity of solvent and then add about 100 mg of silica gel. Stir the mixture until the solvent evaporates and all that is left is a dry powder. Place the dry powder on a piece of folded weighing paper and then move it to the top of the prepared column. Now that a new eluting solvent has been added to the top, we may begin the elution process [189].

As demonstrated in Figure 5.8, no fraction was separated from the column chromatography technique after utilizing our mobile phase; as a result, this mobile phase was not useful for extract separation of the sumac plant.

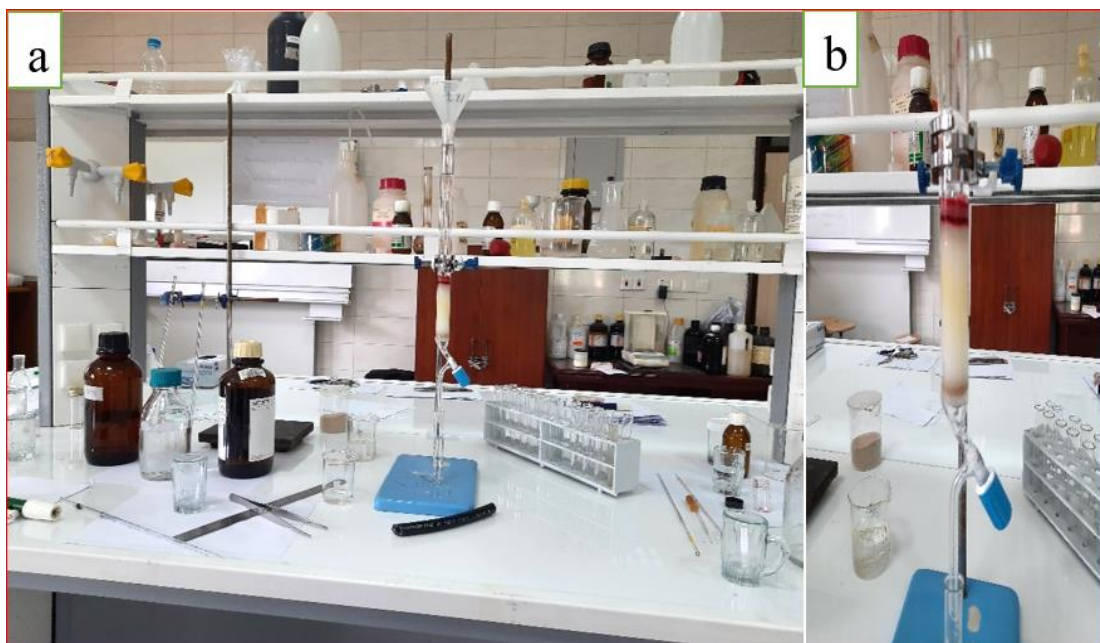


Figure 5.8. Sumac isolation using dry loading with mobile phase (chloroform:MeOH:water, 150:15:15), (a) begin column chromatography elution (b) column chromatography is being performed

5.4.3. Second attempt at selecting a TLC-based solvent for sumac extract

The process of isolating sumac extract using the most effective mobile phase was attempted for the second time due to failure in the dry loading procedure. It is evident from Table 5.10 that several different solvent combinations were attempted. As seen in Figure 5.9, the sixth mobile phase solvents mixture generated four spots, whereas mobile phase solvents mixture number twelve generated just three. Each mobile phase produces more than two spots. However, mobile phase number sixty-two caused the production of six spots; as a result, this mobile phase was employed for column chromatography because it could dissolve sumac extract.

Table 5.10. Mobile phase solvent combinations for isolating sumac extract by TLC for the second attempt

No.	Solvent-1	Solvent-2	Solvent-3	Mixing ratio (v/v)
1	Hexane	EtOAc	MeOH	90:20:20
2	Hexane	EtOAc	MeOH	90:20:30
3	Diethyl ether	EtOAc	MeOH	90:20:20
4	Diethyl ether	EtOAc	MeOH	90:30:20
5	Chloroform	EtOAc	MeOH	80:50:20
6	Chloroform	EtOAc	MeOH	80:30:20
7	Chloroform	EtOAc	MeOH	80:20:20
8	Chloroform	EtOAc	MeOH	80:10:20
9	Acetone	MeOH		100:10
10	Acetone	MeOH		100:50
11	Chloroform	THF		100:10
12	Chloroform	THF		100:5
13	Chloroform	THF		150:5
14	Toluene	EtOAc		100:10
15	Toluene	EtOAc		100:15
16	Toluene	EtOAc	MeOH	100:10:10
17	Toluene	EtOAc	MeOH	100:10:15
18	Toluene	EtOAc	MeOH	100:15:10
19	Toluene	EtOAc		100:30
20	Toluene	EtOAc		100:50
21	Toluene	EtOAc	MeOH	100:30:10
22	Toluene	EtOAc	MeOH	100:15:15
23	Toluene	EtOAc	MeOH	100:10:20
24	Toluene	EtOAc	MeOH	80:10:10
25	Toluene	EtOAc	MeOH	60:10:10
26	Toluene	EtOAc	MeOH	60:15:10
27	Toluene	EtOAc	MeOH	60:10:15
28	Toluene	EtOAc	MeOH	50:10:10
29	Toluene	EtOAc	MeOH	60:15:15
30	Toluene	EtOAc	MeOH	60:20:15
31	Toluene	EtOAc	MeOH	60:30:15
32	Chloroform	MeOH	FA	125:10:3
33	Chloroform	MeOH	FA	125:5:3
34	Chloroform	MeOH	FA	150:10:3
35	Chloroform	MeOH	FA	150:15:3
36	Chloroform	MeOH	FA	125:10:8
37	Chloroform	MeOH	FA	125:15:3
38	Chloroform	MeOH	FA	110:10:3
39	Toluene	Acetone	MeOH	90:10:10

40	Toluene	Acetone		70:30
41	Toluene	Acetone	MeOH	70:30:5
42	Toluene	Acetone	MeOH	80:10:10
43	Toluene	Acetone	MeOH	70:10:10
44	Toluene	Acetone	MeOH	60:40:5
45	Toluene	Acetone		60:40
46	Toluene	THF		100:5
47	Toluene	Acetonitrile	MeOH	90:10:10
48	Toluene	MeOH		90:10
49	Toluene	MeOH		90:20
50	Toluene	EtOAc	FA	4:2:1
51	Toluene	EtOAc	FA	10:2:1
52	Toluene	EtOAc	FA	10:4:3
53	Toluene	EtOAc	FA	4:4:2
54	Toluene	EtOAc	FA	4:2:3
55	Toluene	EtOAc	FA	10:2:4
56	Toluene	EtOAc	FA	10:4:4
57	Toluene	EtOAc	FA	4:4:3
58	Toluene	EtOAc	FA	4:5:2
59	Toluene	EtOAc	FA	10:6:3
60	Toluene	EtOAc	FA	4:5:4
61	Toluene	EtOAc	FA	4:8:1
62	Toluene	EtOAc	FA	4:5:2

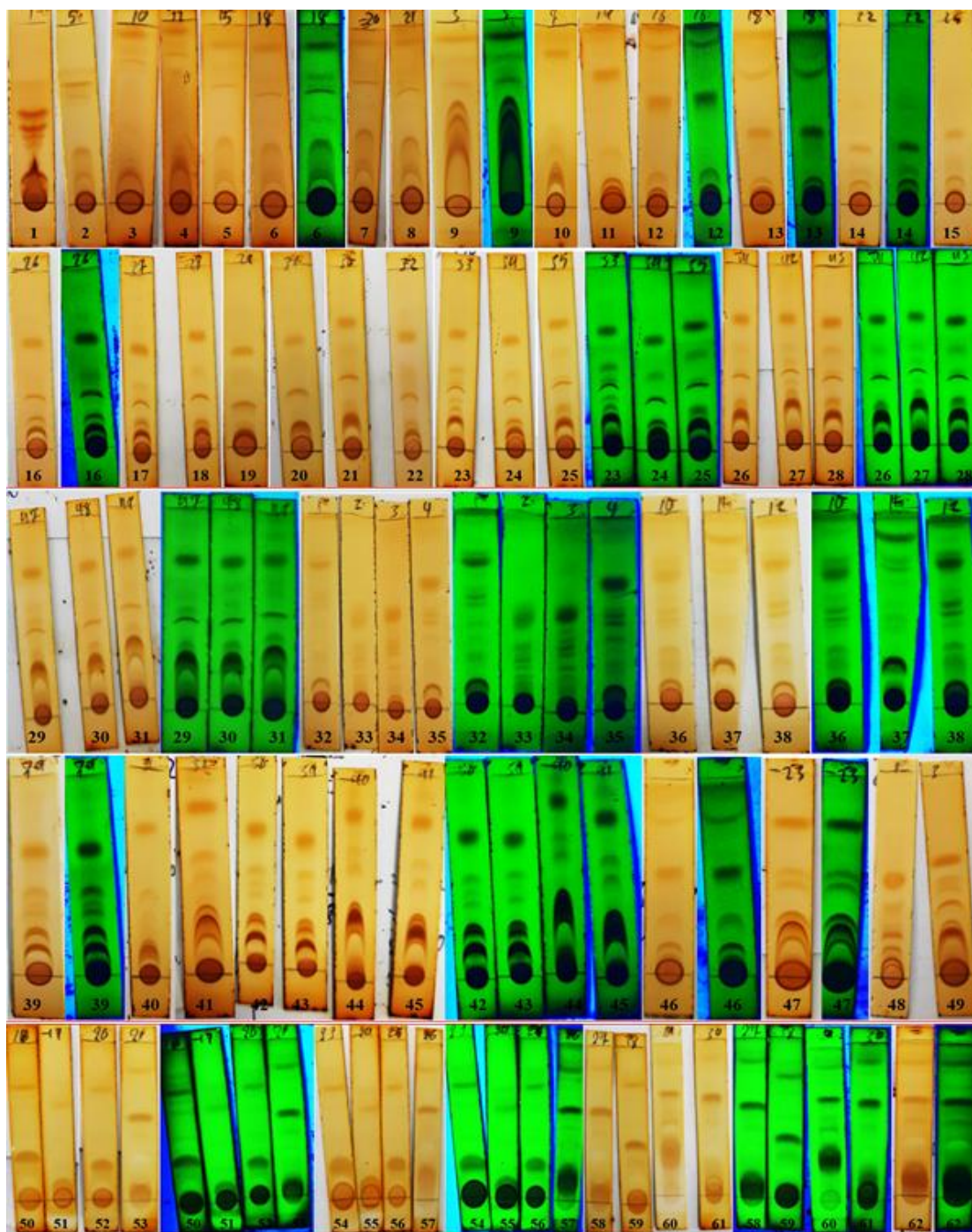


Figure 5.9. Second trial TLC chromatograms of sumac extract resulting from various solvent combinations

5.4.4. Isolation of sumac extract by wet loading Column Chromatography

The second method used was wet-loading column chromatography. This study innovated an appropriate mobile solvent system and after determining the optimal solvent combination for sumac extract using TLC, the solution of toluene:EtOAc:FA in the ratio (4:5:2) was used as the mobile phase for column chromatography of sumac extract. After determining the optimal solvent

combination for sumac extract through TLC, this solution was utilized as the mobile phase for column chromatography (see Figure 5.10).

Therefore, the chromatography was done on an empty glass column filled with activated silica gel and washed with the mobile phase (toluene:EtOAc:FA) in the ratio of (4:5:2). The stationary phase was a silica gel with a mesh size between 70 and 230. It was mixed with a solvent solution and put on the column. A concentrated solution was prepared by dissolving an adequate quantity of sumac extract in the mobile phase solvent combination. This solution was loaded into the column in a single application. The continuous mobile phase was added when the solution reached the sand level. Thus, entrainment and elution of the sumac extract were performed on the column. TLC plates were applied to monitor the collected fractions, and the relevant fractions were mixed and then concentrated under low pressure. Finally, distinct plant extract fractions were available.

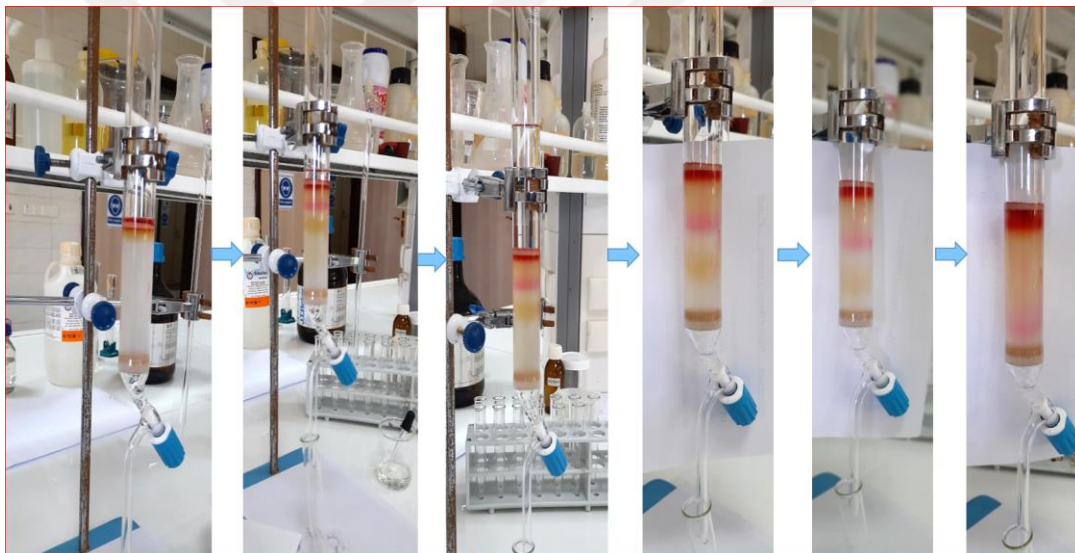


Figure 5.10. Column chromatography progresses after loading the sumac extract solution with toluene:EtOAc:FA (4:5:2) elution

5.4.5. TLC for sumac extract fractions and coded fractions

The eluate exiting the column was collected in a succession of tubes (1-2 mL) as fractions. Seventy different tubes were used for collecting the eluate from the column. Using the mobile phase solvent combination toluene:EtOAc:FA (4:5:2), TLC was performed on each fraction collected in the tubes (see Figure 5.11). Totalling 70 tubes, this collection represents a substantial portion of the whole. They were combining fractions whose R_f values and TLC chromatograms were comparable.

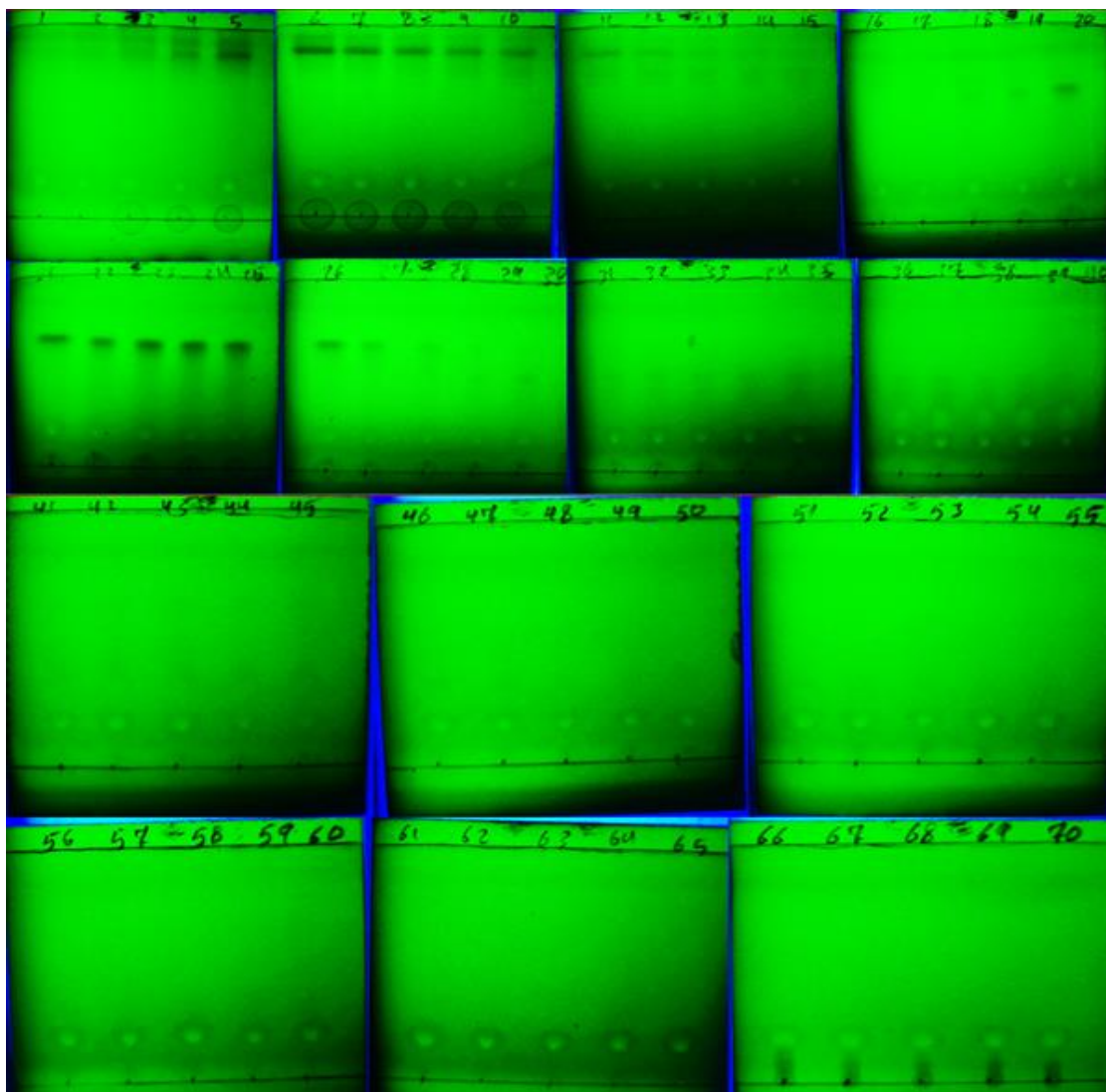


Figure 5.11. TLC for all sumac extract tubes collected following column chromatography elution with toluene:EtOAc: FA (4:5:2)

The combined tubes are coded as follows: fractions 1-2 "Sum1," fractions 3-17 "Sum2," fractions 19-28 "Sum3," fractions 29-35 "Sum4," fractions 36-55 "Sum5," and fractions 50-65 "Sum6". Figure 5.12 summarises the coding of all fractions produced by column chromatography.

