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Crude Lipid Content and Response Surface Methodology-Based Optimization of Phenolics, Flavonoids, and Antioxidant Extraction from Usnea Lichen of Ras Dashen Mountain, Ethiopia

Destaw, Merkebu

BAHIR DAR UNIVERSITY

COLLEGE OF SCIENCE

POSTGRADUATE PROGRAM STUDIES

DEPARTMENT OF CHEMISTRY

MSc THESIS ON:

CRUDE LIPID CONTENT AND RESPONSE SURFACE METHODOLOGY-BASED OPTIMIZATION OF PHENOLICS, FLAVONOIDS, AND ANTIOXIDANT EXTRACTION FROM *USNEA* LICHEN OF RAS DASHEN MOUNTAIN, ETHIOPIA

BY:

DESTAW MERKEBU

JUNE, 2026

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Crude Lipid Content and Response Surface Methodology-Based Optimization of Phenolics, Flavonoids, and Antioxidant Extraction from *Usnea* Lichen of Ras Dashen Mountain, Ethiopia

BY

Destaw Merkebu

A Thesis Submitted to Bahir Dar University, College of Science, Department of Chemistry in Partial Fulfillment of the Requirements for Master of Science in Analytical Chemistry

ADVISOR: MINALESHEWA ATLABACHEW (PhD, PROFESSOR)

CO-ADVISOR: DESTAW DAMTIE (PhD)

JUNE, 2026

BAHIR DAR, ETHIOPIA

DECLARATION

This is to certify that the thesis entitled ‘‘Crude Lipid Content and Response Surface Methodology-Based Optimization of Phenolics, Flavonoids, and Antioxidant Extraction from *Usnea* Lichen of Ras Dashen Mountain, Ethiopia’’, submitted in partial fulfillment of the requirements for the degree of master of Science in Analytical Chemistry of department of Chemistry, Bahir Dar University, is a record of original work carried out by me and has not been submitted previously, either in whole or in part, to this or any other institution for the award of any degree or certificates. All sources of information and assistance received during the course of this investigation have been duly acknowledged.

Destaw Merkebu 10/2018 Bahir Dar

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Approval of Thesis for Defense

I hereby certify that I have supervised, read, and evaluated this thesis titled "Crude Lipid Content and Response Surface Methodology-Based Optimization of Phenolics, Flavonoids, and Antioxidant Extraction from *Usnea* Lichen of Ras Dashen Mountain, Ethiopia" by Destaw Merkebu, prepared under my guidance. I recommend that the thesis be submitted for oral defense.

	Signature	Date
Name of Advisor:		
Minaleshewa Atlabachew (PhD, Professor)	----------	----------
Name of Co-advisor		
Destaw Damtie (PhD)	----------	----------
Department Head	----------	----------

Examiners Approval form

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Approval of thesis for the defense result

As members of the board of examiners, we examined this thesis entitled “Crude Lipid Content and Response Surface Methodology-Based Optimization of Phenolics, Flavonoids, and Antioxidant Extraction from *Usnea* Lichen of Ras Dashen Mountain, Ethiopia” by Destaw Merkebu. We hereby certify that the thesis is accepted as fulfilling the requirements for the award of the degree of “Master of Science in Analytical Chemistry”.

Board of Examiners

External examiner name	Signature	Date
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Internal examiner		
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Chairperson's name		
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ABSTRACT

Lichens are symbiotic organisms consisting of fungi and algae with notable bioactive potential. This study optimized extraction conditions and quantified total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity of Usnea species using Response Surface Methodology (RSM)-Design Expert 13. The Usnea species was collected from three host plant species: Hagenia abyssinica (Kosso), Hypericum revolutum (Amija), and Erica arborea (Wuchena), from the Ras Dashen Mountain region within Simien Mountains National Park (SMNP), northern Gondar, Ethiopia. Methanol concentration (40-100% v/v) and extraction time (60-180 min) were evaluated as independent variables, with optimum yield achieved at 100% methanol and at 115, 130, and 120 minutes for Usnea species collected from Hagenia abyssinica, Hypericum revolutum, and Erica arborea. A UV-Vis spectrophotometer was used to measure the total phenolic and flavonoid contents and the antioxidant activity of the samples. The optimum values of TPC, TFC, and antioxidant activity obtained were 161.06 ± 0.73 mg GAE/100g DW, 413.68 ± 1.82 mg CE/100g DW, and 73.39 ± 0.98 % inhibition (Hagenia abyssinica);111.14 ± 1.24 mg GAE/100g DW, 256.14 ± 1.61 mg CE/100g DW, and 53.52 ± 1.1 % inhibition (Hypericum revolutum); and 123.44 ± 1.07 mg GAE/100 g DW, 429.12 ± 2.19 mg CE/100g DW, and 71.1 ± 0.96 % inhibition (Erica arborea) respectively. Beyond phenolic and antioxidant characterization, lipid content determination revealed that Usnea species exhibited varying lipid levels: 16.34% (w/w) in the sample collected from Hagenia abyssinica, 29.92% (w/w) from Hypericum revolutum, and 15.9% (w/w) from Erica arborea. These findings provide a comprehensive biochemical characterization of Usnea species, demonstrating their potential as a source of antioxidants and nutritionally relevant lipids, and supporting further exploration of their applications in nutritional and pharmaceutical development.

Keywords: Antioxidant activity, Flavonoids, Lipid content, Optimization, Total phenolics.

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ABBREVIATIONS AND ACRONYMS

AAE……. Ascorbic Acid Equivalent

ANOVA……... Analysis of Variance

CCD……..Central Composite Design

F-C……... Folin-Ciocalteu

GAE……. Gallic Acid Equivalent

CE…….....Catechin Equivalent

RSM……..Response Surface Methodology

SMNP…….....Simien Mountains National Park

TFC…….....Total Flavonoid Contents

TPC……...Total Phenolic Contents

UV-Vis……. Ultraviolet-Visible Spectroscopy

CHAPTER 1: INTRODUCTION

1.1 Background of the Study

Lichens are stable symbiotic associations between heterotrophic fungi (mycobionts) and photosynthetic partners such as green algae or cyanobacteria (photobionts) (Garty 2015). The symbiotic association of one mycobiont and one or more photobionts forms a common vegetative structure called a thallus. This thallus usually consists of the upper cortex, photosynthetic layer, medulla, and lower cortex. The symbiotic relationship allows lichens to inhabit extreme and diverse environments. Their ability to colonize extreme habitats underscores both their ecological significance and chemical diversity. Lichens can grow on a wide range of substrates, including minerals, rocks, bare soil, plant wood or leaves, in streams and marine environments, as well as surfaces made of synthetic materials (Morillas et al. 2022).

Historically, lichens have been used as food spices, textile dyes, perfume odorants, and in the cosmetic industry. Several lichens are used as traditional medicines to treat skin, respiratory, and digestive issues, and a few are used as sources of fermentable sugars for the production of ethyl alcohol (Muthu et al. 2021; Zeghina et al. 2024). Lichens have significant ecological functions, including soil formation, nutrient cycling, and bioindication of air quality. In addition to their ecological roles, in recent decades, lichens have received more attention because of their abundant secondary metabolites. There are already over 800 secondary metabolites unique to lichens that are known to exist, and many of them are now recognized to have antibacterial, anticancer, antimutagenic, antifungal, or antiviral properties (Basiouni et al. 2020; Elix 2016; Muthu et al. 2021).

Globally, over 20,000 lichen species have been reported across diverse ecological zones (Belhouala et al. 2024). Ethiopia possesses one of the most diverse and distinctive flora collections on the African continent (Fashing et al. 2022). Unfortunately, there is little information about the nation's cryptogamic flora, and the checklist states that 279 lichen species are available (Yeshitela et al. 2009). Those different lichen species are distributed across different ecological zones, especially in the highland areas characterized by low temperature and variable humidity. Among them, the genus *Usnea* (*Parmeliaceae; lichenized Ascomycetes*), commonly known as beard lichen, is particularly valued for its fruticose morphology and its long

history of ethnomedicinal use. The genus *Usnea* is known to be used in traditional medicines, including treatments for respiratory infections, wound healing, and skin diseases, reflecting its rich reservoir of bioactive metabolites; it is also used as dyes, cosmetics, deodorants, and in spices as a preservative in both eastern and western countries (Crawford 2019).

Research on *Usnea* and its metabolites has confirmed various biological activities, including antioxidant, antimicrobial, antitumor, antiviral, anti-inflammatory, cardioprotective, and hepatoprotective properties (Prateeksha et al. 2016). In addition to these bioactivities, phytochemical analyses have revealed that *Usnea* species contain a wide range of phenolic compounds, flavonoids, depsides, depsidones, dibenzofurans, terpenes, sterols, and polysaccharides. These constituents are central to the lichen's pharmacological potential, with phenolics and flavonoids particularly associated with radical-scavenging and antioxidant activities (Piñeiro et al. 2024).

The inherent structural complexity and extensive chemical heterogeneity of polyphenols present considerable analytical challenges in their isolation, separation, and quantification (Savic, Petrovic, and Knezevic-Jugovic 2026). Several studies have emphasized the importance of optimizing extraction protocols, though their approaches differed in terms of solvent systems and plant species. Therefore, optimizing extraction and separation procedures has become a fundamental step in ensuring the reproducibility and reliability of polyphenol analysis. Conventional optimization methods, which vary one factor at a time, are often inefficient and fail to capture interactions among variables. In contrast, Response Surface Methodology (RSM) is a mathematical and statistical analysis software that is used to fit a polynomial equation to experimental data to optimize the functional relationships between several variables and the responses of interest (Jaafar, Ramli, and Salleh 2020). DINÇER et al. (2021) determined the amount of phenolics present in the *Usnea* longissimi species by supercritical carbon dioxide extraction using RSM. Response Surface Methodology (RSM) was applied in this study because it provides an efficient and systematic approach for optimizing multiple extraction variables simultaneously. Unlike conventional one-factor-at-a-time methods, RSM allows the evaluation of both individual and interactive effects of variables such as methanol concentration and extraction time on the responses. This approach reduces the number of experimental trials

required, thereby saving time, minimizing solvent consumption, and improving experimental efficiency.

Methanol concentration and extraction time strongly influence the extraction efficiency of phenolics and flavonoids (Do et al. 2014). Methanol, due to its intermediate polarity, effectively extracts a wide range of bioactive compounds, while its concentration determines solvent polarity and compound solubility (Gandhi et al. 2022). Extraction time also affects yield, as insufficient time leads to incomplete extraction, whereas prolonged time may cause degradation of sensitive compounds (Ashraf et al. 2024). Therefore, these factors were chosen for optimization using Response Surface Methodology.

Despite the increasing global interest in lichen bioactive compounds, there remains a significant research gap in Ethiopia. To date, no comprehensive study has investigated the crude lipid content and optimized the extraction of phenolics, flavonoids, and antioxidant activity of *Usnea* species from Ras Dashen Mountain using advanced statistical approaches such as RSM. Therefore, this study is novel in that it applied Response Surface Methodology to optimize extraction conditions while also providing a comparative understanding of its advantages over conventional methods. Furthermore, it integrates crude lipid content analysis with phytochemical and antioxidant determination of *Usnea* species, thereby generating baseline scientific data that contributes to the valorization of Ethiopia's underexplored lichen biodiversity and supports future pharmacological, nutraceutical, and biotechnological applications.