Following the chromatographic separation, the portion of the substance that had not been removed from the silica gel was cleaned using MeOH. This section was also given the designation "Sum0".

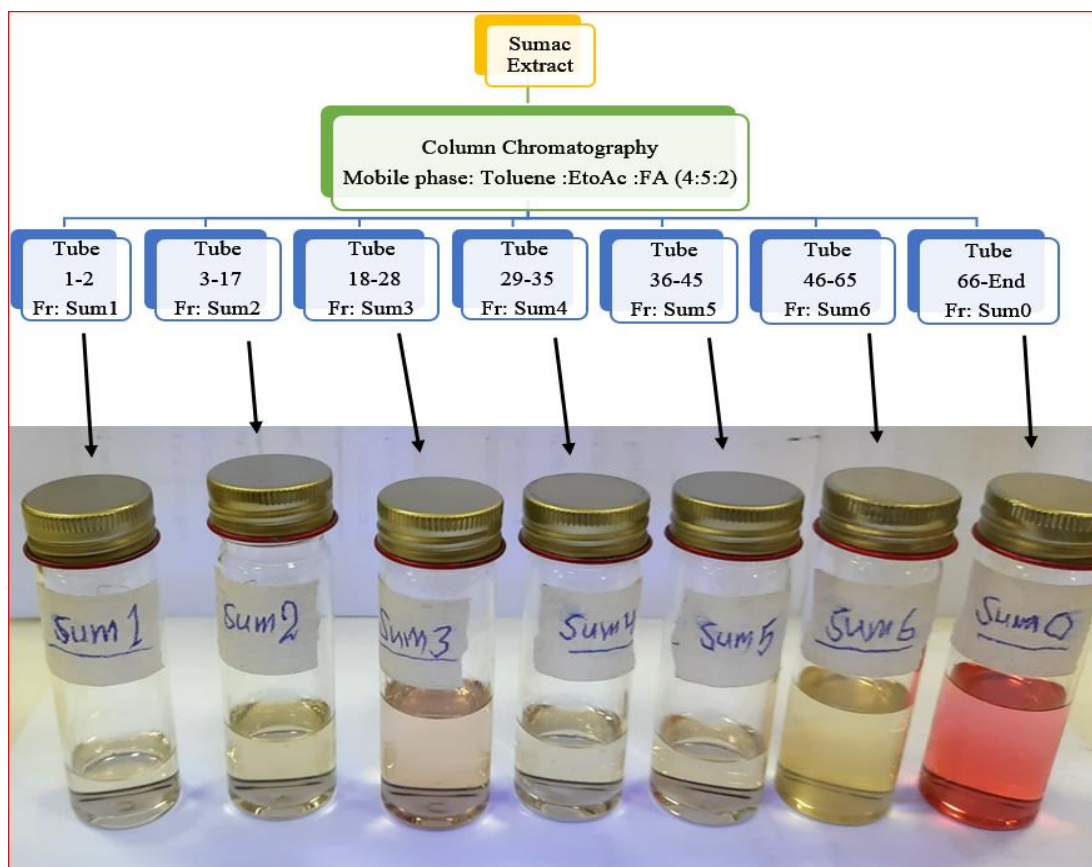


Figure 5.12. Coding of all tubes obtained from column chromatography and sumac extract fractions

Following concentration in a rotary evaporator at 40 °C and decreased pressure and air-drying, the fractions were completed in a vacuum oven. Then, for HPLC analysis, all fractions were stored in clean, labelled vials in the refrigerator at 4-5 °C.

5.4.6. HPLC analysis of sumac extract and fractions

The complexity of natural chemicals makes it difficult to investigate the structure of active substances from different plants and evaluate their antioxidant and biological capabilities. Therefore, it was necessary to identify and characterize some bioactive substances detected in the extract and fractions using high-resolution chromatographic techniques such as HPLC, commonly used to characterize polyphenolic compounds [190]. Efficacy was evaluated using the extract and seven sumac fractions (Sum0–Sum6). As shown in Table 5.11, the extract had at least two major constituents, while the fractions contained between two and four different chemicals.

Table 5.11. The phenolic component concentration of sumac extract and fractions (mg/kg)

No.	Samples	Phenolic compounds					
		Gallic acid	Catechin	p-Coumaric acid	Epicatechin	Rutin	Resveratrol
1	Extract	38.180	N.D.	N.D.	N.D.	11.210	N.D.
2	Sum1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
3	Sum2	N.D.	N.D.	0.430	N.D.	N.D.	N.D.
4	Sum3	172.68	43.820	N.D.	0.357	N.D.	N.D.
5	Sum4	5.280	8.650	N.D.	N.D.	N.D.	0.020
6	Sum5	3.920	3.210	N.D.	N.D.	N.D.	0.110
7	Sum6	4.790	5.880	N.D.	N.D.	N.D.	0.030
8	Sum0	1.070	3.240	N.D.	N.D.	N.D.	N.D.

N.D., Not detected.

5.5. Purslane extract natural ingredients isolation

Following the procedures outlined in Section 4.4.4, the purslane extract was prepared. The purslane extract was collected, and TLC was used to determine the best solvents. The isolation process was carried out using open-column chromatography, and the extract and fractions' polyphonic components were identified using HPLC.

5.5.1. TLC-based solvent system selection for purslane extract isolation

To discover the appropriate solvent or combination of solvents (a "solvent system") for developing a TLC plate or chromatography column containing an unknown mixture, it is necessary to alter the polarity of the solvent in many trials and run a trial-and-error procedure. Observe and document the chromatographic outcomes for each solvent system. As the polarity of the solvent system increases, all the components in the mixture will move faster (and vice versa with lowering the polarity). The optimum solvent system is the one that provides the most significant separation.

Typically, TLC elution patterns transfer to column chromatography elution patterns. TLC is often used to find the optimal solvent system for column chromatography since it is a much quicker technique than column chromatography. For instance, the optimal solvent system for a chromatography technique advances the desired component of the mixture to a TLC R_f of 0.25 to 0.35. It separates it from its closest neighbour by a TLC R_f value difference of at least 0.20. TLC is used to find the optimal solvent(s) for a chromatography method by analyzing a combination.

Frequently, novices do not know where to begin: Which solvents should be taken from the shelf to elute a TLC plate? Typical solvents include hexanes (or petroleum ethers/ligoin) and ethyl

acetate due to concerns about toxicity, cost, and flammability (an ester). However, diethyl ether is usable. It is very combustible and volatile. Alcohols (MeOH, EtOH) may be utilized. Since it is caustic, nonvolatile, very polar, and emits irritating vapours, acetic acid (a carboxylic acid) may be employed as a tiny percentage of the system. Ketone acetone may be used. The halogenated hydrocarbons methylene chloride and chloroform are effective solvents, but they are hazardous and should be avoided wherever feasible. Suppose the performance and toxicity of two solvents are the same. In that case, the more volatile solvent is chosen in chromatography since it will be simpler to remove the target molecule following separation by column chromatography.

The best solvent mixture was found by analyzing the TLC chromatograms. The chromatograms formed by applying various solvent combinations to TLC plates are shown in Figure 5.13 (Table 5.12). On TLC plates, most solvent combinations revealed two or more spots. For example, the mobile phases (hexane:EtOAc:MeOH 100:40:20) and (hexane:DCM:MeOH 70:40:5) generated four spots. However, the mobile phase (hexane:DCM:MeOH 70:50:8) gave six spots and had a high R_f value, as did the mobile phase (hexane:diethyl ether:MeOH 65:35:10). For column chromatography, the mobile phase (hexane:diethyl ether:MeOH, 65:35:10) was selected.

Table 5.12. TLC was applied to identify the purslane extract solvent mixture

No.	Solvent-1	Solvent-2	Solvent-3	Mixing ratio (v/v)
1	EtOAc	Petroleum ether		100:50
2	Hexane	EtOAc		100:5
3	Hexane	EtOAc		100:10
4	Hexane	EtOAc		100:15
5	Hexane	EtOAc	MeOH	100:10:10
6	Hexane	EtOAc	MeOH	100:10:15
7	Hexane	EtOAc	MeOH	100:15:15
8	Hexane	EtOAc	MeOH	100:40:20
9	Hexane	EtOAc	MeOH	100:20:15
10	Hexane	EtOAc	MeOH	100:25:15
11	Hexane	EtOAc	MeOH	100:15:20
12	Hexane	EtOAc	MeOH	100:15:25
13	Hexane	EtOAc	MeOH	100:15:30
14	Hexane	EtOAc	MeOH	100:15:20
15	Hexane	EtOAc	MeOH	80:15:20
16	Hexane	Diethyl ether		100:100
17	Hexane	Diethyl ether		50:10
18	Hexane	Diethyl ether		90:10
19	Hexane	Diethyl ether		80:20
20	Hexane	Diethyl ether		70:30

21	Hexane	Diethyl ether		40:60
22	Hexane	Diethyl ether	MeOH	90:10:10
23	Hexane	Diethyl ether	MeOH	80:20:10
24	Hexane	Diethyl ether	MeOH	70:30:10
25	Hexane	Diethyl ether	MeOH	40:60:10
26	Hexane	Diethyl ether	MeOH	60:40:10
27	Hexane	Petroleum ether	MeOH	70:30:10
28	Hexane	DCM	MeOH	70:30:10
29	Hexane	DCM	MeOH	70:30:5
30	Hexane	DCM	MeOH	70:30:15
31	Hexane	DCM	MeOH	70:30:10
32	Hexane	DCM	MeOH	75:25:10
33	Hexane	Diethyl ether	MeOH	75:25:10
34	Hexane	EtOAc	MeOH	100:10:15
35	Hexane	EtOAc	MeOH	100:15:20
36	Hexane	EtOAc	MeOH	100:15:15
37	Hexane	MeOH		100:10
38	Hexane	MeOH		100:15
39	Hexane	Diethyl ether	MeOH	70:30:10
40	Hexane	Diethyl ether	MeOH	70:30:15
41	Hexane	Diethyl ether	MeOH	70:30:7
42	Hexane	Diethyl ether	MeOH	70:20:10
43	Hexane	Diethyl ether	MeOH	70:40:10
44	Hexane	DCM	MeOH	65:30:10
45	Hexane	DCM	MeOH	80:30:10
46	Hexane	DCM	MeOH	70:20:10
47	Hexane	DCM	MeOH	70:30:7
48	Hexane	DCM	MeOH	70:30:15
49	Hexane	DCM	MeOH	70:40:5
50	Hexane	DCM	MeOH	70:50:5
51	Hexane	DCM	MeOH	70:50:10
52	Hexane	DCM	MeOH	70:50:8
53	Hexane	Diethyl ether	MeOH	75:25:10
54	Hexane	Diethyl ether	MeOH	60:30:10
55	Hexane	Diethyl ether	MeOH	65:35:10
56	Hexane	Diethyl ether	MeOH	70:50:10
57	Hexane	Diethyl ether	MeOH	65:35:10

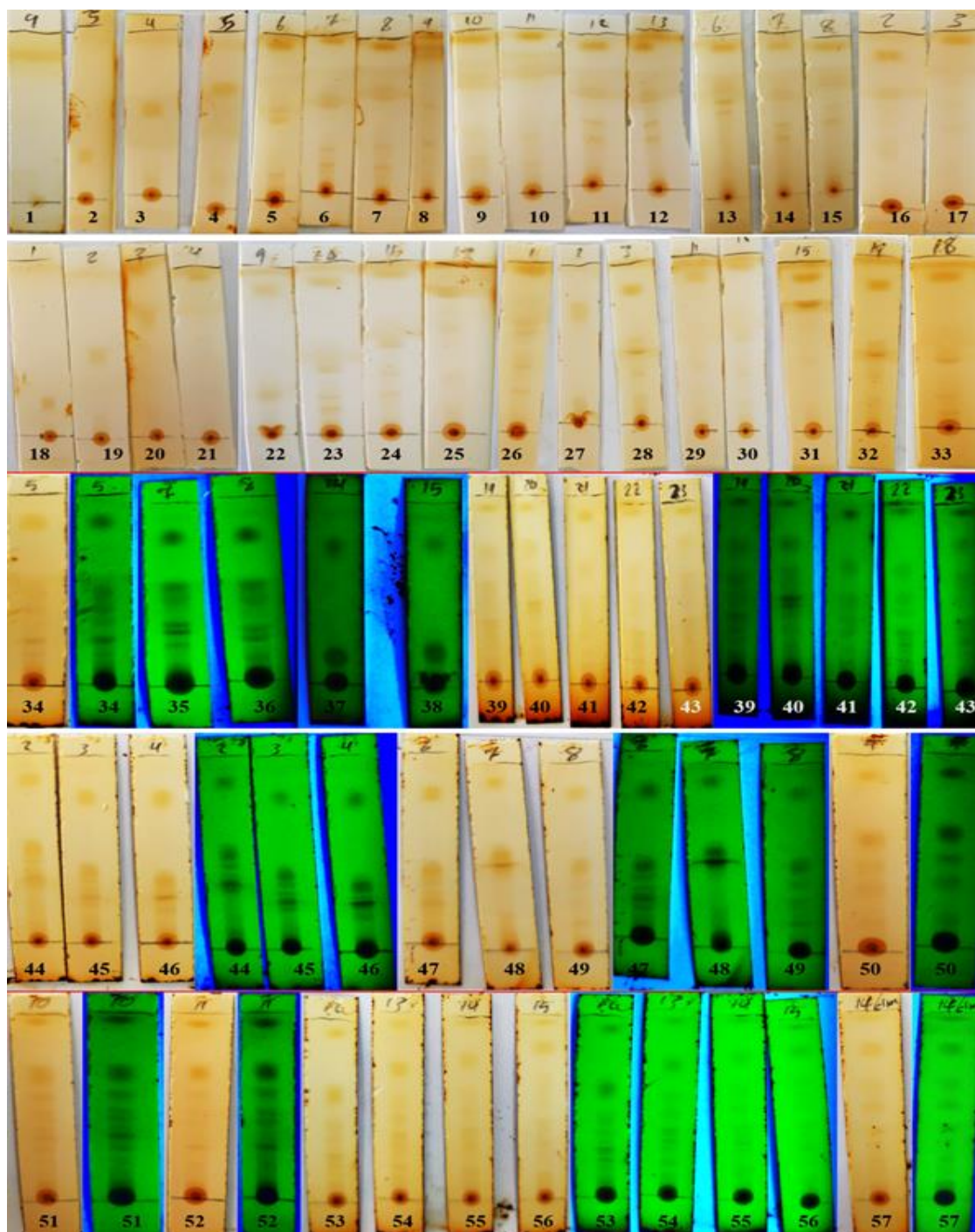


Figure 5.13. Purslane extract TLC chromatograms obtained in various solvent combinations

5.5.2. First attempt purslane extract isolation by column chromatography

Purslane extract dissolves in the mobile phase used for column chromatography (hexane:diethyl ether:MeOH 65:35:10). Therefore, the wet packing approach was attempted for column chromatography. Dissolving enough purslane extract in the mobile phase solvent mixture yielded a concentrated solution. A single application was needed to get this solution into the

column. After the solution reached the sand, the continuous mobile phase was injected (see Figure 5.14). Thus, the purslane extract was separated and eluted from the column. As fractions, the eluate leaving the column was collected in a series of tubes (1-2 mL). There were sixty-three distinct tubes utilized for collecting. The solvent combination hexane:diethyl ether:MeOH (65:35:10) was used in the mobile phase.

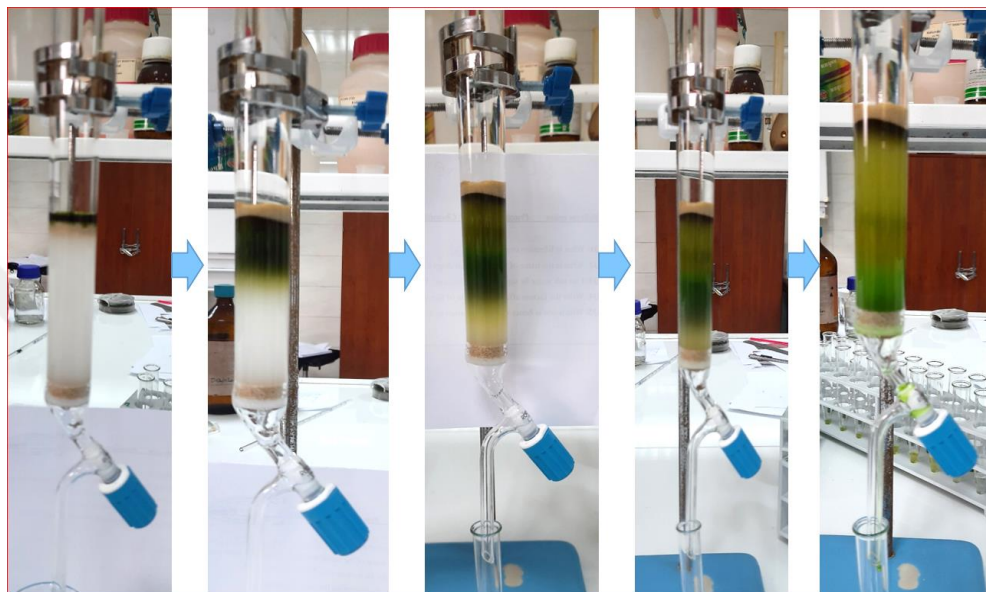


Figure 5.14. Chromatography proceeds after loading the purslane extract solution with the mobile phase hexane:diethyl ether:MeOH (65:35:10).

TLC was carried out on five fractions collected in tubes (see Figure 5.15). For instance, tube number four has one spot, tube numbers nine and sixteen have two spots each, tube number twenty-five has three spots, and tube number sixty-three has two spots. Multiple spots on the TLC chromatograms of our five tubes indicate component mixing in the tubes. Our mobile phase was thus ineffective for isolating purslane extract. Consequently, we could not use this mobile phase for purslane extract separation and sought to identify another mobile phase.