1.2 Statement of the Problem

Ethiopia is one of the most remarkably ecologically diverse countries and is home to a variety of lichen species, with around 279 species recorded (Yeshitela et al. 2009). Lichens play vital roles in ecosystem balance, including soil formation, nutrient cycling, and serving as bioindicators of environmental change. Yet, their chemical composition and bioactive potential are largely unexplored in Ethiopian highlands. Some of these phytochemicals include total phenolics, total flavonoids, antioxidant activity, and lipid content of *Usnea* lichen species from Ras Dashen Mountain. There are reports on the total phenolics, total flavonoids, and antioxidant activities in worldwide *Usnea* species of lichens collected from different countries. Phenolic and flavonoid compounds are known for their strong antioxidant and antimicrobial activities. Lalremruata et al. (2022) reported that phytochemical constituents (flavonoids and phenols) and the antioxidant

activity of the methanol extract of *Usnea* species from India exhibited high total phenolic and flavonoid contents, with strong antioxidant and antibacterial activities. Similarly, Kocakaya et al. (2024) compared the biological potential of *Usnea* species and reported significant levels of usnic acid alongside measurable phenolic and flavonoid concentrations. These findings confirm that *Usnea* species are rich sources of natural antioxidants.

Beyond polyphenols, previous studies on *Usnea* species have focused primarily on profiling fatty acids, revealing diverse compositions that contribute to cellular adaptation and environmental resilience. For instance, UHPLC-MS analysis demonstrated the presence of saturated and unsaturated fatty acids, with antimicrobial and nutritional properties (Salgado et al. 2018). Similarly, Carrasco et al. (2024) reported that *Usnea* species in Arequipa, Peru, exhibited the highest total fatty acid concentration among lichens studied. These findings highlight the richness of *Usnea* in fatty acids. However, to date, there was no published reports on the total lipid content of *Usnea* species, particularly within the Ethiopian highlands. The present study therefore provides the first determination of lipid content, addressing this gap and offering new insights into the ecological and pharmacological potential of Ethiopian lichens. Moreover, scientific data remain insufficient on how these compounds are distributed and influenced by the unique climatic gradients and altitudinal variations of the Ethiopian highlands. Without such information, the ecological and pharmacological significance of Ethiopian lichens has remained underappreciated and underutilized. In this study, optimum parameter conditions of extraction were determined, and total phenolic, flavonoid, and antioxidant activity were quantified. Lipid content was also assessed from *Usnea* species collected from Ras Dashen Mountain, northern Ethiopia. The findings have provided baseline biochemical data essential for future research, conservation planning, and potential biotechnological applications

1.3 Objectives of the Study

1.3.1 General Objective

The main objective of this study was to determine the crude lipid content and to optimize the extraction of phenolics, flavonoids, and antioxidant activity from *Usnea* lichen of Ras Dashen Mountain, Ethiopia, using response surface methodology.

1.3.2 Specific Objective

- To optimize the extraction conditions for total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity of *Usnea* species using Response Surface Methodology.
- To determine the total phenolic content (TPC) and total flavonoid content (TFC) of *Usnea* extracts.
- To determine the antioxidant property of the extracts.
- To determine the crude lipid content of the studied samples.
- To assess the correlation between phenolic and flavonoid contents and antioxidant activity of *Usnea* species collected from three host plants.

1.4 Scope of the Study

The scope of this study was focused on determining the total phenolic content, total flavonoid concentration, antioxidant activity, and lipid content of *Usnea* lichen species collected from the Ras Dashen (Ras Dejen) Mountain regions in the Simien Mountains National Park, northern Ethiopia. It aims to explore the biochemical diversity and adaptive traits of lichens thriving under the mountain's extreme highland conditions. The research included field collection, sample preparation, and optimization using the response surface method, and laboratory analysis using standard analytical methods.

1.5 Significance of the Study

Lichens are the most bioactive compound source of natural resources, yet their biochemical compositions remain poorly understood in Ethiopia. The Ras Dashen (Ras Dejen) mountain, with its extreme climate and unique biodiversity, provides an ideal natural laboratory for exploring how lichens produce and accumulate bioactive compounds such as phenolics, flavonoids, and lipid contents. Understanding these compounds is significant for several reasons. First, phenolics and flavonoids are well known for their antioxidant, antimicrobial, and anti-inflammatory properties, which could have potential applications in pharmaceutical development. Second, lipids play vital roles in maintaining membrane stability and adapting to stress, offering insights into how lichens survive harsh alpine conditions.

Additionally, this study will provide new information on Ethiopian highland lichens, offering valuable data that can support ecological research and biodiversity conservation efforts. The findings may also inform future bio-prospecting initiatives, encourage the sustainable use of local natural resources, and raise awareness of the ecological importance of lichens in mountain ecosystems. Findings not only highlight species-specific metabolic variation but also suggest potential for further pharmacological and industrial exploration.

CHAPTER 2: LITERATURE REVIEW

2.1 Overview of Lichens

2.1.1 Definition and Classification

Lichens are symbiotic relationships between a fungus (the mycobiont) and one or more photosynthetic groups (the photobiont), such as cyanobacteria or green algae (Asplund and Wardle 2017; Zhao, Wang, and Xu 2021). The fungal partner gives structure, water retention, and absorption of minerals from the environment, while the algal or cyanobacterial partner performs photosynthesis to produce carbohydrates. They are the best example of mutualistic relationships (Saini, Nayaka, and Bast 2019).

Lichens are classified as a different taxonomic group. They are mainly classified as crustose (grow in a crust-like state), foliose (broad, leafy-like structure), and fruticose (shrub-like, long and stringy) based on their physical characteristics (Lakhnarayan Kumar Bhagarathi et al. 2022). Further they are also grouped in to the following on the bases of substrate on which they develop and to a habitat subsets as legnicolous (grow on dead wood), ramicolous (growing over twigs), musicolous (growing on mosses), terricolous (inhabit the soil or ground), follicolous (growing over the leave), saxicolous (growing on rocks or stones) and corticolous (growing on the bark of tree) (Singh, Arya, and Vishwakarma 2019).

2.1.2 Ecology and Distribution of Lichens

Lichen species are distributed worldwide and are found to grow in almost all climatic conditions (Pichler et al. 2023), although they are influenced by multiple environmental, climatic, and substrate-related factors (Lõhmus, Motiejūnaitė, and Lõhmus 2023). The type of photobiont is also important for the environmental response and spatial distribution of lichens (Schweiger et al. 2022). Trees, rocks, and soil are the three most prevalent natural substrates. They can absorb and hold moisture from diverse sources, allowing them to grow on exposed substrata such as tree leaves and bark. They can also grow in harsh settings such as hot deserts, barren rocky cliffs, and cold Polar Regions, such as arctic tundra, and even high-altitude situations. Other extreme habitats, such as toxic slag heaps, are also home to lichen species (Chai et al. 2024). Lichens can also grow in marble from antique buildings and monuments (Lücking, Leavitt, and Hawksworth 2021). Since they cannot be found without the symbiotic relationship between a mycobiont and a

photobiont, their reproduction method is generally dual, involving both the fungal and algal partners of sexual and asexual reproduction (Martínez et al. 2012).

Highland ecosystems such as those in Ethiopia expose organisms to unique stress conditions, including high UV radiation, temperature extremes, and nutrient-poor soils. These abiotic pressures have been shown to enhance metabolite diversity, particularly in secondary compounds like phenolics and flavonoids, which serve protective ecological functions. Recent studies on Ethiopian highlands emphasize that such stress-driven adaptations contribute to biodiversity resilience and may result in distinctive phytochemical profiles in lichen species (Muhammed and Elias 2024).

The host substrate or plant species plays a significant role in influencing lichen metabolite composition. Variations in bark chemistry, moisture retention, and surface texture can affect nutrient availability and microenvironmental conditions, leading to differences in phenolic and flavonoid content among the same lichen species growing on different hosts (Aragón et al. 2025).

Altitude is a key environmental factor influencing lichen metabolism. At higher elevations, increased UV radiation and harsh climatic conditions promote the synthesis of secondary metabolites, particularly phenolic compounds, which act as photoprotective and antioxidant defenses (BACHEREAif and Ast 1997).

2.1.3 The Genus *Usnea*

The genus *Usnea* belongs to the large family Parmeliaceae and is commonly referred to as “beard lichens” due to their fruticose, hair-like morphology. These lichens are widely distributed in temperate and tropical regions, often colonizing high-altitude ecosystems where cool temperatures and clean air prevail. Most of the time, these species grow on tree bark (Truong and Clerc 2012).

Different species of the genus *Usnea* exhibit significant variation in their phytochemical composition, including differences in phenolic and flavonoid contents, as well as antioxidant activity. Comparative studies have shown that distinct *Usnea* species contain varying numbers and types of metabolites and display different antioxidant capacities, which are influenced by both genetic variability and environmental conditions such as habitat and altitude (Salgado et al. 2018).

2.1.4 Importance of Lichens

Historically, lichens have been valued for their medicinal properties and have been widely used in traditional medicine, especially across Asian and African cultures. They were commonly employed to treat wounds, coughs, respiratory, gastrointestinal, and digestive issues due to their healing and antimicrobial effects (Shahid et al. 2020).

Ecologically, lichens play a vital role in maintaining ecosystem balance. They contribute to nutrient cycling and soil formation and serve as habitats for various microorganisms and small invertebrates. Because of their sensitivity to environmental factors such as light, humidity, UV-B radiation, temperature, and airborne pollutants like sulfur dioxide (SO_2) and nitrogen oxides (NO_x), lichens are excellent bioindicators for assessing air pollution and climate change impacts (Morillas 2024).

Lichens have been used in medicine preparation commercially, apart from their usage in traditional medicine for centuries. In modern science and pharmaceuticals, lichens are recognized for producing diverse secondary metabolites such as usnic acid, depsides, parietin, and atranorin. These compounds exhibit strong antimicrobial, antiviral, anti-inflammatory, anticancer, and antioxidant properties (Molnár and Farkas 2010; Poulsen-Silva et al. 2023).

Beyond their ecological and medicinal importance, lichens are also used in various industrial and biotechnological applications. Their aromatic compounds are valuable in dyeing, cosmetics, perfume industries, and textile production. Their extracts play a role in enzyme production and the green synthesis of nanoparticles (Hamida et al. 2021).

The genus *Usnea* has long been valued in folk medicine worldwide. Traditionally, species have been consumed to treat diarrhea, ulcers, urinary infections, tuberculosis, pneumonia, stomachache, and fungal diseases in cattle. Previous phytochemical studies have revealed that the dibenzofuran usnic acid is found in all *Usnea* species, and it is one of the most common and abundant metabolites. Usnic acid as a pure substance has been used in creams, toothpastes, mouthwashes, deodorants, and sunscreen products as an active ingredient or as a preservative because of its antimicrobial properties. *Usnea* contains usnic acid, phenolic compounds, and

flavonoids, all of which contribute to its antioxidant, antibacterial, hepatoprotective, antiulcerogenic, antigenotoxic, lipid-regulatory, and anticancer properties (Phi et al. 2023).

2.2 Phenolic Compounds in Lichens

Phenolics are a diverse group of secondary metabolites characterized by the presence of one or more aromatic rings and one or more hydroxyl groups (de la Rosa et al. 2019). They are broadly distributed in the plant kingdom and are the most common secondary metabolites in plants, with over 8,000 known structures ranging from basic compounds like phenolic acid to highly polymerized substances called tannins (Alara, Abdurahman, and Ukaegbu 2021; Dai and Mumper 2010).

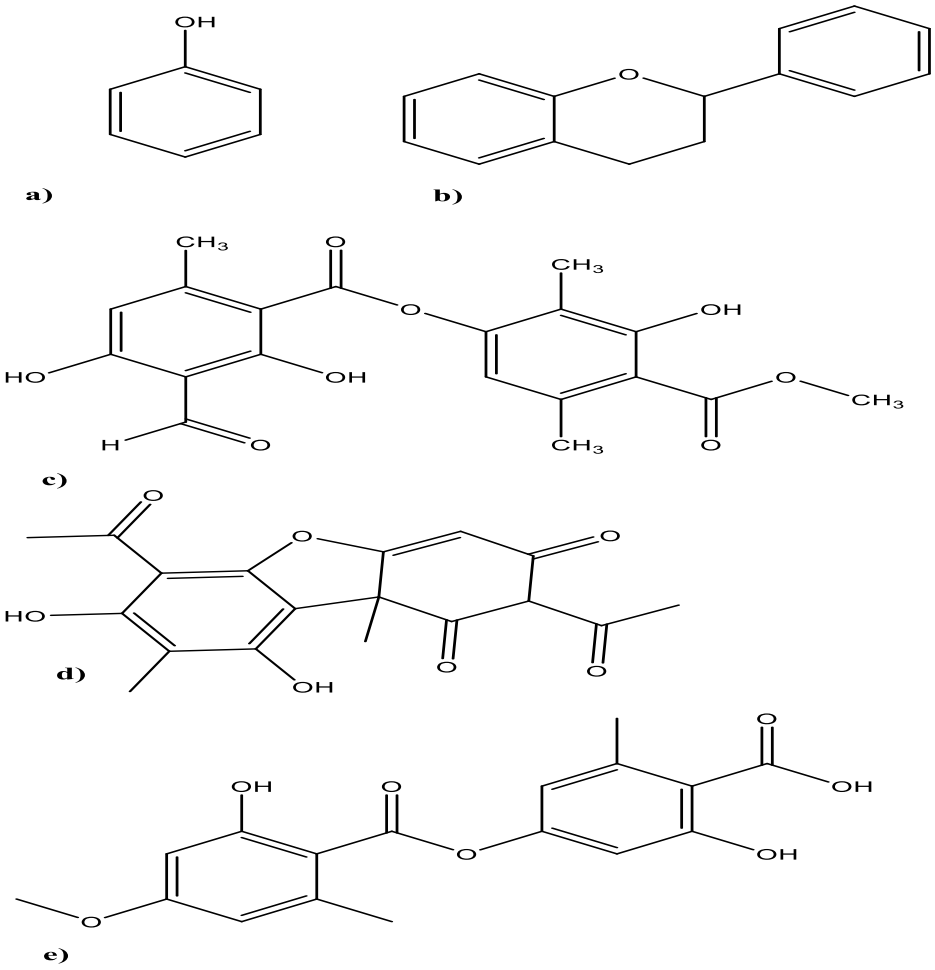


Figure 2.1. Chemical structures of phenol (a), flavonoid (b), depside atranorin (c), dibenzofuran usnic acid (d), and depsidones (e)

In lichens, phenolics serve as protective agents against oxidative stress and UV radiation. Their antioxidant activity is primarily due to their ability to donate hydrogen atoms or electrons to neutralize free radicals (White et al. 2014).

Studies on lichen phenolics have demonstrated significant variation in content depending on species, habitat, climate, and extraction conditions. For example, lichens collected from high-altitude regions often exhibit higher phenolic content, possibly due to increased exposure to environmental stressors (Vega-Bello et al. 2023). From Asia, particularly in the Kashmir region of India, Piñeiro et al. (2024) reported that the methanol extract of the genus *Usnea* contains approximately 48 mg GAE/g of dried extract, indicating a substantial phenolic content. Comparable results were reported by Londoñe-Bailon et al. (2019), who quantified the total phenolic content using methanol extracts. Their study demonstrated values of 19.42 ± 0.32 mg GAE/g in *Usnea aurantiaco-atra* and 22.80 ± 0.08 mg GAE/g in *Usnea Antarctica*.

2.3 Flavonoids in Lichens

Flavonoids are polyphenolic compounds having a flavan nucleus with 15 carbon atoms organized in three rings (C6-C3-C6), two benzene rings (A and B) linked by a three-carbon pyran ring (C), forming **Figure 1b**. They are widely distributed in plants and lichens in excess amounts and contribute to the color, fragrance, and flavor characteristics of plants (Maleki, Crespo, and Cabanillas 2019). They prevent UV damage, resist disease, and affect the formation of root nodules in legumes (Zhang et al. 2022). Because of the position of the B-ring and the position and number of hydroxy groups on it, the antioxidant capacity of flavonoids can be different (D'Amelia et al. 2018). The chemical reaction mechanism of flavonoid antioxidant protection is achieved by the donated electrons through resonance from functional hydroxy groups to stabilize free radicals (Šamec et al. 2021).

Several studies have investigated flavonoids and related compounds in *Usnea* lichens, highlighting their antioxidant, cytotoxic, and pharmacological properties. Jannah et al. (2022) confirm that the genus *Usnea* contains flavonoids, and these compounds have high antioxidant properties. A broader review by Prateeksha et al. (2016) found that this species contains flavonoids and plays important roles in the plant's pharmacological activity, supporting traditional uses in treating infections and inflammation. Together, these findings suggest that flavonoids in *Usnea* are not only secondary metabolites but also key contributors to its

antioxidant, antimicrobial, and potential therapeutic properties. A study was conducted in India by Sharma & Kalikotay. (2012) reported that the methanolic extract of this species contained a total flavonoid content of 1.543 ± 0.0057 µg QE/g dry weight (DW).

2.4 Antioxidant Activity in Lichens

Antioxidant activity refers to the ability of compounds to scavenge free radicals. Phenolic-rich lichen extracts and isolated compounds scavenge free radicals, inhibit lipid peroxidation, and modulate inflammatory mediators in vitro. These properties underpin proposed applications in wound healing, where antioxidant and anti-inflammatory actions can accelerate tissue repair and reduce infection risk (G et al. 2024; Poulsen-Silva et al. 2023). Lichens, including *Usnea*, have demonstrated strong antioxidant activity in various assays such as DPPH, ABTS, and FRAP (Kalra et al. 2021). Although some studies report a negative correlation between antioxidant and flavonoid contents across different solvent extracts, others report a positive correlation between phenolic and flavonoid contents and antioxidant activity in lichens (Kalra et al. 2021). This suggests that these compounds are the primary contributors to lichen antioxidant potential.

Odabasoglu et al. (2004) reported that species within the genus *Usnea* demonstrated notable antioxidant activity. In particular, methanolic extracts of *Usnea florida* and *Usnea longissimi* exhibited inhibition rates of 13.38 and 82.44 %, respectively. The extent of antioxidant activity, however, varied depending on the solvent employed. A strong relation was observed between total phenolic content and antioxidant activity in methanol extracts, whereas no linear correlation was detected in water extracts. In contrast, the methanol extract of *Usnea* species suggested that phenolic compounds do not all exhibit the same antioxidant potential; some demonstrate strong activity, others moderate, and some only weak activity. Similarly, the study by Behera et al. (2006) indicates that the genus *Usnea* is a potential source of natural antioxidants. According to Popovici et al. (2021), this species exhibited notable antioxidant activity when extracted with different solvents. The methanol extract demonstrated the most significant antioxidant effect with a DPPH IC_{50} = 3300 µg/mL. However, the water extract displayed the lowest antioxidant activity, which may be attributed to the limited solubility of compounds in this solvent. A similar study on the genus *Usnea* was conducted by Singleton et al. (1999), and the highest antioxidant activity was observed from the methanol extract (125.2 ± 6.4 mg TE/100 g of dried lichen). All

of these studies show that lichens and lichen substances might be novel sources of natural antioxidants.

Although several studies report high phenolic and antioxidant values in *Usnea* species, the reported results vary widely depending on species type, extraction solvent, and environmental conditions. For instance, methanolic extracts consistently show higher phenolic content compared to aqueous extracts, highlighting the importance of solvent polarity. However, differences among species and habitats suggest that metabolite production is not uniform and requires systematic optimization.

2.5 Lipid Content in Lichens

Lipids are essential biomolecules that serve as structural components of cell membranes, energy reserves, and signaling molecules. In lichens, lipids contribute to physiological adaptation, stress tolerance, and ecological resilience. Although many studies have investigated the fatty acid composition of lichens, for instance, Carrasco et al. (2024) conducted the identification and quantification of fatty acids (FAs) in various lichen species using GC-FID analysis of extracts prepared with a MeOH:$(CH_3)_2$ CO (1:1) solvent mixture. The study shows that butyric acid was the only common FA detected across the species examined, with concentrations ranging from 3.4 to 4.1 mg/g. Within the genus *Usnea*, this species exhibited a comparatively higher total FA concentration **(**5.81 mg/g) than the other lichens analyzed. Reports specifically addressing total lipid content of *Usnea* species are limited. Lipid determination provides an overview of the biochemical reserves in lichen thalli and can serve as a baseline for understanding their nutritional and pharmacological potential. In particular, members of the genus Usnea are recognized for their diverse secondary metabolites, yet their crude lipid content has not been widely documented. Assessing lipid levels in these species offers valuable insight into their biochemical composition and potential applications in nutraceutical and biotechnological fields.

2.6 Analytical Techniques for Total Phenolics, Total Flavonoids, Antioxidant Assay

The Folin-Ciocalteu (FC) assay is a simple and widely used spectrophotometric method for estimating total phenolic content. It works by the reduction of the F-C reagents under alkaline conditions, producing a blue complex measurable at 765 nm. Folin-Ciocalteu reagent acts as the

oxidizing compound. Despite being non-specific (other reducing agents may interfere), the assay remains a benchmark tool due to its sensitivity, reproducibility, and adaptability to diverse biological samples, including lichens. Recent studies confirm its continued relevance for polyphenol quantification in natural products (Dominguez-López, Pérez, and Lamuela-Raventós 2024).

The $AlCl_3$ colorimetric assay is the most widely applied spectrophotometric method for quantifying total flavonoid content in plant and lichen extracts. The principle is based on the formation of a stable complex between aluminum chloride and the keto and hydroxyl groups of flavonoids, producing a yellow coloration. Flavonoids, such as catechin, rutin, and luteolin, also form red complexes with aluminum in the presence of sodium hydroxide and sodium nitrate. The method is valued for its simplicity, reproducibility, and adaptability to diverse biological matrices, though it is limited by interference from other polyphenolic compounds and variability in binding affinity among flavonoid subclasses (Pękal and Pyrzynska 2014; Sultana et al. 2024).

Antioxidant Activity Assays

The DPPH radical scavenging assay is one of the most widely applied methods for evaluating the antioxidant activity of phenolics. DPPH is a stable violet radical that accepts hydrogen atoms or electrons from phenolic hydroxyl groups, resulting in a decrease in absorbance and discoloration measurable at 517 nm (Yamauchi et al. 2024).

2.7 Optimization of Phytochemical Extraction

Extraction represents a critical step in the isolation and characterization of potential bioactive compounds from plant materials. The efficiency of this process is strongly influenced by several parameters, including the type and composition of the solvent, extraction temperature, duration, solid-to-liquid ratio, and the particle size of the plant material. Careful optimization of these factors is essential to maximize yield and ensure the accurate recovery of bioactive constituents (Gao and Mazza 1996; Prasad et al. 2012).

The traditional optimization approach, commonly referred to as the one-factor-at-a-time (OFAT) method, has been extensively employed by researchers to evaluate specific parameters, where a single variable is altered while all other factors are held constant. The major disadvantage is that

it does not include the interactive effects among the variables studied. This technique is also time-consuming and costly because many experiments are being run. Consequently, significant factors influencing the extraction process can be overlooked, thereby reducing the reliability of the results. To overcome this problem, response surface methodology (RSM) has been introduced and is widely used nowadays for optimizing the extraction conditions. RSM is considered to be a powerful mathematical technique used widely in many industries for technological operations in order to optimize certain experimental conditions.