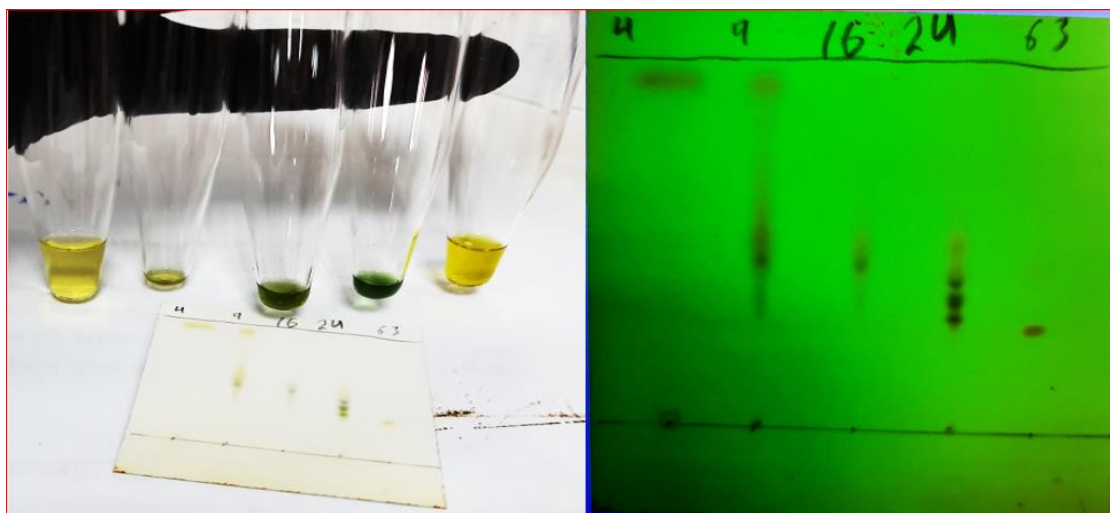


Figure 5.15. TLC for five purslane extract fractions obtained in tubes using mobile phase (hexane:diethyl ether:MeOH 65:35:10)

5.5.3. Second attempt at selecting a TLC-based solvent for purslane extract

TLC was used again to successfully isolate purslane extract, with the most effective mobile phase employed. The results of these tests are summarized in Table 5.13, which presents various solvent combinations. As can be seen in Figure 5.16, the fifth mobile phase generated four spots. However, the phase eighteen mobile phase generated just three spots. In every mobile phase, there were more than two spots generated. However, mobile phase 6 generated six spots. After analyzing the TLC chromatograms, it was determined that the number 6 solvent combination was the one that worked best. Thus, it was employed for column chromatography. After putting this mobile phase through its trials, we discovered that it could dissolve purslane extract.

Table 5.13. Again, the mobile phase for isolating purslane extract was determined using TLC

No.	Solvent-1	Solvent-2	Solvent-3	Mixing ratio (v/v)
1	Hexane	EtOAc	EtOH	75:20:8
2	Hexane	EtOAc	EtOH	75:20:5
3	Hexane	EtOAc	EtOH	90:20:5
4	Hexane	EtOAc	EtOH	75:15:3
5	Hexane	EtOAc	EtOH	75:20:3
6	Hexane	Diethyl ether	EtOH	50:60:5
7	Hexane	Diethyl ether	EtOH	40:60:5
8	Hexane	Diethyl ether	EtOH	60:40:5
9	Hexane	EtOAc	EtOH	90:30:3
10	Hexane	EtOAc	EtOH	110:30:3
11	Hexane	EtOAc	EtOH	75:5:10

12	Hexane	EtOAc	EtOH	75:10:10
13	Hexane	EtOAc	EtOH	75:3:5
14	Hexane	Acetone	EtOH	95:5:10
15	Hexane	Acetone	EtOH	95:20:3
16	Hexane	DCM	EtOH	90:30:3
17	Hexane	DCM	EtOH	50:50:2
18	Hexane	DCM	EtOH	90:35:3
19	Hexane	DCM	EtOH	90:30:8
20	Hexane	DCM	EtOH	90:40:3
21	Hexane	DCM	EtOH	70:35:3

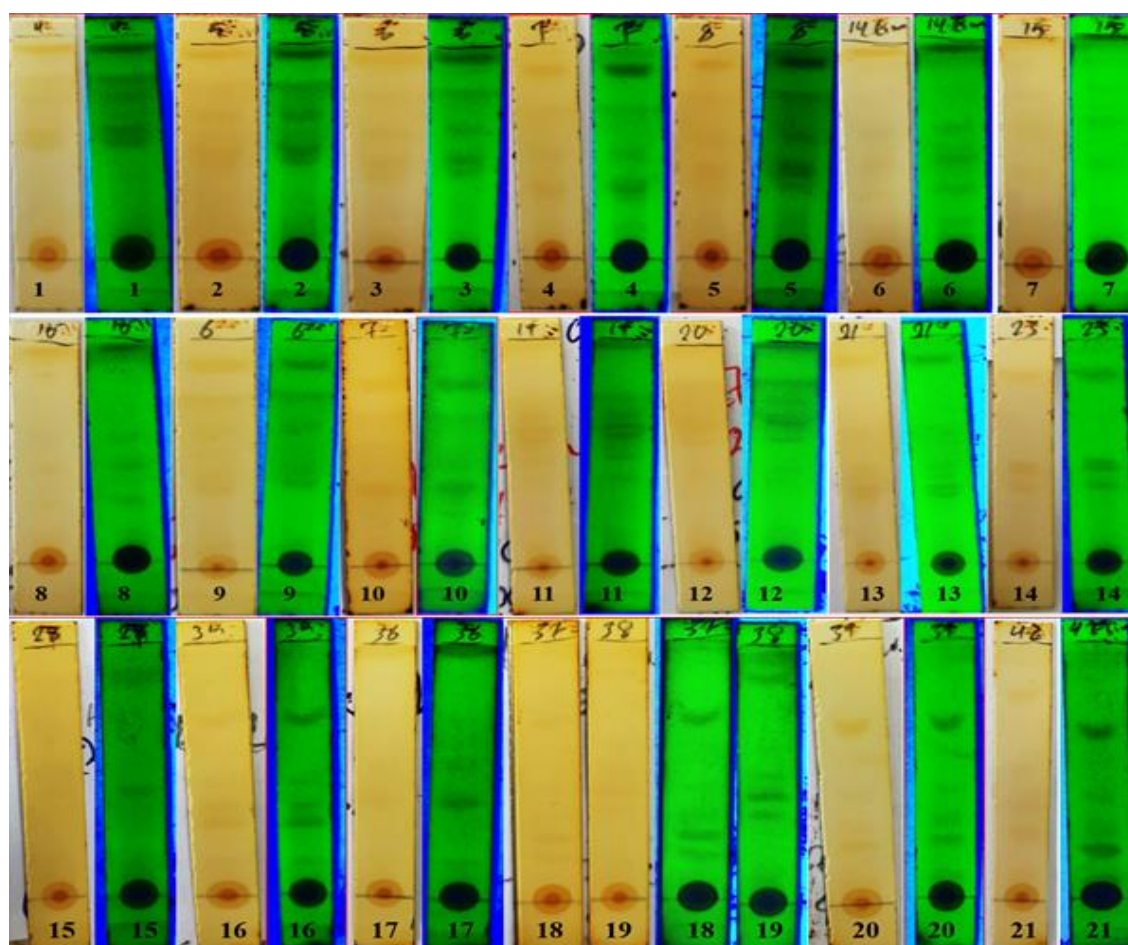


Figure 5.16. Purslane extract TLC chromatograms from the second round of trials showing the effects of different solvent mixtures

5.5.4. Second attempt purslane extract isolation by column chromatography

A column's main component is a glass tube containing the silica gel stationary phase. The stationary phase was placed on top of a frit pad, which was packed at the column's base. Before introducing the soluble extract, the column was filled to within 1-2 cm of sand to prevent any

disruption of the stationary phase. An interruption in the stationary phase causes the separation bands to be asymmetric (adsorbent layer).

Purslane extract was dissolved with a small amount of mobile phase hexane:diethyl ether:EtOH (50:60:5). All the samples were injected into the column once, soaking up near the column's top. Elution can be used to get the sample away from here.

The purslane extract moves through the column at different rates depending on its affinities for the solvent or solvent mixture adsorbent typically collected in solution as it exits the column at different times. With column chromatography, the purslane extract in a solution is adsorbed onto a stationary phase, separating the solution into its parts. Different components of the mobile phase and purslane extract flow through the column at different speeds after being injected at the inlet.

In general, components having less adsorption and affinity to the stationary phase tend to desorb from the stationary phase more rapidly. The faster-moving components are eluted first, then the slower ones.

This technique successfully separates each component from the column (see Figure 5.17). Separation was performed using the mobile phase that was chosen using TLC. After the purslane extract has been separated using column chromatography, it is straightforward to monitor the separation's progress. Then, small fractions of the eluent, approximately 1-2 mL, are consecutively collected in labelled tubes—intricate TLC monitoring of every fraction.

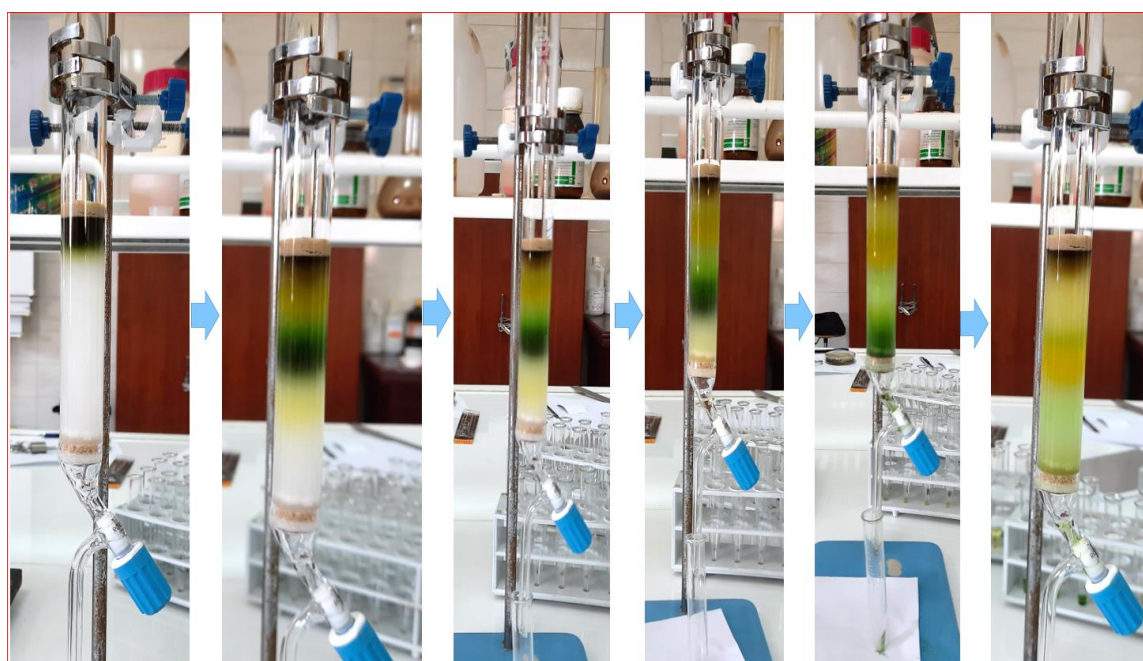


Figure 5.17. Column chromatography progresses after loading the purslane extract solution with hexane:diethyl ether:EtOH (50:60:5) elution

5.5.5. TLC for purslane extract fractions and coded fractions

There were seventy-five unique tubes used during the collecting process. TLC was done on each fraction collected in the tubes using the mobile phase solvent combination hexane:diethyl ether:EtOH (50:60:5) (see Figure 5.18). This collection includes 75 tubes and is a significant fraction of the whole set. They combined fractions whose R_f values and TLC chromatograms were similar.

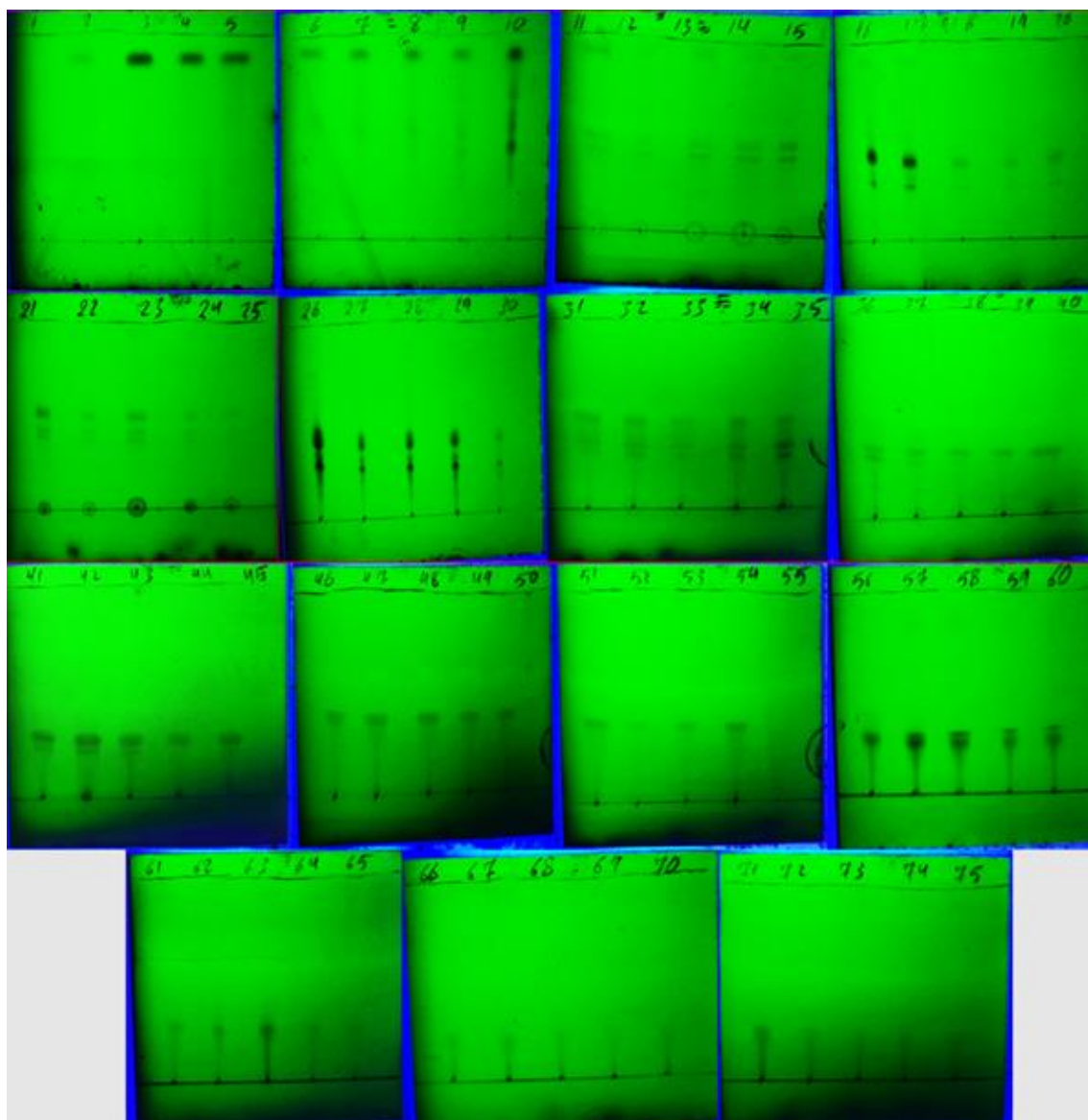


Figure 5.18. Performed TLC for all purslane extract tubes gathered after column chromatography elution with hexane:diethyl ether:EtOH (50:60:5)

The combined tubes have been given the following coding: fractions 2-6 are labelled "Purs1," fractions 7-9 are labelled "Purs2," fractions 10-17 are labelled "Purs3," fractions 18-30 are labelled

" Purs4," fractions 31-35 are labelled " Purs5," fractions 36-55 are labelled " Purs6," and fractions 56-72 are labelled " Purs7." The coding of all fractions obtained by column chromatography is shown in Figure 5.19. Following the chromatographic separation, the remaining material extract on the silica gel was washed using MeOH. This segment was further designated " Purs0."

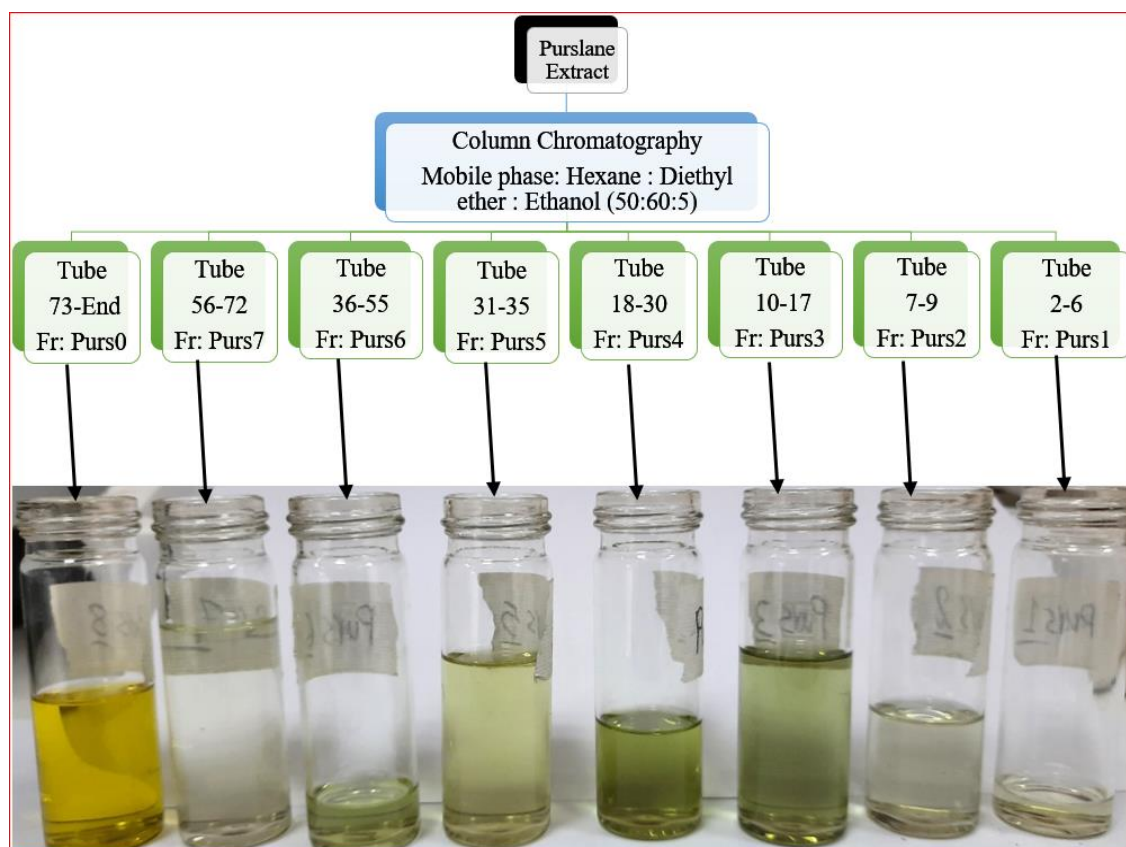


Figure 5.19. Coding of all tubes produced via column chromatography after loading purslane extract and purslane extract fractions

After being concentrated in a rotating evaporator set to 40 °C, under reduced pressure and air drying, the fractions were finished in a vacuum oven. All fractions were then placed in sterile, labelled vials and refrigerated at a temperature of 4–5 °C until HPLC analysis could be performed.