In RSM, the model should be fitted with experimental values to compare the interaction between each variable and predict the optimum values of the independent and response variables. Model fitting refers to the process of developing a mathematical relationship between independent variables (factors) and dependent variables (responses). The models used in RSM are typically polynomial equations, including linear, interaction, and quadratic models. These models describe how changes in variables such as methanol concentration and extraction time affect responses like total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity. By generating response surface plots, central composite design facilitates the identification of optimal conditions for maximizing phytochemical yield and bioactivity (Prasad et al. 2012; Silva, Rogez, and Larondelle 2007).

Extraction efficiency is influenced by several factors, including solvent type, solvent concentration, extraction time, and temperature. Among these, solvent polarity and extraction duration are particularly critical for maximizing phenolic and flavonoid yield (Do et al. 2014). Based on the previous study, total phenolics, total flavonoids, and antioxidant activity have been successfully optimized using the response surface method (Hou et al. 2016; Silva, Rogez, and Larondelle 2007).

2.8 Summary of Previous Studies and Research Gaps

Table 2.1: Summary of previous studies on *Usnea* species

Study	**species**	**location**	**Solvent/ Method**	**Key findings**	**Limitation/ Contribution**
Piñeiro et al. (2024)	*Usnea* sp.	India	Methanol extraction	High phenolic content	No optimization approaches
Londoñe-Bailon et al. (2019)	*Usnea* sp.	Antarctica	Methanol Solvent extraction	Moderate phenolics	No RSM application
Odabasoglu et al. (2004)	*Usnea* sp. (*Usnea florida* and *Usnea longissimi*)	Turkey	Methanol extract	Low to high antioxidant activity (13.38 and 82.44 %)	No interaction studies
Popovici et al. (2021)	*Usnea* sp.	Europe	Methanol and water (DPPH assay)	Variable antioxidant activity (high with methanol)	No Rs application
Present study	*Usnea* sp.	Ethiopia	40-100 % Methanol/water concentration RSM (CCD)	Optimized extraction of TPC, TFC, and antioxidant, lipid analysis	Applies RSM optimization to *Usnea* species

The literature clearly demonstrates that lichens of the genus *Usnea* are important reservoirs of phenolic, flavonoid, and lipid contents, all of which contribute significantly to antioxidant activity. While global studies have highlighted the influence of species, habitat, and extraction conditions on metabolite yield, systematic investigations that apply statistical optimization

remain limited. Moreover, Ethiopian lichens from high-altitude ecosystems such as Ras Dashen Mountain have not yet been comprehensively examined for their phytochemical composition and antioxidant potential. To address this gap, the present study employs Central Composite Design (CCD) within Expert Design software to optimize extraction parameters and evaluate the Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and antioxidant activity of *Usnea* species. This study also fills the gap in the quantification of lipid content of *Usnea* species.

CHAPTER 3: MATERIALS AND METHODS

3.1 Description of the Study Area

Samples of *Usnea* lichen species were collected from Ras Dashen Mountain region, situated within the Simien Mountains National Park (SMNP) in northern Ethiopia. The area lies in the Amhara Regional State, North Gondar Administrative Zone, which is about 865 km north of Addis Ababa and 132 km northeast of Gondar Town. Ras Dashen (Ras Dejen) is the highest peak in Ethiopia and also the fourth-highest mountain in Africa, rising to about 4,620 meters above sea level (13°14′N, 38°22′E). It is a part of the Simien Mountain massif, a UNESCO World Heritage Site recognized for its dramatic landscapes, exceptional biodiversity, and unique ecological conditions. The mountain's topography is characterized by steep escarpments, deep valleys, and extensive plateaus that rise sharply from the surrounding lowlands. Its climate varies markedly with altitude. Daily temperatures fluctuate widely. The main rainy season in SMNP lasts from the end of June to September, while the dry season encompasses December to April. These environmental gradients, combined with high humidity and frequent mist, create favorable microclimates that support rich epiphytic and saxicolous lichen growth on shaded rock surfaces and tree trunks (Asrat and Mogessie 2012).

3.2 Collection, Identification, and Specimen Handling of Lichens

Three *Usnea* lichen samples were collected from three host plant species: *Hagenia abyssinica* (Kosso), *Hypricum revolutum* (Amija), and *Erica arborea* (Wuchena), from Ras Dashen Mountain in October 2025. After collection, the samples were transported to the Bahir Dar University laboratory. The collected samples (**Figure 3.1**) were shade-dried at ambient temperature, and extraneous materials were removed.

a b

c

Figure 3.1. *Usnea* species collected from the host plants (a) *Hagenia abyssinica*, (b) *Hypericum revolutum*, and (c) *Erica arborea*.

3.3 Chemicals and Reagents

All chemicals and reagents used in this study were of analytical grade. Methanol Absolute (Acetone Free), from Reagent Chemical Services Limited, Industrial Estate, Run Corn, Cheshire; sodium carbonate (Anhydrous extra-pure AR grade) from Sisco Research Laboratories Pvt. Ltd., Maharashtra. India; aluminum chloride (97%, Hexahydrate, extra pure) and Folin-Ciocalteu reagent were from LOBA CHEMIE PVT. LTD. India; sodium hydroxide, sodium nitrite, and ascorbic acid from Blulux Laboratories (p) Ltd.; D-Catechin hydrate (96%, HPLC grade), and 2,2-Diphenyl-1-picrylhydrazyl (DPPH); gallic acid monohydrate from Ranchem Industry and trading, CARLO ERBA, France; chloroform (99.5%), and sodium chloride were from Research Lab Fine Chem, Mumbai, India.

3.4 Instruments and Apparatus

An electronic balance (OHAUS Corporation, USA), an electrical blender (FW100 high-speed universal disintegrator, manufacturer not specified on instrument label), a platform shaker (ZHWY-334, ZHICHENG, China), sieve, Whatman No. 1 filter paper, beakers, flasks, micropipettes, test tubes, glass vials, centrifuge (model 800-1, manufacturer not specified on instrument label), refrigerator (RE-100-Pro, digital inverter, China), UV-Vis spectrometry (HACH Company, 5600 Lindbergh Drive, Loveland, Co 8539, Germany) were used in the study.

3.5 Experimental Procedure for Sample Preparation

3.5.1 Sample Preparation

Once dried, the lichen species were pulverized into a fine powder with an electric blender. Then the powdered sample was sieved through a 100-μm pore size sieve. The aliquots of the powdered sample were stored in airtight containers in a cold, dry, and dark area. Proper storage conditions were maintained to prevent mold growth and degradation of secondary metabolites, ensuring the plant materials remain potent until further processing.

3.5.2 Experimental Design and Extraction for Total Phenolics, Flavonoids, and Antioxidant Activity with Central Composite Model

The experimental design was structured using a face-centered central design (CCD) to investigate the influence of extraction parameters on the response. The selection of CCD is its ability to estimate curvature more flexibly. Response Surface Methodology (RSM) was employed to optimize the process conditions. Design Expert software (version 13) was used to design two numerical factors at three levels. In total, 11 randomized experimental runs were conducted per sample, including three replicates at the center point to ensure reliability, as shown in **Table 3.1**. Two parameters were optimized: methanol concentration and extraction time. To facilitate comparison, all variables were normalized and scaled within the range of -1 to +1.

Table 3.1. Summary of experimental design, including factors and levels applied for optimization.

Symbol	Independent variable	Code level		
		-1	0	1
X_1	Solvent concentration (methanol/water %)	40	70	100
X_2	Extraction time (minutes)	60	120	180

The dependent variables were total phenolic contents (TPC), total flavonoid contents (TFC), and DPPH antioxidant activity.

For each run, 0.5 g of the sample was dissolved in 10 ml of solvent at varying methanol/water concentrations (40%, 70%, and 100%). The mixtures were subjected to continuous agitation on a shaker at 200 rpm for extraction times of 60, 120, and 180 minutes. Following extraction, the samples were centrifuged at 400 rpm for 12 minutes, and the supernatant was collected. Finally, the liquid extracts were stored at 4°C for analysis.

3.5.3 Crude Lipid Extraction Using Folch Method

Crude extraction from the *Usnea* lichen powder sample was performed using the Folch method (Agidew et al. 2021) with slight modifications. Briefly, 0.5 g of powdered lichen material was accurately weighed, transferred to a clean test tube, and mixed with 12 mL of chloroform/methanol (2:1). The mixture was extracted for 24 hours on a platform shaker at 300 rpm. The extract was then centrifuged, and the filtrate was collected. The lipid phase was separated by adding 2 mL of 0.73% aqueous sodium chloride, and the upper phase was removed with a micropipette. The lower (chloroform) layer containing the lipid was collected, and the solvent was removed by leaving the extract exposed to air overnight.

Lipid Content Determination

Total lipid content of the *Usnea* lichen samples was determined gravimetrically after extraction using the Folch method. The total lipid content was calculated using the following equation:

$$Lipid\ content(\%) = \left(W_{lipid}/W_{sample}\right) \times 100$$

Where W_{lipid} is the weight of the extracted lipid (g), and W_{sample} is the dry weight of the lichen sample (g).

3.6 Experimental Procedure for Analysis

3.6.1 Total Phenolic Content Determination (Folin-Ciocalteu method)

The total phenolic content of the extracted sample was determined using the Folin-Ciocalteu reagent by the spectrophotometric method (Lalremruata et al. 2022) with some modifications. 0.5 mL of each aliquot of the extracted sample was mixed with 2.5 mL of 10% Folin-Ciocalteu reagent and shaken. After 3 minutes, 2 mL of a 7.5% Na_2CO_3 solution was added, and the mixture was shaken. The mixture was further incubated at room temperature in the dark for 30 minutes. A reagent blank was prepared in parallel using distilled water.

Preparation of standard solutions

A stock solution of gallic acid was prepared by accurately weighing 100 mg of pure gallic acid and dissolving it in 100 mL of distilled water to obtain a concentration of 1000 µg/mL. From this stock, a series of working standard solutions was prepared in the concentration range of 1-100 µg/mL (1, 10, 20, 40, 60, 80, and 100 µg/mL) through appropriate dilution with distilled water.

For the determination of phenolic content, from each series concentration, 0.5 mL of the gallic acid standard was mixed with 2.5 mL of 10% Folin-Ciocalteu reagent. The mixture was shaken and allowed to stand for 3 minutes. Subsequently, 2 mL of 7.5% sodium carbonate (Na_2CO_3) solution was added, followed by gentle mixing. The reaction mixture was incubated at room temperature in the dark for 30 minutes to allow complete color development.

Finally, the absorbance was measured at 765 nm using a UV-Vis spectrometer. The calibration curve was constructed by plotting absorbance vs. concentration. The concentration of total phenolic compounds in the extract was expressed as milligrams of gallic acid equivalents (GAE) per 100 grams of sample (mg GAE/100 g). All samples were analyzed in triplicate to ensure accuracy.

Calculations:

$$\text{Total phenolic content (mg GAE/g)} = \frac{C \times V}{m}$$

Where C = Concentration of phenolics determined from the gallic acid standard curve (µg/mL)

V = Volume of extract used (mL)

m = Mass of the extract used (g)

3.6.2 Total Flavonoid Content Determination (Aluminum chloride method)

The total flavonoid content of the extract was determined using the aluminum chloride ($AlCl_3$) colorimetric method as described by Pradhan et al. (2023) with some modifications. In this procedure, 0.5 mL of each lichen aliquot of the extracted sample was transferred into a test tube and mixed with 1.5 mL of distilled water. To this mixture, 0.25 mL of 5% sodium nitrite solution was added, and the mixture was allowed to stand for 5 minutes. Subsequently, 0.15 mL of 10% $AlCl_3$ was introduced, and after 1 minute, 0.5 mL of sodium hydroxide (1 M) was added. The reaction mixture was then diluted to a final volume of 5 mL with distilled water. The mixture was incubated at room temperature for 30 minutes.