5.5.6. HPLC analysis of purslane extract and fractions

Due to the complexity of natural compounds, it is challenging to analyze the structural makeup of active substances derived from various plant sources and assess these substances' antioxidant and biological capacities. Because of this, it was required to identify and describe several bioactive chemicals discovered in the extract and fractions using high-resolution chromatographic methods such as HPLC, often employed to characterize polyphenolic compounds.

The extract and eight other purslane fractions (Purs0–Purs7) were used to conduct the efficacy analysis. According to Table 5.14, the extract had a minimum of two primary ingredients, while the fractions comprised anything from two to four unique compounds.

Table 5.14. Purslane extract and fractions' phenolic component content (mg/kg)

No.	Samples	Phenolic compounds						
		Catechin	p-Coumaric acid	Epicatechin	Rutin	Resveratrol	Naringin	Chlorogenic acid
1	Extract	14.446	N.D.	N.D.	N.D.	0.303	N.D.	N.D.
2	Purs1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
3	Purs2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
4	Purs3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
5	Purs4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
6	Purs5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
7	Purs6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
8	Purs7	N.D.	N.D.	N.D.	N.D.	0.008	N.D.	N.D.
9	Purs0	N.D.	0.257	0.155	N.D.	0.304	4.189	N.D.

N.D., Not detected.

5.6. Blackberry extract natural ingredients isolation

The blackberry extract was prepared following the steps described in Section 4.4.4. The optimum solvents were chosen using TLC after the blackberry extract was obtained. Open-column chromatography was used for the isolation method, and HPLC was used to determine the polyphenolic components of the extract and fractions.

5.6.1. TLC-based solvent system selection for blackberry extract isolation

TLC is a powerful tool for observing chemical processes. It also decides the solvent system for a particular column chromatography separation. TLC is used to analyze substances in a mixture by separating them. TLC may help determine how many substances make up a combination, what those substances are, and how pure they are. TLC is an efficient analysis method since it requires less time and can detect changes in microgram (0.000001 g) levels in about 5–10 minutes.

The three stages of TLC are spotting, development and visualization. Initially, a very dilute (approximately 5%) solution was made by dissolving the blackberry extract in a volatile (quickly evaporated) solvent like MeOH. The spotting procedure involves applying a drop of this diluted

solution to one end of a TLC plate comprised of powdered silica gel coated onto an aluminium sheet. A small amount of material is left behind when the spotting solution evaporates.

The development process involves putting the bottom of the TLC plate in a small pool of a development solvent, which then moves up the plate through capillary action. As the solvent advances the plate, it moves across the initial area. The silica gel plate and the developing solvent for the spotted substance engage in a competition. Extremely polar silica gel attempts to keep the spot in its original location while the solvent moves it up the plate. The equilibrium of three polarities determined the result: the plates, the development solvents, and the spot materials. The spot will shift from its initial position if the development solvent is sufficiently polar. Differently polarized components of the original spot will travel varying distances from the original spot's position and appear as distinct spots. When the solvent has nearly reached the top of the plate, it is withdrawn, the solvent front is indicated with a pencil, and the solvent is allowed to evaporate.

Spots from coloured compounds are easily seen following development, making visualization a breeze. Most chemicals, however, are colourless. Therefore, some other mode of vision is required. After placing the TLC plates in a UV light source, the spots may be marked by drawing around them with a pencil. Alternatively, it might expose the plate to iodine vapours for a few minutes to get a clearer picture. When combined with iodine, most organic molecules produce a black complex. In case a compound does not show up with one approach, having a backup is a brilliant idea.

The R_f value was used to measure the material flow along the plate. R_f equals the distance travelled by the substance divided by the solvent's distance travelled. Its value is always between zero and one. Comparing these R_f values to the R_f values of recognized substances may allow for preliminary identification. Note that three dots indicate only the presence of at least three components in a combination. Some components may have polarities so similar that they cluster together after development.

How far up the plate the solvent can take the extract depends on where the intermolecular forces are in equilibrium. In other words, does the spot prefer to travel with the solvent or stay on the plate? The equilibrium is determined by: first, the polarity of the TLC plate (which is constant and high); second, the polarity of the development solvent (which may be changed by using various solvents); and third, the polarity of the compounds in the spot, all of which contribute to the equilibrium (this varies depending upon what compounds are in a spot). If a sample has two components, one more polar than the other, the more polar component will gravitate toward the plate, while the less polar component will float freely in the solvent. A more polar development solvent might hasten the process for both. The best solvent or solvent combination may be guessed if the approximate structures of the solutes are known. However, in reality, the optimal solvent or solvent combination for separating a specific mixture of chemicals is selected by trial and error.

Through the use of the TLC chromatograms, the optimal solvent combination was determined. Figure 5.20 displays the chromatograms obtained by applying a variety of solvent mixtures on TLC plates to create them (Table 5.15). On TLC plates, most solvent combinations showed the presence of two or more spots. For instance, the mobile phases (EtOAc and MeOH), with the proportions of 200:5 and 250:5, respectively), produced four spots. Despite this, the mobile phase (DCM:MeOH:EtOH, 150:3:3) generated six spots and had a high R_f value, identical to the mobile phase (DCM: MeOH, 200:10). For column chromatography, a mobile phase of 150:3:3 (DCM:MeOH:EtOH) and 200:10 (DCM:MeOH) was used.

Table 5.15. The blackberry extract solvent combination was identified using TLC

No.	Solvent-1	Solvent-2	Solvent-3	Mixing ratio (v/v)
1	Chloroform	MeOH		90:10
2	Chloroform	MeOH	Water	90:10:10
3	Chloroform	MeOH		90:15
4	Chloroform	MeOH		120:15
5	EtOAc	Chloroform	MeOH	90:10:10
6	EtOAc	Chloroform	MeOH	50:50:10
7	EtOAc	Chloroform	MeOH	10:90:10
8	EtOAc	MeOH		90:10
9	EtOAc	Chloroform	MeOH	90:10:30
10	EtOAc	Chloroform	MeOH	50:50:30
11	EtOAc	Chloroform	MeOH	10:90:30
12	EtOAc	MeOH	Water	90:10:10
13	Hexane	EtOAc		90:10
14	Hexane	EtOAc		50:50
15	Hexane	EtOAc		10:90
16	DCM	Toluene	MeOH	90:10:10
17	DCM	Toluene	MeOH	50:50:10
18	DCM	Toluene	MeOH	10:90:10
19	DCM	MeOH		90:10
20	Toluene	MeOH		90:10
21	DCM	MeOH		90:20
22	Toluene	MeOH		90:20
23	Hexane	DCM	MeOH	90:90:10
24	Hexane	DCM	MeOH	50:50:10
25	Hexane	DCM	MeOH	10:90:10
26	EtOAc	Toluene		90:10
27	EtOAc	Toluene		50:50
28	EtOAc	Toluene		10:90

29	EtOAc	DCM		90:10
30	EtOAc	DCM		50:50
31	EtOAc	DCM		10:90
32	EtOAc	Chloroform	MeOH	5:95:30
33	EtOAc	Chloroform	MeOH	10:100:30
34	EtOAc	Chloroform	MeOH	10:110:30
35	Chloroform	DCM	MeOH	50:50:20
36	Hexane	EtOAc		80:20
37	Hexane	EtOAc		70:30
38	Hexane	DCM	MeOH	10:100:10
39	Hexane	DCM	MeOH	20:80:10
40	Hexane	DCM	MeOH	30:70:10
41	EtOAc	Chloroform	MeOH	10:110:20
42	EtOAc	Chloroform	MeOH	10:110:10
43	EtOAc	Chloroform	MeOH	10:130:30
44	EtOAc	Chloroform	MeOH	10:150:30
45	EtOAc	Chloroform	MeOH	10:90:30
46	EtOAc	Chloroform	MeOH	10:90:40
47	EtOAc	Chloroform	MeOH	10:140:40
48	EtOAc	Chloroform	MeOH	10:160:40
49	Chloroform	MeOH		150:10
50	Chloroform	MeOH		200:10
51	EtOAc	MeOH		200:5
52	EtOAc	MeOH		250:5
53	DCM	MeOH		150:5
54	DCM	MeOH		150:10
55	DCM	EtOH		150:10
56	DCM	MeOH	EtOH	150:3:3
57	DCM	MeOH		200:10

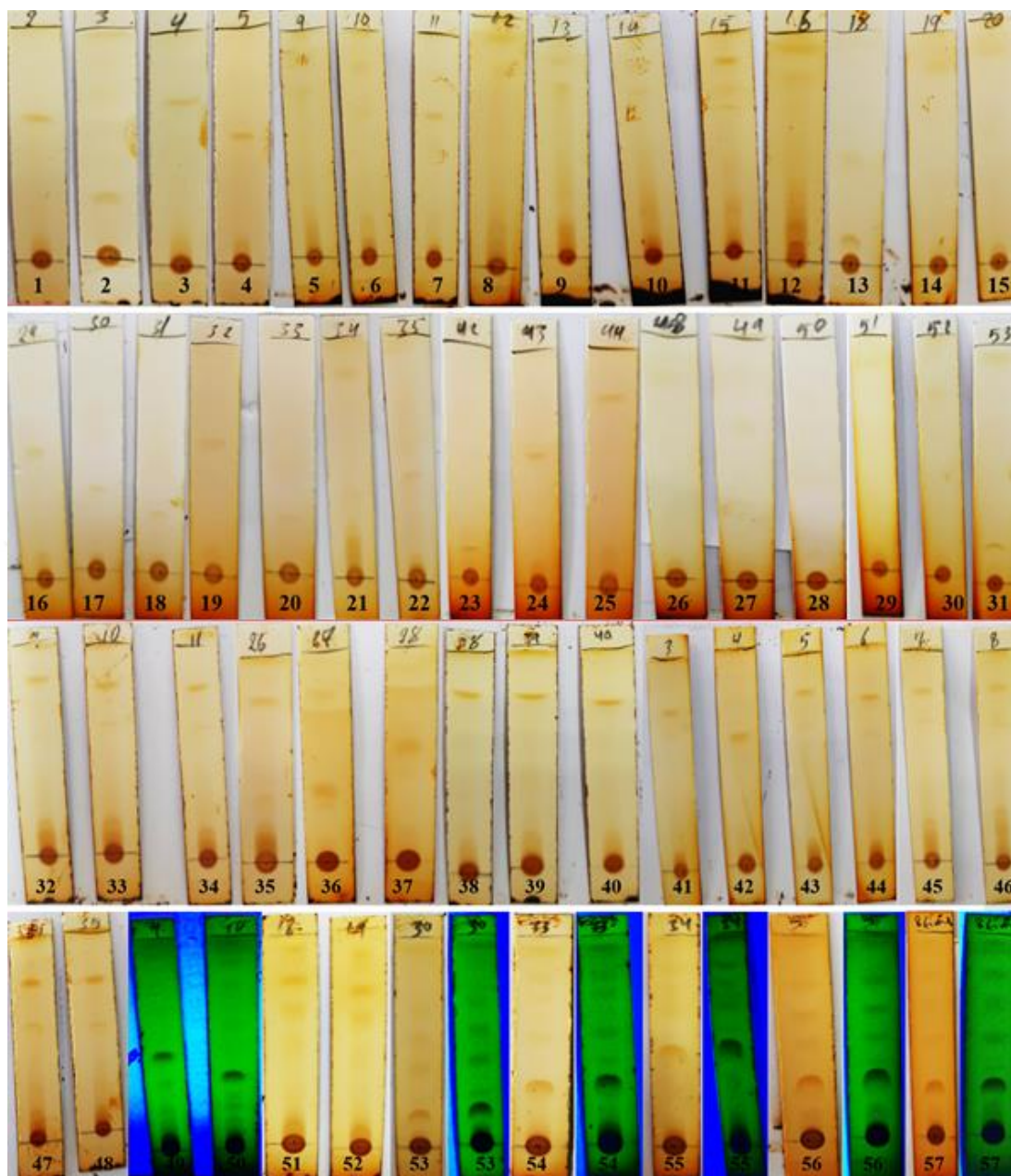


Figure 5.20. TLC chromatograms of blackberry extract produced with different solvent combinations

5.6.2. Dry loading column chromatography for blackberry extract isolation

The blackberry extract did not dissolve when trying column chromatography using the mobile phase (DCM:MeOH:EtOH) in the ratio of 150:3:3 and (DCM:MeOH) in the ratio of 200:10. Consequently, an effort was made to conduct column chromatography utilizing the dry packing method.

"Procedure for dry loading." After the desired extract has been dissolved with the minimum amount of solvent possible, about 100 mg of silica gel should be added. Stir the mixture continuously until all that is left is a dry powder and the solvent evaporates. After positioning the

dry powder on a piece of folded weighing paper, transfer it to the top of the column that has been created. Now that a chosen eluting solvent has been added to the top, you may start the elution process. After filling the column with the chosen mobile phase two times, there was no extract eluted as a fraction from the column. Since our mobile phase did not result in the separation of any fraction using the column chromatography method, as shown in Figure 5.21, we could not use this mobile phase for the extraction separation process and instead look for another mobile phase.

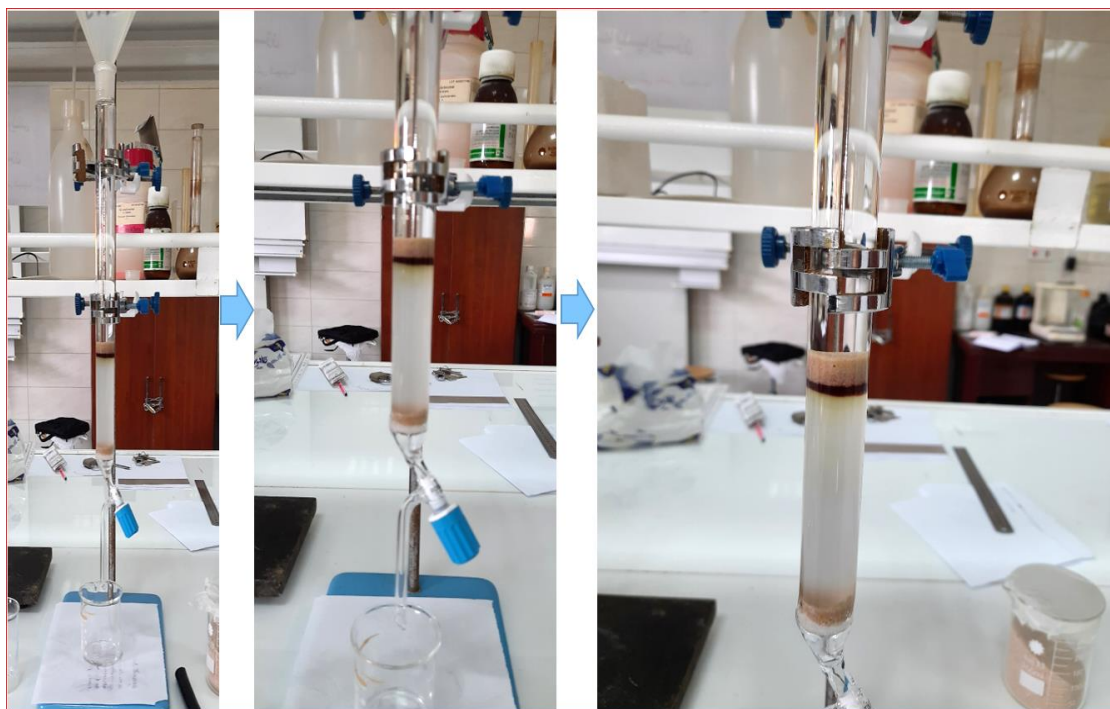


Figure 5.21. Isolation of blackberry extract by dry loading with mobile phase (DCM:MeOH:EtOH) in the ratio of 150:3:3.

5.6.3. Second attempt at selecting a TLC-based solvent for blackberry extract

As before, the most efficient mobile phase was utilized with TLC to separate blackberry extract effectively. Table 5.16 provides a quick overview of the many different solvent combinations tested. Figure 5.22 shows that four spots were produced during the fifth mobile phase. However, there were just three spots produced by the mobile phase 26. More than two spots are produced in every iteration of the mobile phase. Four spots were produced during mobile phase 36. The TLC chromatograms were examined, and the optimum solvent combination was found to be the 36th tested. This meant it could be used in column chromatography.

Table 5.16. TLC was used again to select the mobile phase for isolating the blackberry extract

No.	Solvent-1	Solvent-2	Solvent-3	Mixing ratio (v/v)
1	Chloroform	EtOAc	MeOH	90:10:40
2	Chloroform	EtOAc	MeOH	160:10:50
3	Chloroform	EtOAc	MeOH	140:10:50
4	EtOAc	Toluene		100:10
5	Toluene	EtOAc	FA	100:125:50
6	EtOAc	Toluene		100:20
7	Toluene	EtOAc	FA	80:125:50
8	Toluene	EtOAc	FA	120:125:50
9	Toluene	EtOAc	FA	100:100:50
10	Toluene	EtOAc	FA	100:150:50
11	Toluene	EtOAc	FA	100:125:10
12	Toluene	EtOAc	FA	100:125:25
13	Toluene	EtOAc	FA	100:125:60
14	Toluene	EtOAc	FA	100:125:20
15	Toluene	EtOAc	FA	100:80:50
16	Toluene	EtOAc	FA	100:80:10
17	Toluene	EtOAc	FA	70:40:20
18	EtOAc	MeOH	Water	160:10:15
19	Acetone	MeOH	Water	100:5:5
20	Acetone	MeOH	Water	100:10:5
21	EtOAc	MeOH	Water	125:10:5
22	EtOAc	MeOH	Water	125:5:10
23	Acetone	Water		200:5
24	Toluene	EtOAc	FA	65:45:25
25	Toluene	Acetone	Water	10:40:5
26	Toluene	Acetonitrile	FA	50:50:5
27	Toluene	Acetone	FA	50:50:5
28	Toluene	Acetone	FA	100:50:5
29	Toluene	Acetone	FA	100:50:7.5
30	Toluene	Acetone	FA	75:50:5
31	Toluene	Acetone	FA	100:75:7.5
32	Toluene	Acetone	FA	100:25:7.5
33	Toluene	Acetone	FA	100:100:7.5
34	Toluene	Acetone	Water	15:40:3
35	Toluene	Acetone	Water	15:40:6
36	Toluene	EtOAc	FA	50:50:25

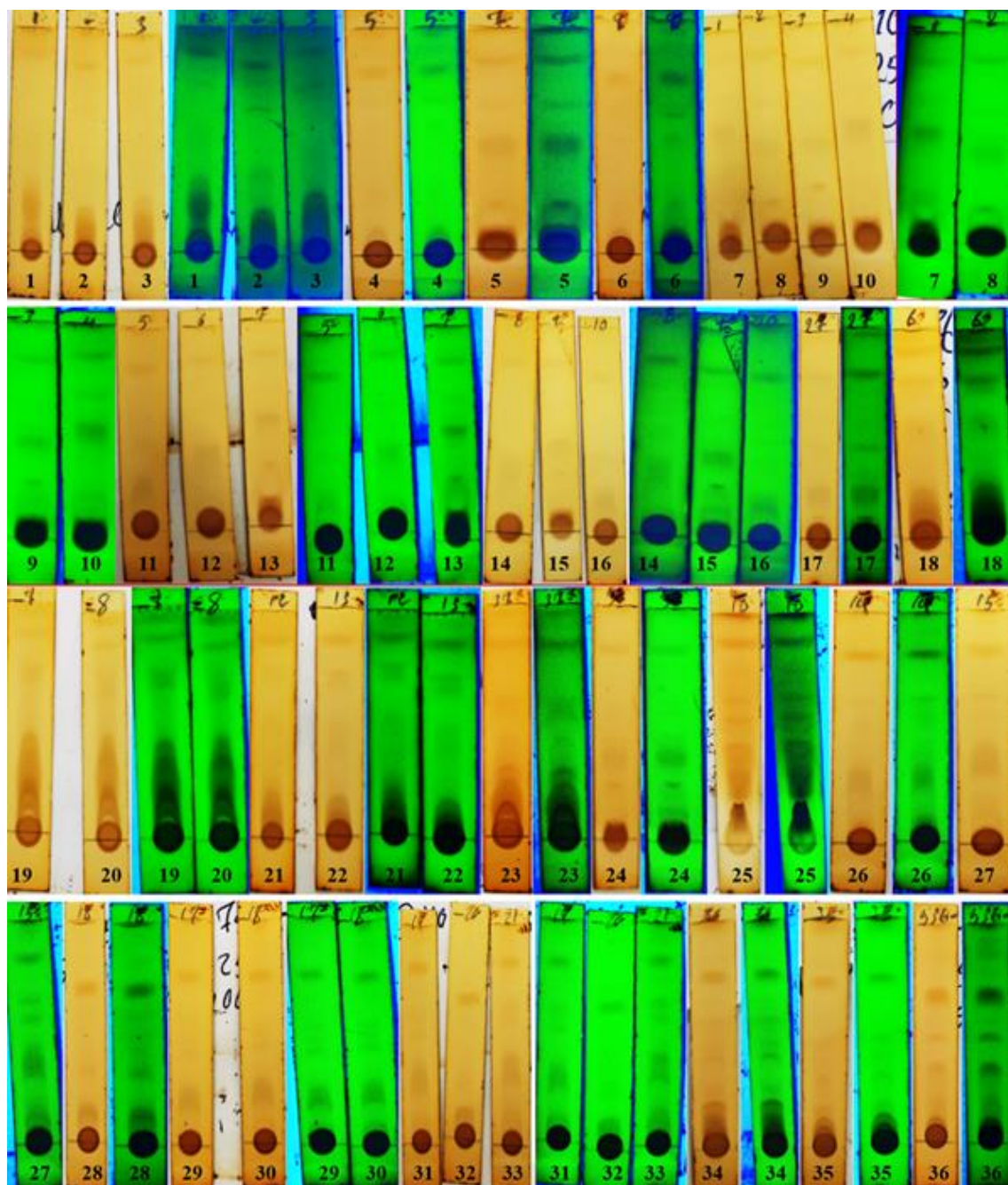


Figure 5.22. TLC chromatograms of blackberry extract from the second set of testing demonstrate the impact of various solvent mixes

5.6.4. Isolation of blackberry extract by wet loading Column Chromatography

The mobile phase (toluene:EtOAc:FA, 50:50:25) employed in column chromatography is appropriate for blackberry extract. Since this was the case, wet packing was tried for column chromatography. Blackberry extract was successfully dissolved in the mobile phase solvent combination, resulting in a concentrated solution. Only one input was required to add this solution to the column. The continuous mobile phase was injected once the solution had reached the sand

(see Figure 5.23). This resulted in the blackberry extract being isolated and eluted from the column. The column's effluent, known as eluate, was separated into fractions and collected in separate tubes (1-2 mL).

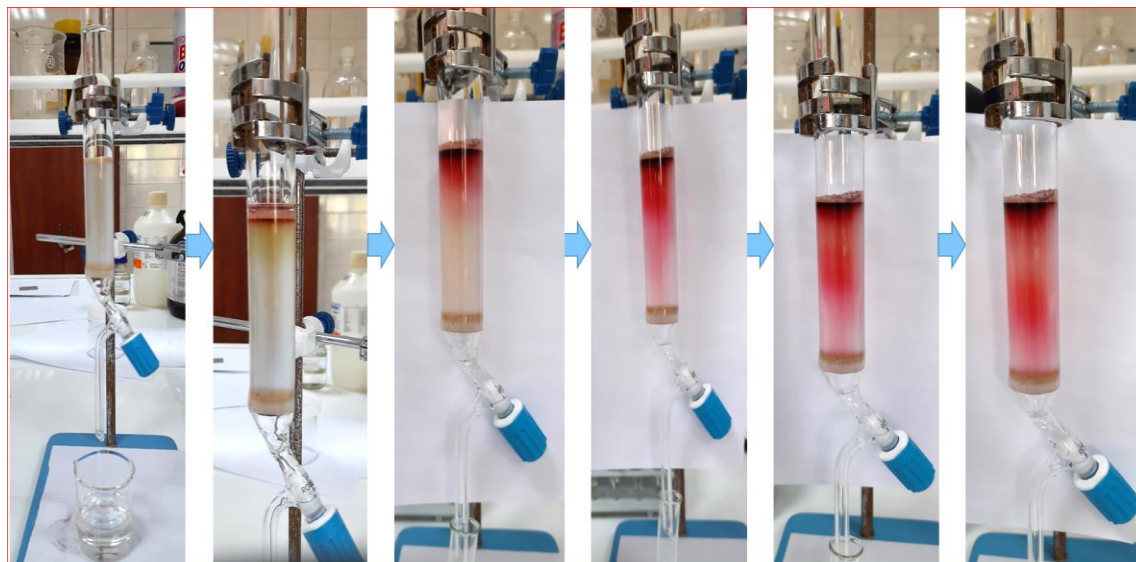


Figure 5.23. Isolation of blackberry extract using wet loading with mobile phase (toluene:EtOAc:FA) at a 50:50:25 ratio

During the collection procedure, a total of sixty-nine different tubes were used. TLC was performed on the starting fraction collected in the tubes at thirty and the latest ones at sixty-five and sixty-nine using the mobile phase solvent combination (toluene:EtOAc:FA) at 50:50:25 ratios. The total number of tubes in this collection is 69, making it a portion of the whole set.

Because the spots were hard to see and were not shown in Figure 5.24 in an obvious way, the TLC analysis did not show clear evidence of separation. Since toluene:EtOAc:FA (50:50:25) was not in the mobile phase, the blackberry extract could not be effectively separated. To separate the blackberry extract, we will employ a different solvent mixture.

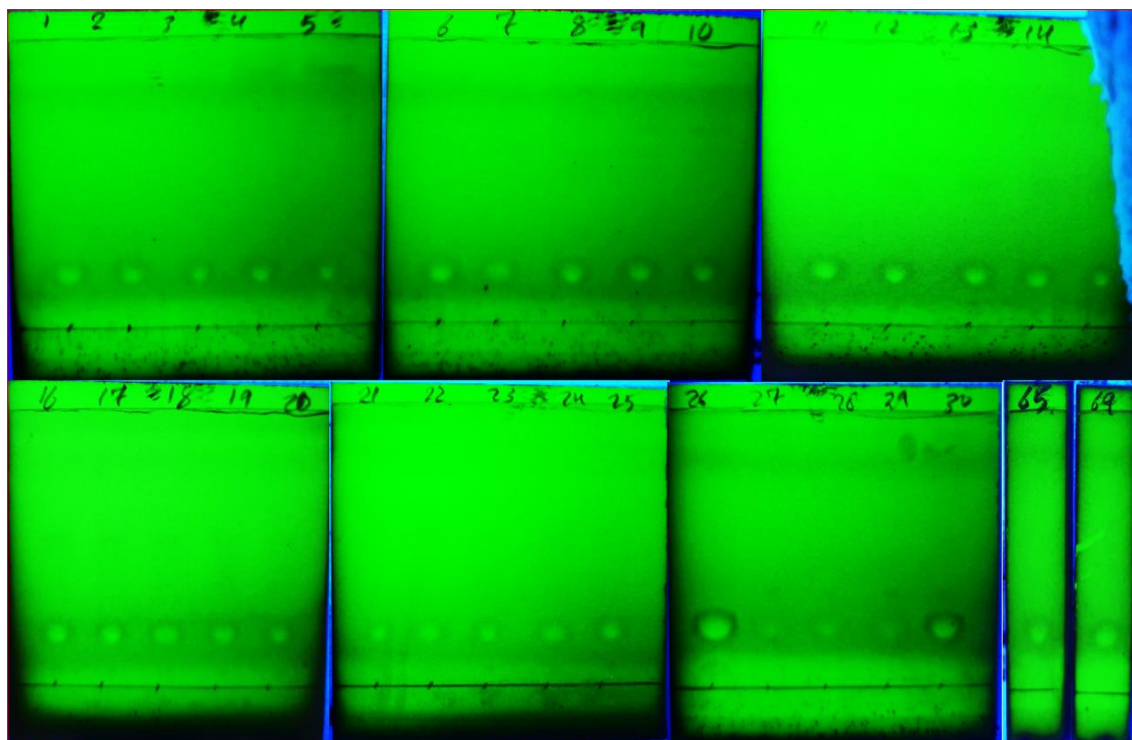


Figure 5.24. Performed TLC on thirty-two blackberry extract tubes following column chromatography in toluene:EtOAc:FA (50:50:25)

Figure 5.22 shows that the mobile phase number 24 (toluene:EtOAc:FA, 65:45:25) has four spots that work for column chromatography with blackberry extract. Given this, wet packing was attempted for column chromatography. The solvents for the mobile phase could be mixed with blackberry extract to make a concentrated solution.

The glass tube that holds the silica gel stationary phase is essential to the column. The frit pad was packed at the column's base, and the stationary phase was put on top. Since the column was filled to within 0.5–1 cm sand of the top. A modest quantity of the mobile phase toluene:EtOAc:FA (65:45:25) was used to dissolve blackberry extract. After dissolving the sample in the mobile phase, the sample was injected into the column just once.

Sample extract moves through the column at different speeds and leaves at different times due to its different affinities for the solvent or solvent combination (see Figure 5.25). After being injected into the inlet of the column, the mobile phase and blackberry extract move through it at different rates. In a chromatography column, the blackberry extract in a solution sticks to a stationary phase (silica gel), separating the parts of the solution. Components that do not stick as well or as well to the stationary phase tend to move away from the stationary phase more quickly. The components that move more slowly are eluted after the ones that move more quickly.

Because of this method, we can effectively isolate each column constituent. The mobile phase was selected by TLC and used for separation. Once the blackberry extract has been separated

using column chromatography, it is easy to keep an eye on the separation process. After that, the eluent is split into smaller tubes of about 1-2 mL and put into labelled tubes one at a time for careful TLC analysis.

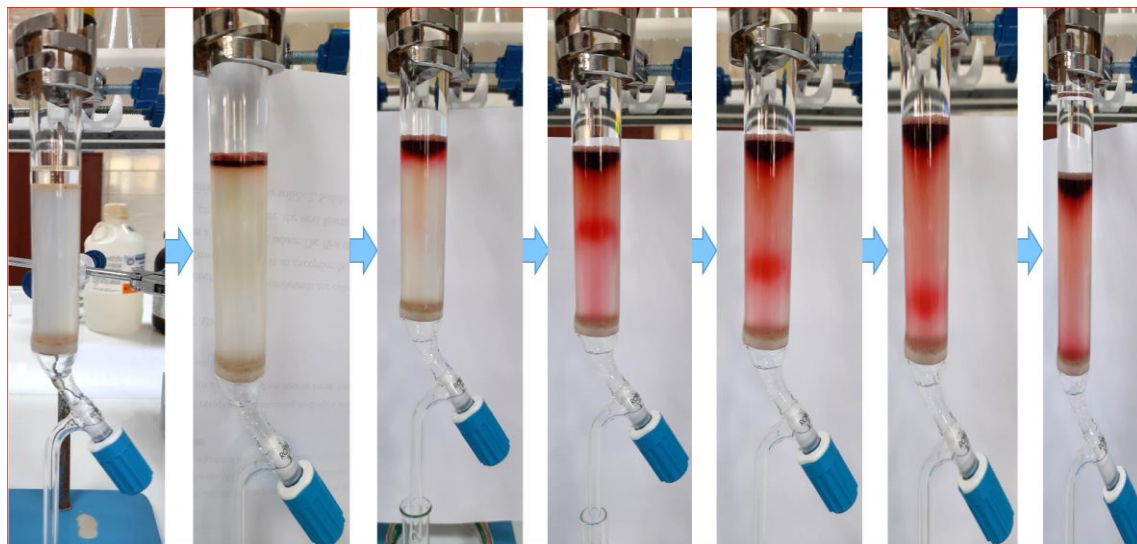


Figure 5.25. Wet loading with mobile phase (toluene:EtOAc:FA) at 65:45:25 ratios to isolate blackberry extract

5.6.5. TLC for blackberry extract fractions and coded fractions

During the collection process, a total of ninety-five different tubes were utilized. Using a mixture of toluene, EtOAc, and FA (65:45:25) as the mobile phase solvent, TLC was done on each fraction put in a tube (see Figure 5.26). The fractions with similar R_f values and TLC chromatograms were combined.

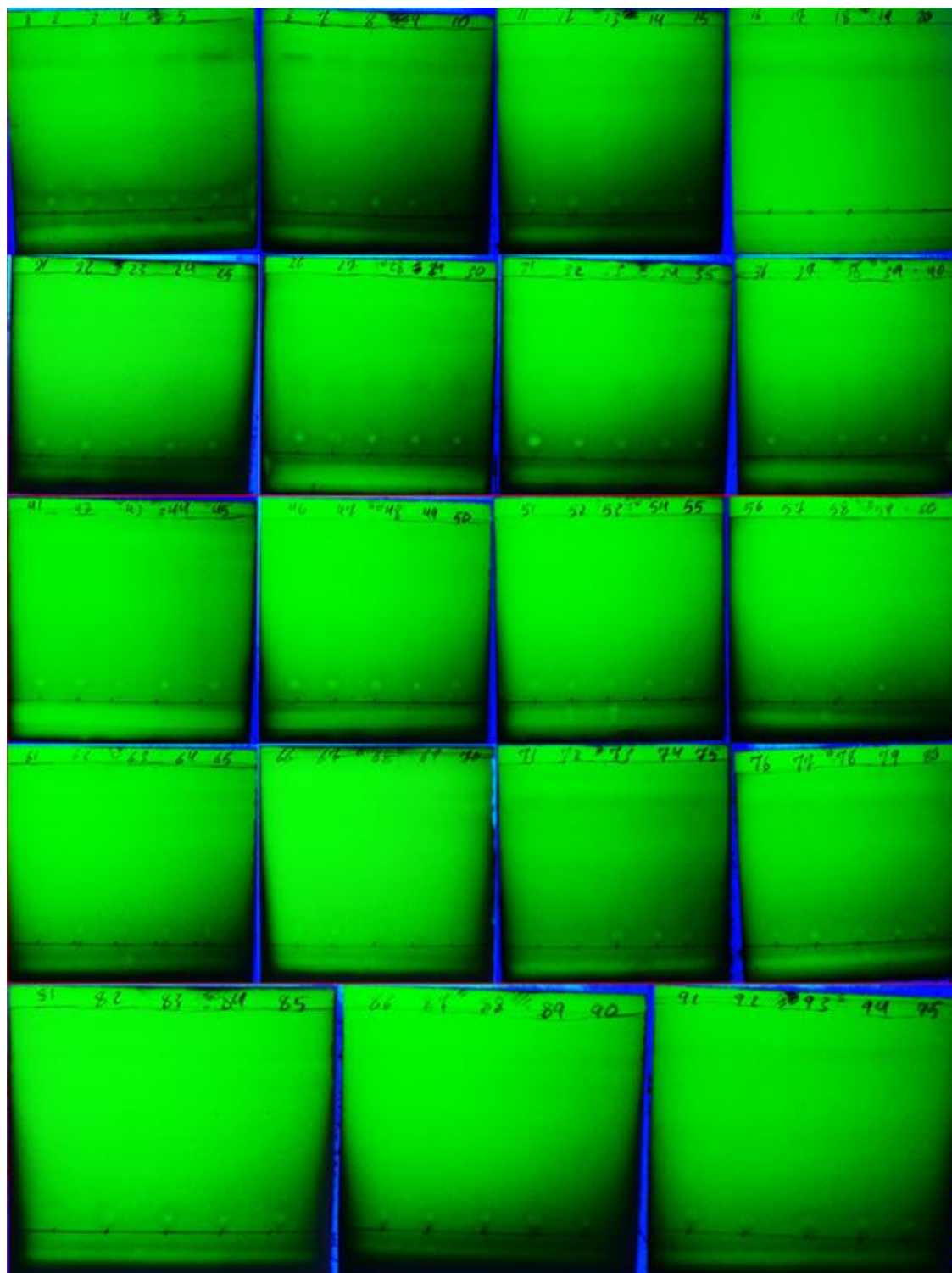


Figure 5.26. TLC for 95 blackberry extract tubes after column chromatography with mobile phase toluene:EtOAc:FA (65:45:25)

The combined tubes were coded as follows: Black1 includes portions 1-9, Black2 includes portions 10-22, Black3 includes portions 23-50, Black4 includes portions 51-70, Black5 includes portions 71-82, black6 includes portions 83-85, and Black7 includes portions 86-94. Column

chromatography yielded several different fractions, shown in Figure 5.27, with their respective codes. After chromatographic separation, the silica gel with the remaining extract was washed with MeOH to eliminate any leftover pieces. This part of the extract was subsequently labelled Black0.