Preparation of standard solutions

(+)-Catechin hydrate standard solutions were prepared by accurately weighing 10 mg of (+)-catechin hydrate and dissolving it in 25 mL of methanol to obtain a stock solution concentration of 400 µg/mL. The concentration was corrected to catechin equivalent based on the molecular weight difference between (+)-catechin hydrate and anhydrous catechin, giving an effective concentration of 376.8 µg/mL. From this stock, a series of working standards was prepared in the range of 4.71-282.60 µg/mL.

For the determination of flavonoid content, from each concentration series, 0.5 mL of the catechin standard was mixed with 1.5 mL of distilled water in a test tube. To this mixture, 0.25 mL of 5% sodium nitrite solution was added, and the mixture was allowed to stand for 5 minutes. Subsequently, 0.15 mL of 10% $AlCl_3$ was introduced, and after 1 minute, 0.5 mL of sodium hydroxide (1 M) was added. The reaction mixture was then diluted to a final volume of 5 mL with distilled water. The mixture was incubated at room temperature in the dark for 30 minutes to allow complete color development. All standards were measured in triplicate, and reagent blanks were included to ensure accuracy and reproducibility of the assay. Finally, the absorbance was measured at 510 nm against a blank reagent using a UV-Vis spectrometer. The calibration curve was constructed by plotting absorbance vs.

concentration. Results were expressed as milligrams of catechin equivalent (CE) per 100 grams of dry weight (mg CE/100 g dry weight) of the sample.

Calculations:

$$\text{Total Flavonoid Content (mg CE/g)} = \frac{C \times V}{m}$$

Where C = Concentration of flavonoids determined from the catechin standard curve (µg/mL)

V = Volume of extract used (mL)

m = Mass of the extract used (g)

3.6.3 Antioxidant Activity (DPPH free radical scavenging assay)

The antioxidant activity of the sample extract was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay, as described by Ayele et al. (2022), with slight modifications.

Preparation of DPPH solution

0.1 mM DPPH solution was prepared by dissolving 9.86 mg of DPPH in 250 mL of methanol in a volumetric flask. The solution was then stored in the dark.

The control setup: the control solution was prepared by mixing 0.3 mL of solvent, 0.7 mL of methanol, and 1 mL of the DPPH solution, then incubated in the dark for 30 minutes.

Preparation of ascorbic acid stock solution

A stock solution of 500 µg/mL was prepared by dissolving 50 mg of ascorbic acid in 100 mL of methanol. To construct a calibration curve, a series of concentrations (1, 5, 10, 15, 20, 25, and 30 µg/ml) was prepared from this stock. For each series concentration, 0.3 mL of the standard solution was mixed with 0.7 mL of methanol and 1 mL of DPPH, then the test tube was covered with aluminum foil and incubated in the dark for 30 minutes. Finally, the absorbance of the unreacted DPPH was measured spectrophotometrically at 517 nm. Then, the percent of radical-scavenging activity of ascorbic acid vs. ascorbic acid concentration was plotted.

DPPH Assay Procedure

0.3 mL of each lichen extract was pipetted into a test tube, mixed with 0.7 mL of methanol, and then 1 mL of DPPH solution was added. The mixtures were incubated under the same conditions as the standards (dark, 30 minutes).

Absorbance was recorded at 517 nm using UV-Vis spectrometry. The percentage of the radical scavenging activity was calculated using the formula:

$$I\% = \frac{A_0 - A}{A_0} \times 100$$

Where I% is the percent of DPPH inhibition, A_0 is the absorbance of the DPPH control solution, and A is the absorbance of the DPPH solution in the presence of the sample extract. All measurements were performed in triplicate, and results were expressed as milligrams of ascorbic acid equivalent per gram of sample (mg AAE/g sample).

3.7 Methods for Data Analysis

Statistical analysis of data

Data were expressed as the mean plus standard deviation (SD) of triplicate determinations. The Design Expert 13.0 program (Version 13, Stat Ease Inc., Minneapolis, USA) was used to conduct optimization and statistical analysis. The importance of the model and its terms was evaluated using analysis of variance (ANOVA), which included the lack-of-fit test and the coefficient of determination (R^2). Calibration lines were made using Microsoft Excel. Pearson's correlation test was used to calculate the correlation coefficient (r) between TPC, TFC, and antioxidant activities. Finally, to validate the optimum extraction condition generated by RSM, the significance level of both predicted and experimental values of TPC, TFC, and DPPH was assessed using a one-sample t-test in IBM SPSS Statistics (Version 23.0, IBM Corp., Armonk, NY, USA).

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Determination of Total Phenolic Content (TPC), Flavonoid Content (TFC), and Antioxidant Inhibition Activity

4.1.1 Estimation of Total Phenolic Content (TPC)

Gallic acid was used as a standard to determine the total phenolic content of three samples of *Usnea* species. The total phenolic content of the lichen samples was expressed as gallic acid equivalents (mg GAE/100 g) using the regression equation $y = 0.0084x + 0.0102$, $R^2 = 0.9986$, derived from the gallic acid calibration curve (**see Appendix B**). The TPC values of all sample extracts are presented in **Table 4.1a, b, and c**.

Table 4.1. Design matrix and experimental responses for central composite design of *Usnea* species collected from different host plants.

a) *Usnea* species collected from *Hagenia abyssinica* (host plant)

	Independent variables		Dependent variables		
Run	Methanol concentration (%)	Extraction time (min.)	TPC (mg GAE/100 g DW)	TFC (mg CE/100 g DW)	DPPH assay (% inhibition)
1	40	60	57.93 ± 0.51	44.21 ± 5.57	41.15 ± 1.53
2	40	120	68.29 ± 1.01	62.81 ± 1.61	54.2 ± 3.41
3	40	180	70.67 ± 0.67	66.67 ± 2.65	61.29 ± 0.86
4	70	60	69.36 ± 0.51	99.3 ± 1.22	65.1 ± 0.15
5[a]	70	120	89.48 ± 0.17	97.19 ± 1.61	76.9 ± 0.41
6[a]	70	120	92.69 ± 0.34	99.65 ± 2.65	77.23 ± 0.76
7[a]	70	120	89.12 ± 0.17	92.63 ± 3.16	78.57 ± 0.83
8	70	180	84.83 ± 0.17	97.19 ± 4.98	75.23 ± 0.73
9	100	60	142.57 ± 0.34	402.81 ± 5.8	61.29 ± 0.86
10	100	120	164.12 ± 0.84	422.11 ± 4.82	69.63 ± 0.38
11	100	180	138.05 ± 0.34	389.83 ± 3.7	64.55 ± 0.73

Runs marked with [a] are the center point; GAE = gallic acid equivalent; CE = catechin equivalent; DW = dry weight. Values are mean ± standard deviation, n = 3.

b) *Usnea* species collected from *Hypericum revolutum* (host plant)

	Independent variables		Dependent variables		
Run	Methanol concentration (%)	Extraction time (min.)	TPC (mg GAE/100 g DW)	TFC (mg CE/100 g DW)	DPPH assay (% inhibition)
1	40	60	22 ± 1.01	14.39 ± 2.19	35.91 ± 2

2	40	120	34.21 ± 0.51	16.14 ± 1.61	39.8 ± 3.97
3	40	180	40.71 ± 0.67	15.79 ± 2.11	42.29 ± 2.12
4	70	60	58.64 ± 0.84	36.84 ± 4.82	48.19 ± 0.07
5[a]	70	120	63.31 ± 0.67	36.84 ± 1.82	55.8 ± 0.59
6[a]	70	120	66.6 ± 1.68	35.09 ± 1.61	57.29 ± 0.4
7[a]	70	120	65.4 ± 1.35	41.4 ± 3.99	56.14 ± 0.52
8	70	180	62.81 ± 1.35	41.4 ± 3.04	53.4 ± 0.42
9	100	60	106.86 ± 0.67	238.6 ± 5.4	46.68 ± 2.67
10	100	120	112.93 ± 1.18	252.98 ± 4.86	50.7 ± 4.07
11	100	180	98.6 ± 1.18	262.11 ± 6.57	49.86 ± 4.06

Runs marked with 'a' are the center point; GAE = gallic acid equivalent; CE = catechin equivalent; DW = dry weight. Values are mean ± standard deviation, n = 3.

c) *Usnea* species collected from *Erica arborea* (host plant)

	Independent variables		**Dependent variables**		
Run	Methanol concentration (%)	Extraction Time (min.)	TPC (mg GAE/100g DW)	TFC (mg CE/100g DW)	DPPH assay (% inhibition)
1	40	60	56.74 ± 2.19	52.98 ± 1.22	40.84 ± 1.13
2	40	120	59.12 ± 0.51	69.12 ± 2.19	54.85 ± 1.13
3	40	180	60.07 ± 1.18	57.19 ± 3.22	55.78 ± 0.08
4	70	60	69.12 ± 1.85	76.14 ± 6.77	61.4 ± 0.24
5[a]	70	120	73.17 ± 1.52	89.12 ± 2.19	74.74 ± 1.22
6[a]	70	120	72.19 ± 1.18	91.93 ± 1.61	61.4 ± 0.24
7[a]	70	120	75.31 ± 2.19	90.53 ± 1.05	77.4 ± 0.99
8	70	180	70.55 ± 1.85	79.65 ± 1.22	69.45 ± 1.02
9	100	60	123.64 ± 1.18	419.65 ± 4.98	61.24 ± 1.11
10	100	120	131.62 ± 0.67	440 ± 3.16	67.53 ± 2.15
11	100	180	111.38 ± 1.68	410.18 ± 6.69	66.23 ± 1.18

Runs marked with 'a' are the center point; GAE = gallic acid equivalent; CE = catechin equivalent; DW = dry weight. Values are mean ± standard deviation, n = 3.

The results presented in **Table 4.1** clearly show that methanol concentration and extraction time significantly influenced the extraction yield of phenolics, flavonoids, and antioxidant activity. In general, higher methanol concentrations (100%) resulted in increased TPC and TFC values across all host plants. However, antioxidant activity (DPPH inhibition) did not always follow the same trend; this may indicate that factors other than phenolic concentration also contribute to antioxidant potential.

Furthermore, noticeable variations in total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity (DPPH inhibition) were observed among lichens collected from

different host plants. This variation may be attributed to the influence of the host substrate (phorophyte) on lichen metabolism and chemical composition. Previous studies have demonstrated that the chemical properties of the host tree, particularly bark chemistry, nutrient availability, and phenolic composition, play a crucial role in shaping the diversity and concentration of lichen secondary metabolites (Paukov et al. 2022). Moreover, lichens exhibit a strong preference for specific host substrates, and this substrate specificity leads to differences in their physiological and biochemical responses.

Therefore, the observed differences in TPC, TFC, and antioxidant activity in the same sample and among *Usnea* samples collected from *Hagenia abyssinica*, *Hypericum revolutum*, and *Erica arborea* can be explained by the combined effects of solvent concentration, extraction time, host plant chemistry, substrate characteristics, and environmental conditions. This confirms that host plant type is an important factor influencing the phytochemical composition and bioactivity of lichen species.

Fitting the models

Fitting the models is crucial in interpreting the accuracy of the RSM mathematical models for the prediction of the TPC, TFC, and DPPH radical scavenging activity. The experimental design was based on a Central Composite Design (CCD), which is suitable for exploring quadratic response surfaces. Among the tested models, the quadratic model was selected as the most appropriate for describing the relationship between the independent variables and the responses, based on the experimental data results. The suitability of the model in fitting the experimental data was confirmed through analysis of variance and lack-of-fit testing. The lack-of-fit testing was used to verify the adequacy and suitability of the fit (Ozkan et al. 2025). The ANOVA for the lack-of-fit test did not indicate inadequacy of the model for the response variable.

The ANOVA results for total phenolic contents (TPC) in all three samples demonstrated that the quadratic model was significant ($p < 0.05$), while the lack-of-fit test was not significant ($p > 0.05$), as shown in **Table 4.2**, confirming that the model adequately fits the experimental data. High values of determination (R^2: 0.9853, 0.9969, and 0.9882) of the response variability of TPC for all three samples indicate that the selected or recommended model was suitable. The predicted R^2 of 0.8495, 0.9739, and 0.8858 are in reasonable agreement with the Adjusted R^2 of 0.9706, 0.9938, and 0.9764; i.e., the difference is less than 0.2, respectively, in the three samples.

The C.V. of 6.22%, 3.48%, and 5.03%, which were less than 10%, indicates that the response's reliability and precision were very high according to (Jaafar, Ramli, and Salleh 2020). Thus, the quadratic model was considered statistically valid and suitable for predicting the responses within the experimental domain.

Table 4.2. Analysis of variance (ANOVA) results for the total phenolic content (TPC) extraction of ***Usnea*** species in three host plants, fitted to a quadratic polynomial model under the Central Composite Design.

Sample	Source	Sum of Squares	df	Mean Square	F-value	p-value	
Usnea (H. abyssinica)	Model	12178.23	5	2435.65	66.95	0.0001	Significant
	Lack of Fit	174.16	3	58.05	15.03	0.0630	not significant
	Pure Error	7.73	2	3.86			
	R^2	0.9853					
	Adjusted R^2	0.9706					
	Predicted R^2	0.8495					
	C.V. %	6.22					
Usnea (H. revolutum	Model	8563.87	5	1712.77	320.09	< 0.0001	significant
	Lack of Fit	21.21	3	7.07	2.55	0.2941	not significant
	Pure Error	5.54	2	2.77			
	R^2	0.9969					
	Adjusted R^2	0.9938					
	Predicted R^2	0.9739					
	C.V. %	3.48					
Usnea (E. arborea)	Model	7132.50	5	1426.50	83.83	< 0.0001	significant
	Lack of Fit	80.00	3	26.67	10.47	0.0884	not significant
	Pure Error	5.09	2	2.55			
	R^2	0.9882					
	Adjusted R^2	0.9764					
	Predicted R^2	0.8858					
	C.V. %	5.03					

Influence of methanol concentration and extraction time on the total phenolic content

Studies confirmed that both solvent concentration and extraction time significantly influence the yield of phenolic compounds (Do et al. 2014). The present study effectively assessed the impact

of methanol concentration and extraction time on the total phenolic contents present in *Usnea* species. The results reflected that the TPC experiences differences in quantity, ranging from **57.93** to **164.12, 22** to **112.93,** and **56.74** to **131.62 mg GAE/100 DW** of *Usnea* samples collected from *Hagenia abyssinica, Hypericum revolutum,* and *Erica arborea,* respectively, when the extraction factors vary as methanol concentration from 40 to 100% and extraction time from 60 to180 minutes as shown in **Table 4.1a, b,** and **c**. The variation in TPC values is due to differences in extraction conditions and host plant substrate. Methanol concentration and extraction time influence the solubility and diffusion of phenolic compounds, while host plant characteristics affect metabolite biosynthesis. The use of RSM further enhances extraction efficiency by optimizing these variables simultaneously, resulting in higher TPC values compared to conventional methods.

The effects of methanol concentration and extraction time were quantitatively evaluated using a fitted quadratic regression model derived from the Central Composite Design. The relationship between these variables and the extraction response is represented by the following equations:

$$Y_{TPC1} = 90.55 + 41.31X_1 + 3.95X_2 - 4.31X_1X_2 + 25.48X_1^2 - 13.63X_2^2 \quad (\mathbf{1})$$

$$Y_{TPC2} = 65.55 + 36.91X_1 + 2.42X_2 - 6.74X_1X_2 + 7.36\,X_1^2 - 5.53X_2^2 \quad (\mathbf{2})$$

$$Y_{TPC3} = 74.33 + 31.79X_1 + 1.25X_2 - 3.90X_1X_2 + 19.87X_1^2 - 5.66X_2^2 \quad (\mathbf{3})$$

Where $Y_{TPC1,}$ Y_{TPC2}, and Y_{TPC3} are the total phenolic content responses of the three samples of *Usnea* species collected from *Hagenia abyssinica, Hypericum revolutum,* and *Erica arborea,* respectively. X_1 and X_2 represent the coded variables for methanol concentration and extraction time.

The coded equations serve as a valuable tool for assessing the relative influence of individual factors, since the magnitude and sign of the coefficients allow direct comparison of their contributions to the response.

The model reveals that methanol concentration exerts the most significant influence on total phenolic content (TPC), with positive coefficients in both linear (X_1) and quadratic terms (X_1^2) contributing positively, as described in equations 1, 2, and 3. This indicates that increasing methanol concentration enhances phenolic extraction and maximizes phenolic yield, and the

quadratic effect suggests further improvement at higher levels of all three samples. In the present study, we found that the mixed extracts showed the lowest TPC. This could be due to the low solubility of lichen compounds in more polar solvents, because most lichen compounds are insoluble in water and must be extracted with organic solvents (Gandhi et al. 2022).

Extraction time shows a weaker linear effect on total phenolic contents, as shown by the coefficient of X_2 in equations 1, 2, and 3, implying that prolonged extraction slightly reduces TPC. A negative quadratic effect of X_2 was obtained for the three samples of TPC values, indicating that there is a maximum in the TPC extraction at a certain time, and the yield in TPC starts to decrease above this optimal duration. This is due to the non-interaction of solvent and phenolic compounds at a short time, and degradation of compounds above the optimal duration. The interaction term between methanol concentration and extraction time is negative, suggesting that simultaneous increases in both factors at a certain optimum point diminish TPC compared to their independent contributions when the independent factors are increased. Solvent concentration is the most important parameter for extraction yield and total phenolic contents under the same extraction time (Do et al. 2014). Overall, the model demonstrates that maximizing methanol concentration while carefully optimizing extraction time is essential for achieving the highest phenolic content.

The 3D plots in **Figure 4.1** also show that total phenolic content (TPC) increased steadily with increasing methanol concentration. Different colors in the visualization indicate different TPC values. High TPC values are shown in red. It can be observed from the 3D plot that TPC increases linearly with increases in extraction time until the optimum extraction time is reached. The maximum TPC values predicted by the software were **157.273**, **109.878**, and **127.126** mg GAE/100g DW when the optimum methanol concentration was **100%** and the extraction time was **122**, **119, and 92** minutes for *Usnea* species collected from *Hagenia abyssinica*, *Hypericum revolutum*, and *Erica arborea*, respectively.

Longer extraction times generally increase phenolic recovery, but excessive duration can degrade sensitive compounds, while solvent polarity determines which phenolic subclasses are extracted most efficiently.

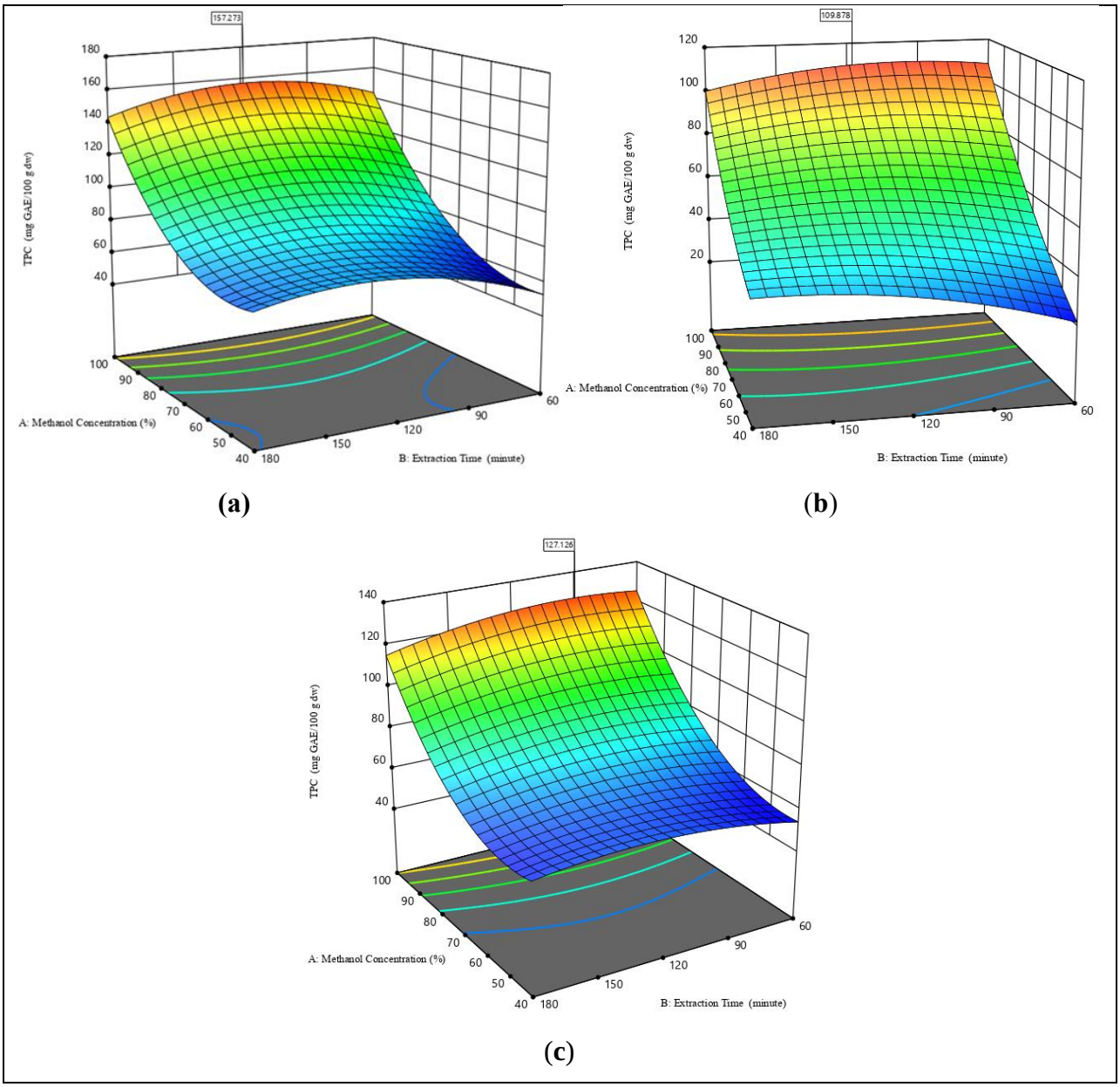


Figure 4.1. Response surface 3D plots showing the interaction effects of methanol concentration (%) and extraction time (min) on the TPC of *Usnea* species collected from (a) *Hagenia abyssinica*, (b) *Hypericum revolutum*, and (c) *Erica arborea*.

4.1.2 Estimation of Total Flavonoid Content (TFC)

In this work, the total flavonoid content (TFC) of the three sample extracts was quantified using the aluminum chloride colorimetric assay, with catechin as the standard. The total flavonoid content (TFC) of the *Usnea* lichen species extracts was subsequently calculated using the regression equation derived from the catechin calibration curve, y = 0.0019x + 0.003, with a

correlation coefficient (R^2) of 0.9978 **(see Appendix B)**. The TFC values for the three samples are shown in **Tables 4.1a, 4.1 b, and 4.1c**.