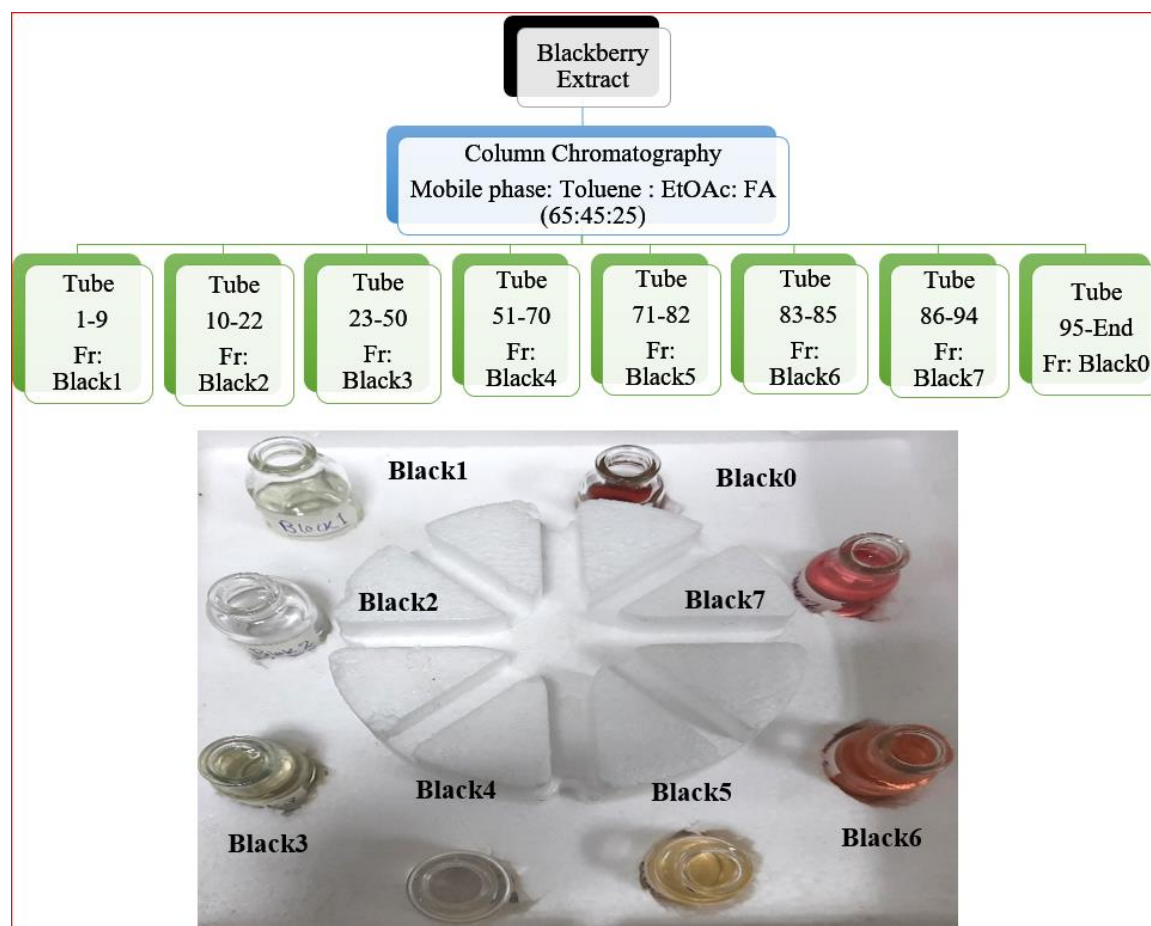


Figure 5.27. The coding of every tube that was acquired from column chromatography and blackberry extract fractions

After the fractions were concentrated in a rotating evaporator at 40 °C with less pressure and air drying, they were put in a vacuum oven to finish. Before HPLC analysis, all fractions were placed into sterile, labelled vials and kept in the fridge at 4–5 °C.

5.6.6. HPLC analysis of blackberry extract and fractions

It is hard to figure out the structures of active substances made from different types of plants, and it is also hard to figure out what antioxidant and biological effects these substances have. Because of this, it was necessary to use high-resolution chromatographic techniques like HPLC to find and describe several bioactive chemicals in the extract and fractions. These kinds of approaches are often used to define polyphenolic compounds. The effectiveness was tested with

the blackberry extract and eight different blackberry fractions, which were given Black0–Black7. Based on the data shown in Table 5.17, it is clear that no phenolic compounds were found in the extract. However, the Black2 fraction includes p-coumaric and chlorogenic acid, and the Black4 and Black5 fractions contain rutin.

Table 5.17. HPLC analysis of the phenolic component content (mg/kg) of blackberry extract and fractions

No.	Samples	Phenolic compounds						
		Catechin	p-Coumaric acid	Epicatechin	Rutin	Resveratrol	Naringin	Chlorogenic acid
1	Extract	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
2	Black1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
3	Black2	N.D.	0.961	N.D.	N.D.	N.D.	N.D.	0.084
4	Black3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
5	Black4	N.D.	N.D.	N.D.	0.404	N.D.	N.D.	N.D.
6	Black5	N.D.	N.D.	N.D.	3.134	N.D.	N.D.	N.D.
7	Black6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
8	Black7	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
9	Black0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

N.D., Not detected.

5.7. Fenugreek extract natural ingredients isolation

Section 4.4.4 outlines the procedure for producing the fenugreek extract. The TLC was used to choose the best solvents once the fenugreek extract was produced. The isolation process used open-column chromatography, and HPLC was employed to identify the polyphenolic compounds of the extract and fractions.

5.7.1. TLC-based solvent system selection for fenugreek extract isolation

The plant extract is complicated and may contain many different natural products. Some of these natural products must be taken out or cleaned up before the active fraction, or purely natural products can be obtained. The criteria for separating them are unique to each natural product and its physical or chemical traits. Chromatography, mainly column chromatography, separates natural compounds from a complex mixture [191].

TLC's key benefits are that it is inexpensive and may provide results quickly. Low- or no-cost materials are sufficient for TLC. These include a development chamber, chromatographic

plates, solvents, detection reagents, and standards. TLC can also be used to find and identify a large number of different substances.

TLC's primary function is to separate or resolve constituents of mixtures. Chemical separations occur when molecules react with the TLC's stationary and mobile phases. Components of samples that need to be separated move through the stationary phase because of capillary forces. They move through the growing solvent system, which is also called the mobile phase. As the mobile phase moves up through the plate, it affects the parts of the solvent system because it pushes them, and the sorbent slows them down. Components are pushed in the direction of the flow of the mobile phase by the driving force, but they are pushed back by the resistive forces, which pull them out of the path of the mobile phase and keep them stuck to the stationary phase by adsorption.

Consequently, each molecule takes a "stop-and-go" route across the sorbent layer. By the end of development, each component point has travelled a certain distance. Components that adhere more strongly to the sorbent layer will travel a shorter distance, while components that dissolve more quickly in the mobile phase will travel a longer distance. Variations in the motion of the individual molecules cause the spots to grow in size. An essential parameter, the R_f value, can describe how a chromatogram's different parts move. It is found by comparing how far the solute (the component) moved to how far the front of the mobile phase moved [192].

Methods for preparing the sample vary widely according to the substance being examined. The type of solvent used will depend on whether or not the sample dissolves. By mixing the fenugreek extract with a solvent that evaporates quickly, like MeOH, a fragile solution (about 5%) was made.

As the first step of the procedure, the sample solution is dropped onto the ready TLC plate. In a capillary glass tube, the sample solution is drawn. The baseline of the plate is marked with 0.1 to 1.0 μL volumes of the sample solution. Often, a template or ruler is used as a guide to ensure that the points along the baseline are all the same distance apart. Application of the spot is a significant step that often requires experience to get sample spots that are small and round. A way to make tiny spots on the origin is to apply sections of the whole volume and dry the area between each application with a cool-setting air gun.

When the sample spots have dried, the plate is placed into a development chamber with a solvent or solvent combination. Solvent vapours must be introduced into the chamber in advance. The typical timeframe for this procedure is between ten and fifteen minutes. The chamber is tightly sealed to keep the volatile solvents from evaporating and the vapour space complete. In a chamber, the plate is produced for a certain amount of time, often four centimetres. Afterwards, you take the plates out of the chamber and put them under a fume hood to dry at an ambient temperature. The amount of time it takes to dry depends on the solvent solution, but it usually takes about 5 minutes.

The TLC plate separates the many chemical components that make up the sample. They can be colourless or colourful compounds. It must be made apparent if they do not have any colour. Identifying the specific personal reaction of the components with a chemical reagent helps assemble the parts to generate noticeable patches under conventional or UV light. To get a more distinct image, the plate may be subjected to iodine vapours for some time. The majority of organic compounds, when combined with iodine, form a dark complex. It is an excellent idea to have a backup strategy in place if one method does not reveal the presence of a chemical.

Following detection, the plate is checked to verify the position of the components. The plate is initially examined under typical lighting conditions. Any discolouration pencil is used to highlight potential dividing zones gently. Next, ultraviolet light is used to analyze the plate. Short-wavelength UV light (centred at 254 nm) and long-wavelength UV light (centred at 365 nm) are used. Once again, fluorescence is noted with a pencil.

Documentation is meticulous for the chromatogram. Documentation comprises a record of the materials tested, the type of plate used, the solvent system, the chamber, and other factors. In addition, pictures are taken of the TLC plate for a permanent record. The plate is captured on black-and-white print film and colour slide film. Finally, the position of each spot is measured and recorded, and the associated R_f values are calculated for each component.

A TLC solvent system is a liquid mobile phase with at least two solvents that can mix. The solvent system competes with the analyte that has been dissolved. These are the "active" parts of the sorbent, and they must be chosen carefully so that the different parts can be separated well. The equilibrium between the solvent, the solutes, and the sorbent layer is used to choose the type of solvent system to use. Most of the time, solvent solutions are chosen by trial and error or by reading about similar uses in the literature.

One of the most important things to consider when choosing a solvent system is keeping the different parts of the mixture separate. The best mobile phase depends on the separated compounds and their behaviour. The quantity and composition of the analyte's functional groups may impact the strength of the analyte's interactions with the mobile phase or the sorbent. For a highly polar molecule to move on the TLC plate, there must be a strong interaction between the mobile phase and the sorbent layer.

Other considerations in selecting a solvent system may include the cost, availability, quality, toxicity, volatility, and miscibility of the selected solvent or solvents. One-or two-solvent systems are preferable over more complicated mixes of solvents. Any impurities can make the separation much less selective and repeatable, so only the cleanest solvents should be used.

The method begins with a low-polar solvent. For example, hexane or toluene can be mixed to make two different solvents by increasing the amount of the more polar solvent. Each combination has a steadily increasing concentration of the more polar solvent. When more of the

polar solvent is used, the eluent is made to be stronger. Many other combinations were tried with the fenugreek extract sample (Table 5.18). Three spots were produced by the mobile phase (toluene:EtOAc:FA, 36:100:36), and the R_f value was high (see Figure 5.28). This way, a mobile phase consisting of (toluene:EtOAc:FA, 36:100:36) will be used for column chromatography.

Table 5.18. TLC discovered fenugreek extract solvent combinations

No.	Solvent-1	Solvent-2	Solvent-3	Mixing ratio (v/v)
1	Hexane	Acetone		20:80
2	Toluene	EtOAc	MeOH	15:2:2
3	Toluene	EtOAc	FA	6.5:2:0.1
4	Toluene	EtOAc	FA	5:4:1
5	EtOAc	Chloroform	EtOH	1:1:2
6	Toluene	EtOAc	FA	5:4:1.2
7	Toluene	EtOAc	FA	1:1:1
8	Toluene	EtOAc	FA	5:1:1
9	Toluene	EtOAc	FA	1:2:1
10	Toluene	EtOAc	FA	1:5:1
11	Toluene	EtOAc	FA	5:1:0.5
12	Toluene	EtOAc	FA	1:5:0.5
13	Toluene	EtOAc	FA	10:10:1
14	Toluene	EtOAc	FA	15:10:1
15	Toluene	EtOAc	FA	10:15:1
16	Toluene	EtOAc	FA	20:10:1
17	Toluene	EtOAc	FA	1:2.5:1
18	Toluene	EtOAc	FA	1:2.75:1
19	Toluene	EtOAc	FA	1:2.25:1
20	Toluene	EtOAc	FA	1:2.3:1
21	Toluene	EtOAc	FA	1:2.35:1
22	Toluene	EtOAc	FA	1:2.4:1
23	Toluene	EtOAc	FA	50:100:5
24	Toluene	EtOAc	FA	50:100:10
25	Toluene	EtOAc	FA	33:100:5
26	Toluene	EtOAc	FA	10:100:5
27	Toluene	EtOAc	FA	45:100:10
28	Toluene	EtOAc	FA	45:100:20
29	Toluene	EtOAc	FA	45:100:45
30	Toluene	EtOAc	FA	40:100:40
31	Toluene	EtOAc	FA	36:100:36

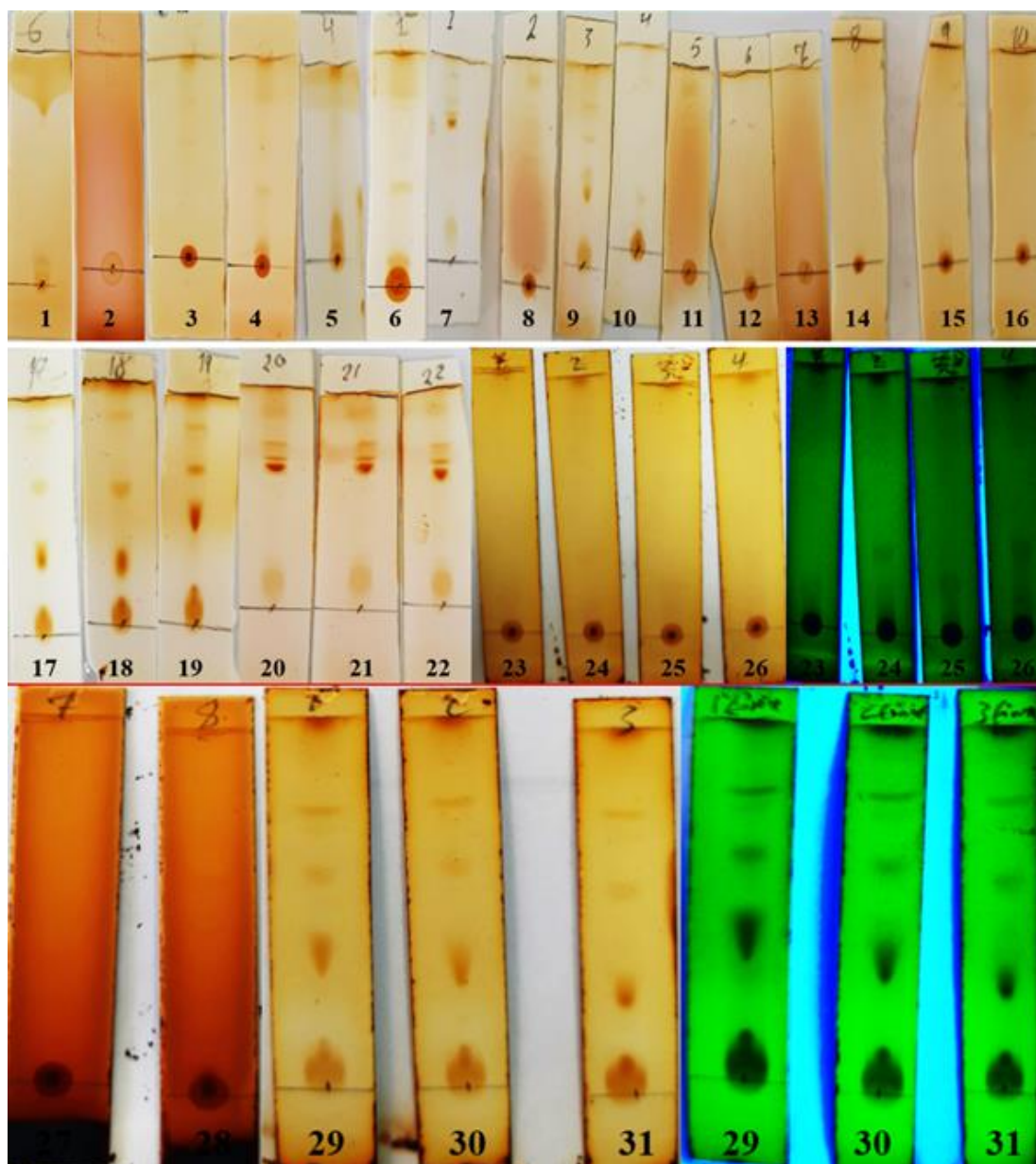


Figure 5.28. Fenugreek extract TLC chromatograms with various solvent combinations

5.7.2. Isolation of fenugreek extract by Column Chromatography

Simple, high capacity and cheap costs of adsorbents like silica gel contribute to adsorption column chromatography's widespread application in the first step of natural product separation. The separation is based on the fact that the natural products and the surfaces of the adsorbents have different affinities for adsorption. Adsorbents (stationary phase) and mobile phases (moving phase) must be carefully chosen to effectively separate natural products, get the most target compounds back, and stop target compounds from getting stuck on the adsorbents that cannot be undone.

Most phytochemical research projects employ silica gel as their adsorbent of choice. Almost 90% of phytochemical separation (on a preparative scale) was based on activated silica gel. Silica

gel has silanol groups, making it a polar absorbent. Hydrogen bonding and dipole-dipole interactions let the silica gel hold onto the molecules. Because of this, silica gel columns retain natural polar compounds longer than nonpolar ones. Chemisorption may be irreversible for certain polar natural compounds [191].

The TLC is used to choose the solvents for the column elution. Before beginning column chromatography, determine a solvent system that will separate the components. Solvents that work well for producing separation on a TLC plate for a given combination will also achieve separation on a column of the same adsorbent (silica) in column chromatography. When comparing the TLC plate to the column, the R_f values should be in the same sequence.

The column consists mainly of a glass tube filled with a stationary silica phase. Before packing the stationary phase, a frit was positioned at the column's base. Add enough sand (0.5-1 cm) to fill the curved section of the column after clamping it to the ring support. The adsorbent (silica) and the mobile phase were mixed to make a slurry, which was then put into the column for wet packing. It is considered the optimal packing method. Pour the slurry into the column quickly but gently. The mixture needs to be stirred and dumped quickly to get the most silica into the column instead of keeping it in the beaker. Tap the side of the column with a rubber stopper or tube to help the silica settle evenly. Utilize a Pasteur pipet to remove any silica clinging to the column's sides. While eluent continues to flow into the flask, let the silica settle. Carefully add sand to the top of the column after the silica has settled. Sand weighs more than silica. If the silica has not settled, the sand may sink into it rather than create a layer on top. Sand that adheres to the column's side must be rinsed away.

A minor mobile phase was used to dissolve the fenugreek extract, which is often a mix of different components. Drain the column's effluent until no solvent remains above the sand's surface. Using a pipette, add the extracted sample to the column with care. Drain effluent from the column until no sample remains above the sand's surface. Use about 1 mL of the eluent to rinse the pipet and container. This millilitre of the sample must be added to the sand. Drain effluent from the column until no liquid remains above the sand's surface. Transfer the extracted sample to the silica gel by repeating this step twice. After rinsing the extracted sample on the silica, slowly pour the eluent into the column. Instead of pouring solvent directly onto the sand, it is better to pipet a few inches of solvent onto the column to avoid disturbing the top. Add more eluent as needed. The eluent composition may be altered as the column advances. Individual tubes (1-2 mL) may be isolated from this zone using elution (see Figure 5.29).

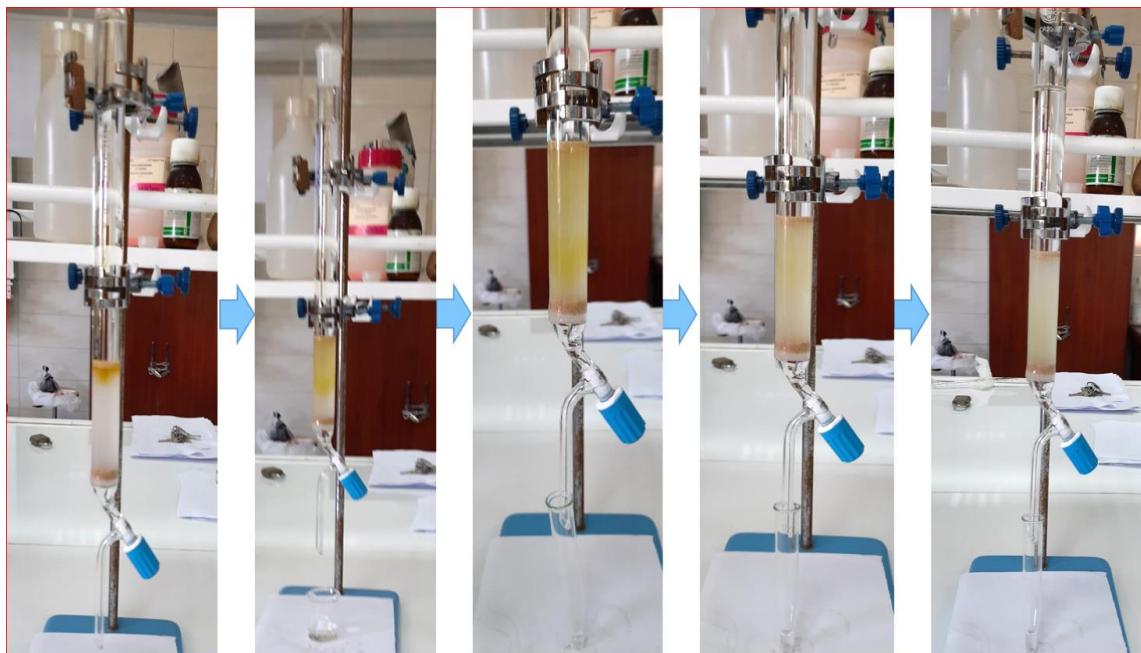


Figure 5.29. Wet loading with mobile phase (toluene:EtOAc:FA) at 36:100:36 ratios to isolate fenugreek extract

5.7.3. TLC for fenugreek extract fractions and coded fractions

Throughout the collecting procedure, 57 unique fraction tubes were gathered. Use TLC on the tubes of fractions to count how many spots there are in each, and then merge the tubes whose chromatograms are comparable. TLC was performed on each fraction tube using a solvent combination of toluene, EtOAc, and FA (36:100:36) as the mobile phase solvent (see Figure 5.30).

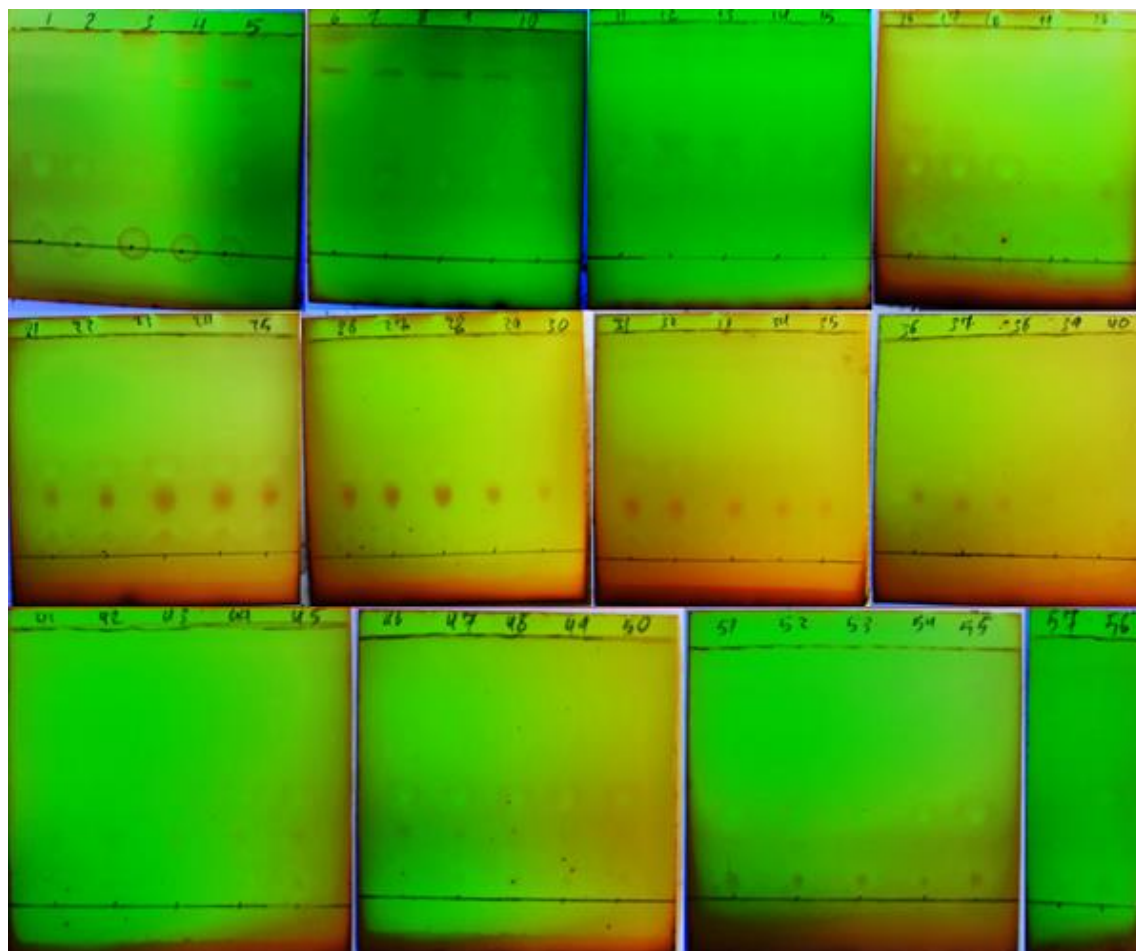


Figure 5.30. TLC for 57 fenugreek extract tubes after column chromatography with mobile phase toluene:EtOAc:FA (36:100:36)

After performing TLC on collected tubes, tubes with comparable TLC chromatograms were merged. The combined tubes were coded as follows: Fen1 contains pieces 3–10, Fen2 contains portions 11–17, and Fen3 contains portions 19–49. The fractions produced by column chromatography are shown in Figure 5.31 with their corresponding codes. Following chromatographic separation, the silica gel containing the residual extract was rinsed with MeOH to remove any lingering fragments. This section of the extract was later labelled Fen0.

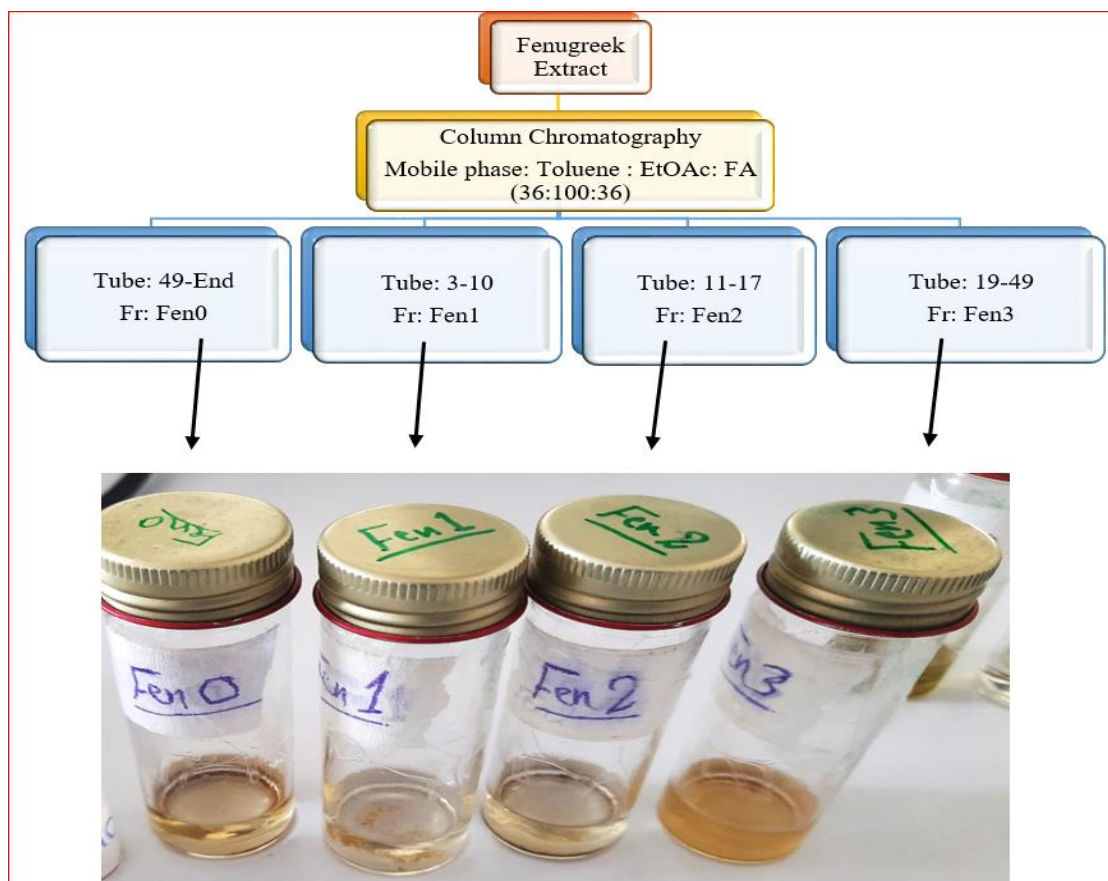


Figure 5.31. The coding of each tube obtained from column chromatography and fenugreek combined fractions

First, the fractions were concentrated in a rotating evaporator at a temperature of 40 degrees Celsius and a lower pressure. Then, they were dried by air before the last step, which was to put them in a vacuum oven. A sterile, labelled vial was used for each fraction. The vials were then put in the fridge at a temperature between 4 and 5 °C while they waited for HPLC analysis.

5.7.4. HPLC analysis of fenugreek extract and fractions

It is challenging to understand the structures of active chemicals derived from various plants, and it is also challenging to comprehend the antioxidant and biological effects of these molecules. Because of this, high-resolution chromatographic methods like HPLC were needed to find and describe several bioactive compounds in the extract and the fractions. Methods similar to this are often used when defining polyphenolic substances. Effectiveness tests were done on the fenugreek extract and the four different fenugreek fractions, which were given the numbers Fen0 through Fen3. Based on the information in Table 5.19, it is clear that the fenugreek extract was made up of only catechin phenolic components. On the other hand, the fractions Fen1, Fen2, and Fen3 contain resveratrol.

Table 5.19. HPLC analysis of the phenolic component concentration (mg/kg) of fenugreek extract and fractions

No.	Samples	Phenolic compounds						
		Catechin	p-Coumaric acid	Epicatechin	Rutin	Resveratrol	Naringin	Chlorogenic acid
1	Extract	8.772	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
2	Fen1	N.D.	N.D.	N.D.	N.D.	0.135	N.D.	N.D.
3	Fen2	N.D.	N.D.	N.D.	N.D.	0.065	N.D.	N.D.
4	Fen3	N.D.	N.D.	N.D.	N.D.	0.092	N.D.	N.D.
5	Fen0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

N.D., Not detected.

6. CONCLUSIONS

As people become more interested in the therapeutic properties of medicinal plants, scientists are studying their phytochemical activities, their ability to fight free radicals, and their ability to be separated using chromatography with a glass column on silica gel. One example is sumac (*Rhus coriaria* L.), commonly used as a spice and flavour in the Mediterranean region. Another is purslane (*Portulaca oleracea* L.), an annual plant found globally as a weed. Fenugreek (*Trigonella foenum graecum*), grown for food in various countries, is also used medicinally. Last, blackberry (*Rubus* genus in the Rosaceae family) is widely farmed worldwide. In this study, they used herbs that grew naturally in the town of Koysinjak/Erbil in the north of Iraq. For this reason, these plants were gathered in October 2020. Only dried fenugreek seeds were available in northern Iraq's Koysinjak/Erbil market.

This study aimed to look at the phytochemical composition and antioxidant properties of four medicinal plants and isolate and test the bioactive parts of the extract with the most activity. The maceration in MeOH was used to extract powdered plant components effectively, and the process was accomplished. Polar solvents, like MeOH, could get the best yield for compounds that could be extracted. For example, when sumac was extracted using the maceration method with MeOH, the sample percentage yield was 39.3%. This was followed by blackberry (34.4%) and fenugreek (11.6%) sample percentage yields. In conclusion, purslane had the lowest percentage yield of all the crops tested (10.1%)

The gallic acid equivalent (mg/g) was used to determine how many phenolic compounds were in sumac, purslane, fenugreek, and blackberry MeOH extracts. Total phenolic content was most significant in sumac extract (138.46 ± 1.44 mg GAE/g), followed by blackberry (38.08 ± 0.19 mg GAE/g) and fenugreek (33.58 ± 1.39 mg GAE/g). On average, purslane extract had the lowest phenolic content of all the tested extracts, at 16.13 ± 1.20 mg GAE/g.

The total flavonoid content of sumac, purslane, fenugreek and blackberry extracts made with MeOH was measured as mg of quercetin per g of crude extracts. Fenugreek extract had the most effective total flavonoid content (16.11 ± 0.71 mg QE/g), whereas sumac extract had a statistically lower flavonoid content (5.54 ± 0.47 mg QE/g), followed by blackberry (14.58 ± 0.83 mg QE/g) and purslane (12.91 ± 0.88 mg QE/g).

The content of flavonols in sumac, purslane, fenugreek, and blackberry crude extracts was determined using milligrams of rutin equivalents per gram. Out of the four, fenugreek extract had the highest TF content (21.81 ± 0.59 mg RE/g), followed by sumac (10.15 ± 0.58 mg RE/g) and purslane (8.07 ± 0.23 mg RE/g). Blackberry extract has a substantially lower flavonol concentration (6.95 ± 0.76 mg RE/g).