The analysis of variance (ANOVA) for total flavonoid content (TFC) confirmed that the quadratic polynomial model provided a statistically reliable fit. The model terms were significant ($p < 0.05$), whereas the lack-of-fit was not significant ($p > 0.05$), as presented in **Table 4.3**. This outcome demonstrates that the experimental data were well described by the quadratic model. Strong coefficients of determination (R^2 values: 0.9984, 0.9995, and 0.9997) close to unity further support the adequacy of the model in capturing the influence of methanol concentration and extraction time on TFC. Moreover, the predicted R^2 values (0.9877, 0.9971, and 0.9971) were consistent with the adjusted R^2 (0.9968, 0.9991, and 0.9993), with differences of less than 0.2 across the three samples. The less than 10% value of C.V. (5.02%, 3.48%, 2.45%) of the three samples indicates high reliability and precision of response. Collectively, these statistical indicators validate the robustness and fitting of the quadratic model for predicting flavonoid extraction responses within the studied domain.

Table 4.3. Analysis of variance (ANOVA) results for the total flavonoid content (TFC) extraction of *Usnea* species in three host plants, fitted to a quadratic polynomial model under the Central Composite Design.

Sample	Source	Sum of Squares	df	Mean Square	F-value	p-value	
Usnea (H. abyssinica)	Model	2.302E+05	5	46048.64	628.33	< 0.0001	significant
	Lack of Fit	341.06	3	113.69	8.96	0.1021	not significant
	Pure Error	25.38	2	12.69			
	R^2	0.9984					
	Adjusted R^2	0.9968					
	Predicted R^2	0.9877					
	C.V. %	5.02					
Usnea (H. revolutum)	Model	1.083E+05	5	21657.43	2200.15	< 0.0001	significant
	Lack of Fit	27.99	3	9.33	0.8793	0.5710	not significant
	Pure Error	21.22	2	10.61			
	R^2	0.9995					
	Adjusted R^2	0.9991					
	Predicted R^2	0.9971					
	C.V. %	3.48					
Usnea (E. arborea)	Model	2.653E+05	5	53068.31	3031.35	< 0.0001	significant
	Lack of Fit	83.58	3	27.86	14.11	0.0669	not significant
	Pure Error	3.95	2	1.97			
	R^2	0.9997					
	Adjusted R^2	0.9993					

	Predicted R^2	0.9971
	C.V. %	2.45

Influence of methanol concentration and extraction time on the total flavonoid content

The solvent concentration and extraction time are crucial factors for achieving optimal flavonoid content (Do et al. 2014). From this study, it can be seen that the amount of total flavonoid content varies in a range from **44.21** to **422.11, 14.39** to **262.11,** and from **52.98** to **440 mg CE/100 DW** of *Usnea* species collected from *Hagenia abyssinica, Hypericum revolutum,* and *Erica arborea*, respectively, as shown in **Table 4.1a, b, and c** as the concentration of methanol vary from 40 to 100% and extraction time from 60 to 180 minutes.

The regression equations **4**, **5**, and **6** describe the influence of methanol concentration and extraction time on the total flavonoid content. It shows that methanol concentration exerts a stronger effect with large positive linear and quadratic coefficients for X_1 across all models. This could be because of the high solubility of flavonoids in methanol (Ben El Hadj Ali et al. 2014). The amount of TFC increases as the extraction time increases, but at a lower rate than the solvent, as can be seen from the linear coefficient of X_2. The coefficient of the quadratic term of X_2 was negative, which indicates that at a certain time there is an optimum amount of TFC, but beyond that the amount of TFC declines. Interaction terms X_1X_2 also differ in sign and magnitude, suggesting that the combined influence of methanol concentration and extraction time depends on the specific sample conditions. Overall, these equations highlight that optimizing solvent composition is the primary driver for maximizing flavonoid extraction, and an upper limit of extraction time must be respected to avoid degradation of thermo-sensitive flavonoids.

$$Y_{TFC1} = 100.35 + 173.51X_1 + 1.23X_2 - 8.86X_1X_2 + 136.32X_1^2 - 7.90X_2^2 \quad (\mathbf{4})$$

$$Y_{TFC2} = 38.45 + 117.90X_1 + 4.91X_2 + 5.53X_1X_2 + 95.11X_1^2 - 0.33X_2^2 \quad (\mathbf{5})$$

$$Y_{TFC3} = 91.99 + 181.76X_1 - 0.29X_2 - 3.42X_1X_2 + 160.39X_1^2 - 16.28X_2^2 \quad (\mathbf{6})$$

Where $Y_{TFC1,}$ Y_{TFC2}, and Y_{TFC3} are the total flavonoid content responses of the three samples of *Usnea* species collected from *Hagenia abyssinica*, *Hypericum revolutum*, and *Erica arborea*, respectively. X_1 and X_2 represent the coded variables for methanol concentration and extraction time.

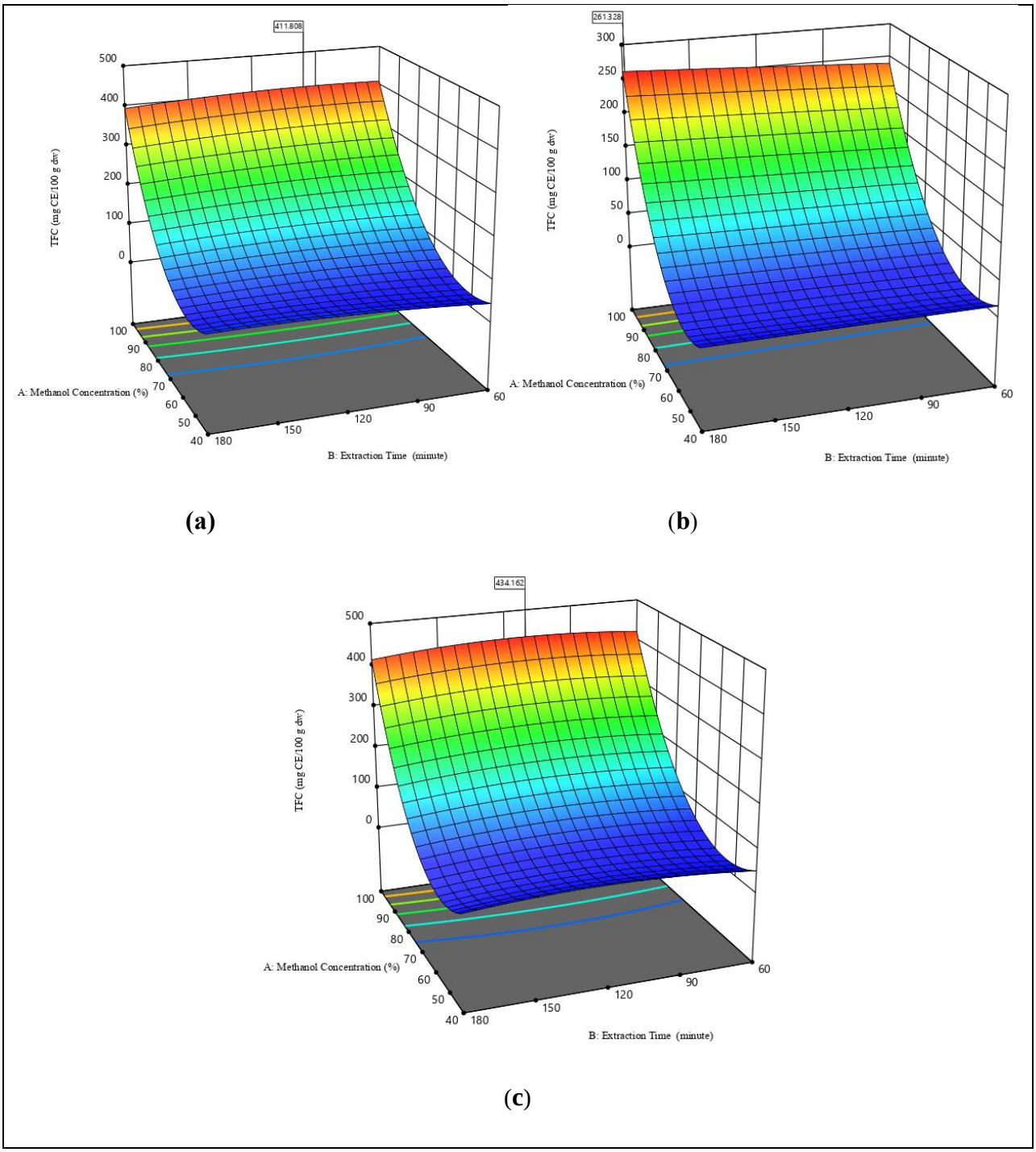


Figure 4.2. Response surface 3D plots showing the interaction effects of methanol concentration (%) and extraction time (min) on the TFC of *Usnea* species collected from (a) *Hagenia abyssinica*, (b) *Hypericum revolutum*, and (c) *Erica arborea*.

The effect of the independent variables on TFC was verified in the 3D plot presented in **Figure 4.2**. TFC increases with increasing methanol concentration and extraction time, but at longer extraction times, it decreases. This may be due to the degradation of some compounds at prolonged extraction times. The red color indicates the highest value of TFC. The predicted optimum values are (**411.81, 261.328,** and **434.162 mg CE/100g DW**) when the solvent reaches **100%** methanol, and the extraction times are **92, 179,** and **112** minutes for *Usnea Hagenia abyssinica*, *Hypericum revolutum,* and *Erica arborea*, respectively.

4.1.3 Antioxidant Inhibition Activity

The antioxidant activity of the extracts was determined using the DPPH radical scavenging assay. The regression equation was obtained as $y = 2.9486x + 1.5022$, $R^2 = 0.9992$ **(see Appendix B)**. The percentage inhibition values for all tested samples are presented in **Table 4.1a, b, and c**.

Similarly, the analysis of DPPH radical scavenging activity confirmed that the quadratic model was the most appropriate choice for describing the data, as evidenced by the model fitting results presented in **Table 4.4**. The model is significant ($p < 0.05$), and the lack of fit is non-significant ($p > 0.05$). Strong coefficients of determination (R^2 values: 0.9901, 0.9745, and 0.9787) close to unity further support the adequacy of the model. The predicted and adjusted values are close. The model was fitted to the experimental data, and the results indicate that the model adequately describes the relationship between the variables. Therefore, the extraction process can be effectively explained by the regression model. The coefficient of variation (C.V.) values of 2.4%, 3.26%, and 3.47%, all of which are less than 10%, indicate high reliability and precision of the experimental responses.

Table 4.4. Analysis of variance (ANOVA) results for DPPH radical scavenging activity extraction of *Usnea* species in three host plants, fitted to a quadratic polynomial model under the Central Composite Design.

Sample	Source	Sum of Squares	df	Mean Square	F-value	p-value	
Usnea (H. abyssinica)	Model	1269.60	5	253.92	99.75	< 0.0001	significant
	Lack of Fit	11.16	3	3.72	4.76	0.1786	not significant
	Pure Error	1.56	2	0.7822			
	R^2	0.9901					

	Adjusted R^2	0.9801					
	Predicted R^2	0.9367					
	C.V. %	2.40					
Usnea (H. revolutum)	Model	482.56	5	96.51	38.18	0.0005	significant
	Lack of Fit	11.42	3	3.81	6.24	0.1412	not significant
	Pure Error	1.22	2	0.6097			
	R^2	0.9745					
	Adjusted R^2	0.9490					
	Predicted R^2	0.8322					
	C.V. %	3.26					
Usnea (E. arborea)	Model	1142.81	5	228.56	46.29	0.0003	significant
	Lack of Fit	20.53	3	6.84	3.30	0.2414	not significant
	Pure Error	4.15	2	2.08			
	R^2	0.9789					
	Adjusted R^2	0.9577					
	Predicted R^2	0.8628					
	C.V. %	3.47					

Influence of solvent concentration and extraction time on the antioxidant activity of the DPPH method

Previous research has demonstrated that the choice of extraction solvent, extraction methodology, and extraction duration significantly influence the yield and composition of phenolic compounds in lichens, as well as their associated antioxidant activity (Naama et al. 2023). The DPPH assay is a widely used method to evaluate antioxidant activities in a relatively short time compared to other methods (Sharma and Kalikotay 2012).