The milligrams of cy-3-glu equivalents per kilogram of dried extracts were used to determine the anthocyanin content in each plant. The pH differential method is quickly and easily applied by colourimetry. Blackberry extract has the highest TAC (7.73 ± 1.17 mg cy-3-glu/kg dry extract weight), followed by sumac (5.33 ± 1.67 mg cy-3-glu/kg dry extract weight) and fenugreek (1.87 ± 1.69 mg cy-3-glu/kg dry extract weight). The amount of anthocyanin in purslane extract was meagre (0.34 ± 1.87 mg cy-3-glu/kg dry extract weight).

When the FRAP of sumac, purslane, fenugreek, and blackberry extracts with MeOH was examined, the results were given in milligrams of quercetin equivalents per gram of dry extracts. While the FRAP of sumac extract was the greatest (92.31 mg QE/g dry extract), the power of purslane extract was statistically the lowest (6.45 mg QE/g dry extract), followed by blackberry (20.53 mg QE/g dry extract) and fenugreek (11.07 mg QE/g dry extract).

The amount of MCA in sumac, purslane, fenugreek and blackberry extracts was measured in milligrams of ferrous sulfate equivalent per gram of dry extracts during an experiment with MeOH. The sumac extract had the highest MCA (638.46 ± 0.89 mg Fe²⁺/g dry extract). In comparison, the purslane extract had the lowest (63.71 ± 0.12 mg Fe²⁺/g dry extract), followed by the blackberry (182.12 ± 0.26 mg Fe²⁺/g dry extract) and the fenugreek (76.35 ± 0.25 mg Fe²⁺/g dry extract).

Sumac, purslane, fenugreek, and blackberry extracts were tested for their ability to inhibit α -amylase using the quantitative starch-iodine method. The inhibition of α -amylase produced by the purslane extract was the greatest ($89.09 \pm 1.16\%$). In contrast, the sumac extract had the lowest level of AAI ($65.71 \pm 1.90\%$), followed by the blackberry ($76.01 \pm 1.57\%$), and then the fenugreek ($84.21 \pm 0.99\%$).

According to these results, the sumac plant had the most effective overall phenolic content, the fenugreek plant had the highest flavonoid and flavonol content, and the blackberry plant had the highest anthocyanin level. The results of antioxidants showed that sumac was most effective at decreasing ferric and binding to metals, while purslane was most effective at suppressing α -amylase.

The data shows that the plants' methanolic extract has powerful antioxidant properties. Based on the findings, it can be concluded that the plant extracts under investigation have varying levels of antioxidant activity.

Related to validation parameters, standard deviations were given in the Tables of the results obtained. The LOD values were found from the calibration graphs to be 5 μ g/mL for TPC, TFC, and TF. For FRAP and MCA analyses, LODs were determined as 20 and 100 μ g/mL, respectively. For accuracy, the recovery tests were examined, and it was found that those values are greater than 90%.

For the first time, TLC, one of the fastest and most widely used ways to separate natural products, was used to find the best solvent mobile phase for the four plants being studied. In particular, the mobile phase toluene:EtOAc:FA (4:5:2) was optimal for sumac extract fractionation. Hexane, diethyl ether, and EtOH in a ratio of 50:60:5 worked best to separate purslane extract. Toluene:EtOAc:FA (65:45:25) was optimal for blackberry extract and mobile phasing fractionation. Toluene:EtOAc:FA (36:100:36) was the optimal solvent for fractionating fenugreek extract.

Column chromatography is a well-known method for fractionating and isolating bioactive natural substances. After discovering the mobile phase using TLC, this method was implemented. Silica gel was used as the stationary phase to separate or isolate bioactive chemicals, while a solvent or solvent of choice was used as the mobile phase. After elution, TLC is used to look at the collected fractions, and fractions with similar properties are put together for later use. An HPLC analysis was performed on the main fractions to check for phenolic chemicals.

After applying column chromatography for sumac extract with mobile phase toluene:EtOAc:FA (4:5:2), a total of 70 tubes were generated. These were merged into seven fractions based on the similarity of their TLC. Because natural compounds are so complicated, it is hard to figure out how the active molecules in different plants are put together and their antioxidant and biological effects. So, high-resolution chromatographic methods like HPLC often used to describe polyphenolic compounds, were needed to find and describe some bioactive chemicals in the extract and fractions. The crude extract and seven sumac fractions (Sum0-Sum6) were all examined for efficacy. Two to four compounds made up the chromatographic fractions, while the methanolic sumac extract had at least two main ingredients (gallic acid and rutin). Sum2 had p-coumaric acid, Sum3 had gallic acid, catechin, and epicatechin, Sum4-6 had gallic acid, catechin, and resveratrol, and Sum0 had gallic acid and catechin.

When purslane extract was put through column chromatography with hexane, diethyl ether, and EtOH (50:60:5) as the mobile phase, 75 tubes were made. They were combined into eight fractions based on the similarities in TLC. We employed HPLC, often used to describe polyphenolic compounds, to analyze the crude extract and eight purslane fractions (Purs0-Purs7) to search for and characterize bioactive molecules. The components ranged from two to four, depending on the significant fraction. The methanolic purslane extract only had two components: catechin and resveratrol. Purs1, Purs2, Purs3, Purs4, Purs5, or Purs6 have yet to discover a phenolic component, whereas Purs0 contained (p-Coumaric acid, epicatechin, resveratrol, and naringin) and Purs7 contained (resveratrol).

Blackberry extract was used in column chromatography with toluene:EtOAc:FA (65:45:25) as the mobile phase to make 95 tubes. They were put into eight groups based on what their TLC had in common. HPLC was used to examine the crude extract and eight blackberry fractions

(Black0-Black7) to find and describe bioactive compounds. However, a methanolic blackberry extract has found no phenolic compounds like Black1, Black3, Black5, Black6, Black7, and Black0. While Black4 and Black5 only had rutin, Black2 had p-coumaric acid plus chlorogenic acid.

For the chromatography of fenugreek extract on glass columns, a mobile phase of toluene, EtOAc, and FA (36:100:36) was used to make 57 tubes. Similarities in TLC allowed for their classification into four distinct fractions. We used HPLC to examine the crude extract and four fenugreek fractions (Fen0-Fen3) to find and describe the bioactive components. A fenugreek methanolic extract contains just catechin. Only Fen1, Fen2, and Fen3 contained the chemical resveratrol. Contrary to Fen0, no phenolic compounds were identified.

Considering all the results obtained in the present study, the sumac plant had the highest total phenolic content and the best ability to bind to metals. It also had the best ability to reduce ferric. Similarly, HPLC research confirmed the presence of seven of the most prevalent phenolic chemicals in sumac extract and fractions. So, the sumac plant may be called a powerful antioxidant. The plant with the most flavonoids and flavonols was fenugreek, while the plant with the most anthocyanins was blackberry. Purslane was the best at inhibiting α -amylase.

The amounts of seven different phytochemicals (polyphenols) in the extracts and fractions were calculated based on the HPLC results. Gallic acid (172.68 mg/kg) and catechin (43.82 mg/kg) were found in the highest amounts in the Sum3 fraction. There was just 0.008 mg/kg of resveratrol in the purs7 fraction

As the results of this study show, selected medicinal plants provide information on possible sources of natural phytochemicals and antioxidants. In addition, all isolated fractions contain significant amounts of bioactive compounds.

RECOMMENDATIONS

The following recommendations are suggested for future work:

Plants extracted with different solvents have different amounts of phenolics, flavonoids, and antioxidative properties. Other solvents are recommended for future plant extraction initiatives.

Screening for the phytochemical content and antioxidant properties of isolated fractions responsible for the high antioxidant activity is recommended.

TLC and glass column chromatography were used to fractionate the extracts. Further purification of fractions is indicated to get pure compounds, and isolated pure compounds should be characterized by nuclear magnetic resonance (NMR), mass spectrometry (MS), and Fourier transform infrared spectroscopy (FTIR).

Because of a lack of standards for polyphonic compounds, HPLC analyzed only eight polyphonic compounds in each extract and fraction. Recommendations for further study of other polyphonic compounds aside from these eight polyphonic compounds.

Evaluating the antibacterial effects of different extracts and fractions of chosen plants is recommended. More research is needed to find, separate, and describe the antibacterial parts of plant extracts and fractions that make them work against bacteria.

Further investigation on the extract plants for in vivo studies

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APPENDICES

APPENDIX- 1:

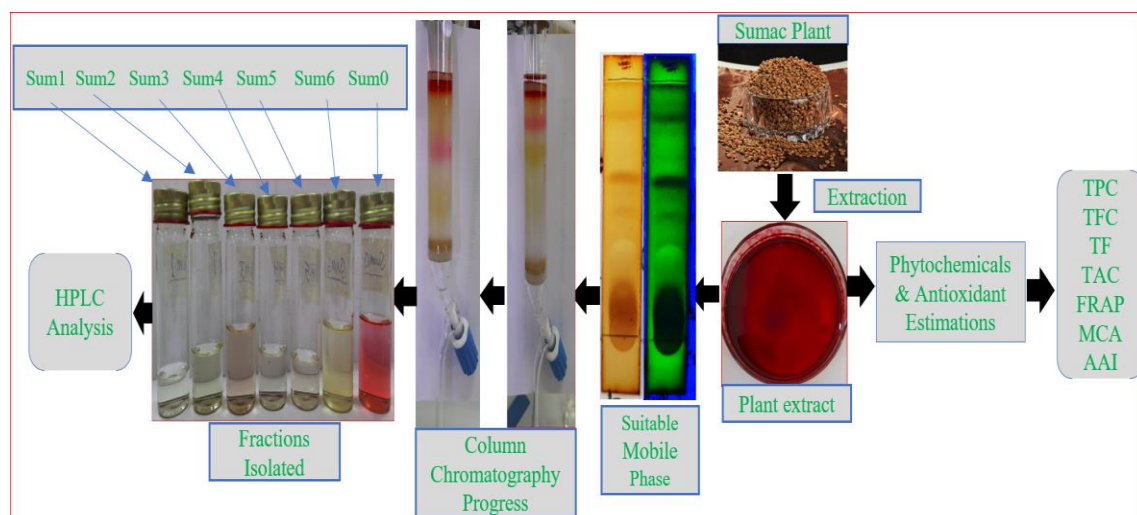


Figure A1. 1. Overall sumac extraction protocol until HPLC analysis, phytochemical screening, and antioxidant estimation

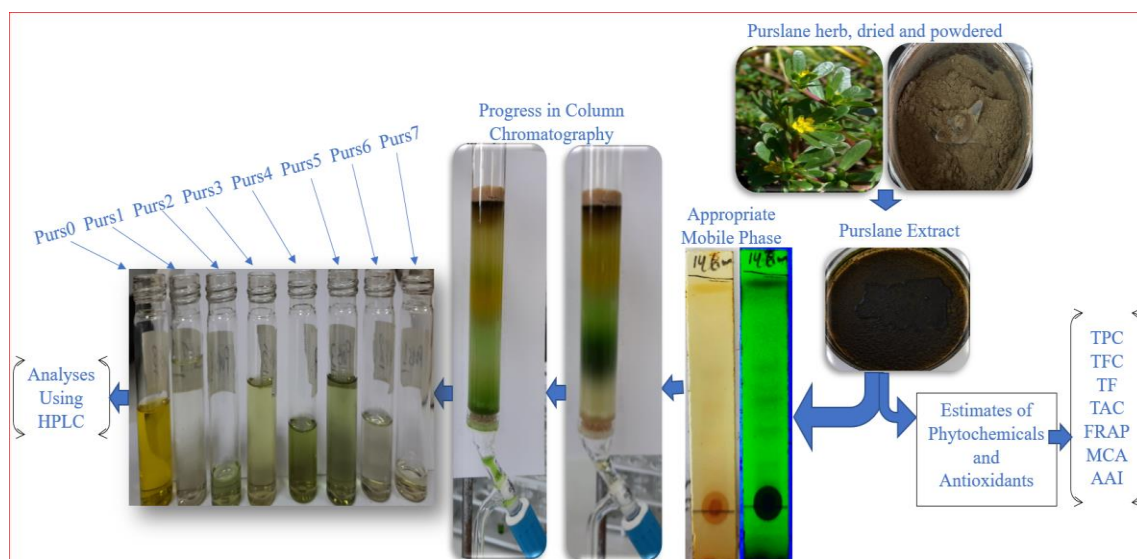


Figure A1. 2. Overall purslane extraction protocol until HPLC analysis, phytochemical screening, and antioxidant estimation

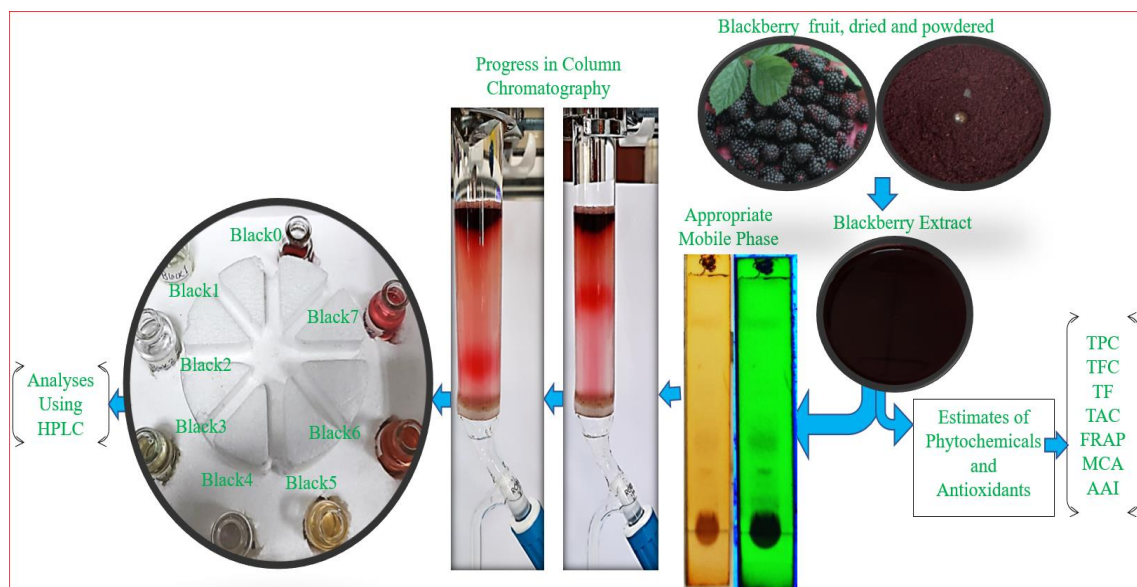


Figure A1. 3. Overall blackberry extraction protocol until HPLC analysis, phytochemical screening, and antioxidant estimation

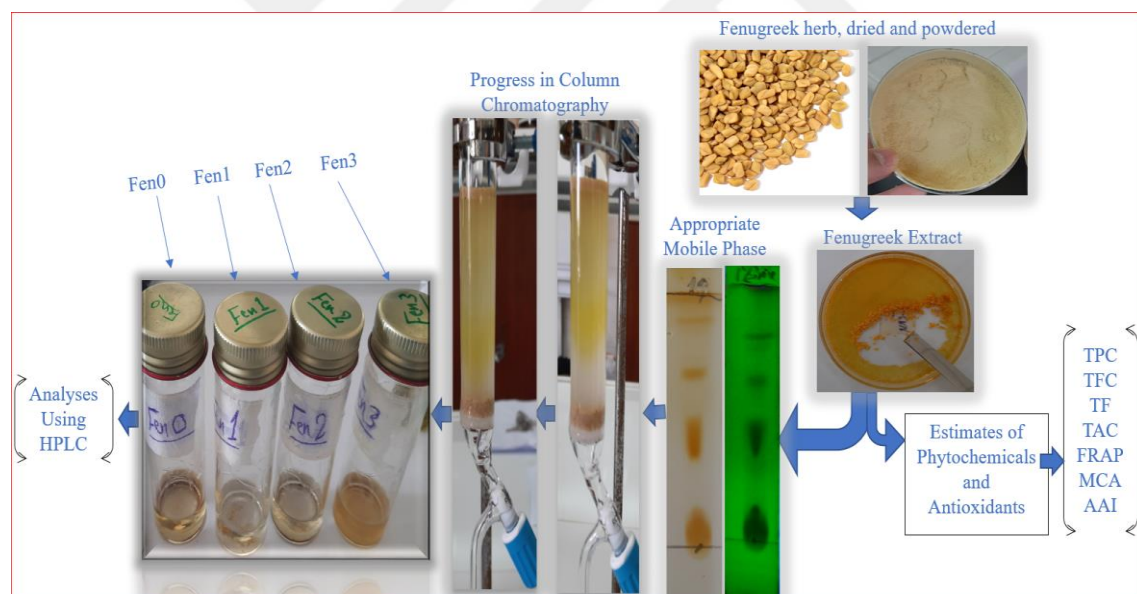


Figure A1. 4. Overall fenugreek extraction protocol until HPLC analysis, phytochemical screening, and antioxidant estimation

CURRICULUM VITAE

Aryan Fathulla QADER

ACADEMIC ACTIVITIES

Paper:

1. Qader, A. and Yaman, M. (2022). Phytochemical Screening, Antioxidant Activity, and Chromatographic Fractionation of Fenugreek (Trigonella Foenum Graecum) Seeds Extract, 41st

World Conference on Applied Science, Engineering and Technology, Ethiopia, Virtual Conference.

2. Qader, A. and Yaman, M. (2022). Phytochemical Screening, Antioxidant Activity, and Chromatographic Fractionation of Fenugreek (*Trigonella Foenum Graecum*) Seeds Extract, *Journal of Pharmaceutical Negative Results*, Vol. 13, pp. 2096–2101.
3. Qader, A. and Yaman, M. (2022). Phytochemical Screening, Antioxidative Activity, Chromatographic Fractionation, and HPLC Analysis of Phenolic Components in Blackberry (*Rubus fruticosus* L.) Extract, *4th International Congress on Analytical and Bioanalytical Chemistry*, Turkey, Online.
4. Qader, A. and Yaman, M. (2020). Comparison methods to determine antioxidant activities: what they mean, *2nd International Congress on Analytical and Bioanalytical Chemistry*, Antalya, Turkey.