In this study, the measured antioxidant activity (DPPH radical scavenging) showed distinct variations across the tested conditions, with values ranging from **41.15** to **78.57, 35.91** to **57.29,** and from **40.84** to **77.4** of % of inhibition of *Usnea* species collected from *Hagenia abyssinica*, *Hypericum revolutum*, and *Erica arborea*, respectively, as the concentration of methanol varied from 40 to 100% and extraction time from 60 to 180 minutes.

The antioxidant activity, measured by DPPH radical scavenging capacity, was modeled using second-order polynomial regression equations. The fitted models are expressed by equations 7, 8, and 9. In equations 7, 8, and 9, methanol has a positive linear coefficient (X_1), indicating that as the concentration of methanol increases, the DPPH radical scavenging activity also increases. Extraction time also has a positive linear coefficient (X_2), suggesting DPPH inhibition activity increases as the extraction time increases. But the quadratic coefficient is negative in both variables (X_1 and X_2), suggesting reduced efficiency at elevated factor levels. This result may indicate that antioxidant activity does not depend solely on total phenolics and flavonoid contents. Other bioactive constituents may play vital roles in the antioxidant activity.

Generally, these models demonstrate that methanol concentration (X_1) consistently exerted a stronger influence on antioxidant activity than extraction time (X_2), while the negative interaction and quadratic terms highlight the nonlinear nature of the response and the importance of optimizing extraction conditions for each species. According to Bilgin et al. (2018), solvent concentration is the most influential parameter for their natural plant extraction system.

$$Y_{DPPH1} = 76.72 + 7.46X_1 + 4.60X_2 - 5.71X_1X_2 - 13.52X_1^2 - 5.27X_2^2 \quad (\mathbf{7})$$

$$Y_{DPPH2} = 55.56 + 4.87X_1 + 2.46X_2 - 0.8X_1X_2 - 9.03X_1^2 - 3.48X_2^2 \quad (\mathbf{8})$$

$$Y_{DPPH3} = 74.66 + 7.26X_1 + 4.66X_2 - 2.49X_1X_2 - 11.85X_1^2 - 7.61X_2^2 \quad (\mathbf{9})$$

Where $Y_{DPPH1,}$ Y_{DPPH2}, and Y_{DPPH3} are the DPPH scavenging activity responses of the three samples of *Usnea* species collected from *Hagenia abyssinica*, *Hypericum revolutum,* and *Erica arborea*. X_1 and X_2 represent the coded variables for methanol concentration and extraction time.

The 3D response surface plots (**Figure 4.3)** illustrate the combined effects of methanol concentration and extraction time on DPPH radical scavenging activity. As shown, increasing both parameters generally enhanced antioxidant activity, with the surface reaching a maximum predicted inhibition of approximately **78.0%, 56.54%,** and **75.66%** with optimum methanol concentration (**77, 78,** and **79%**) and extraction time (**140, 140,** and **136** minutes) for *Usnea* species collected from *Hagenia abyssinica, Hypericum revolutum,* and *Erica arborea*, respectively. After the optimum extraction conditions (X_1 and X_2), the 3D plot shows curvature and decline at higher levels, indicating decreased inhibition activity.

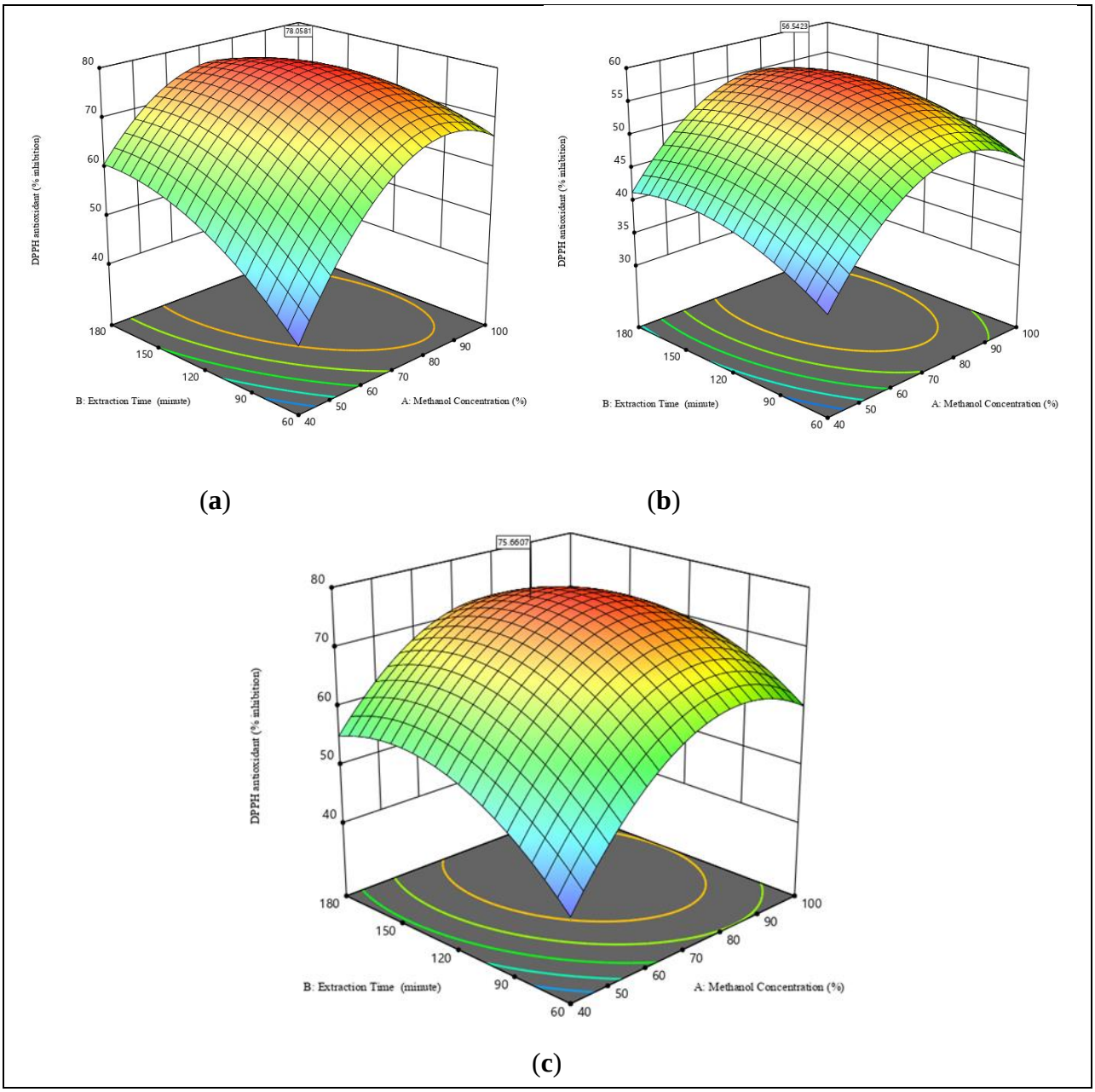


Figure 4.3. Response surface 3D plots showing the interaction effects of methanol concentration (%) and extraction time (min) on DPPH radical scavenging activity of *Usnea* species collected from (a), *Hagenia abyssinica,* (b), *Hypericum revolutum,* and (c), *Erica arborea*, respectively.

The results showed that DPPH radical scavenging capacity first increased and then decreased with higher methanol concentration and longer extraction times. The polarity of solvents is very important to increase the solubility of antioxidant compounds. Thus, it was significant to

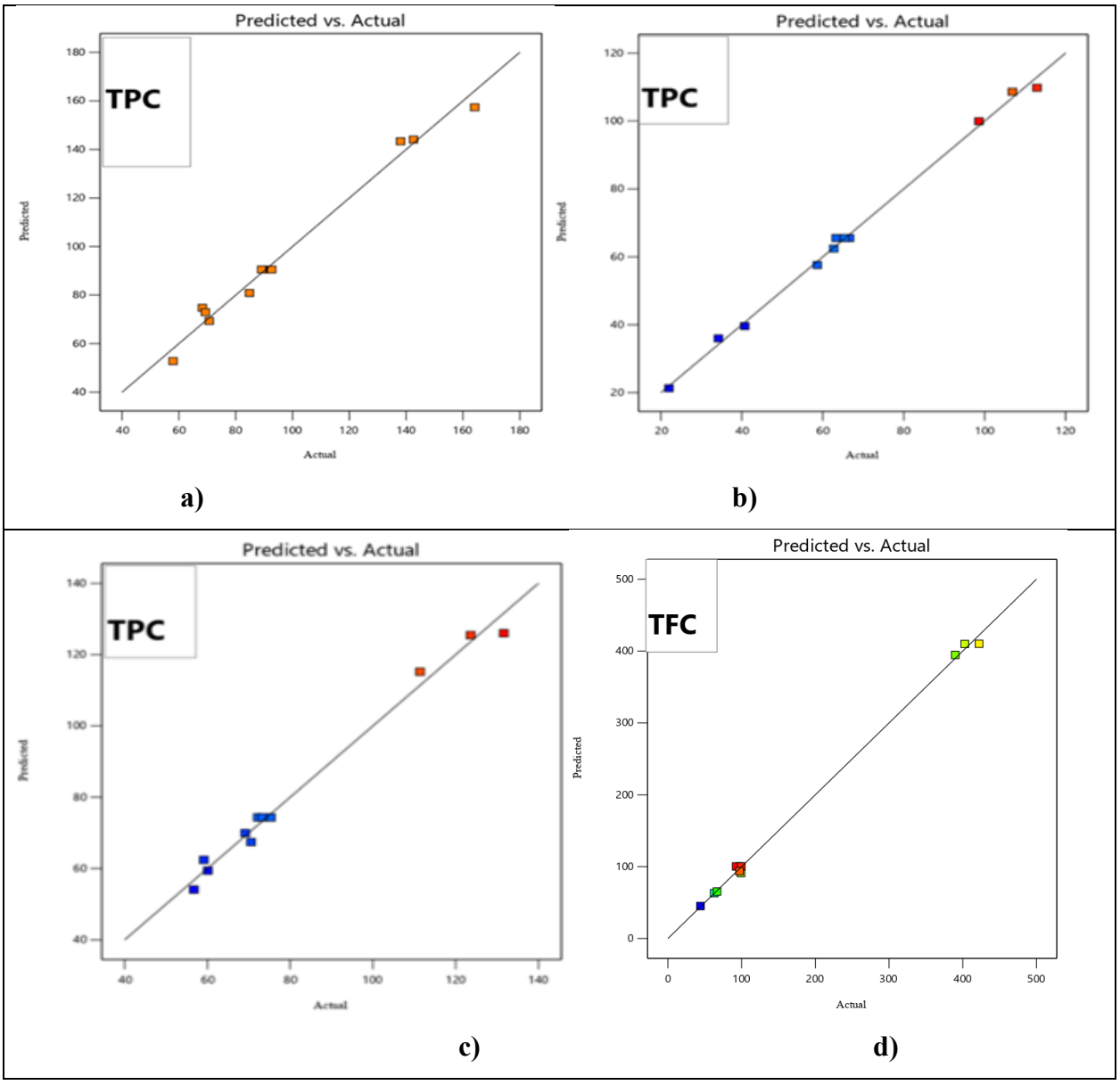
measure the process to validate a good solvent in antioxidant compound extraction. This validation can be optimized to get the maximum antioxidant activity for a certain sample.

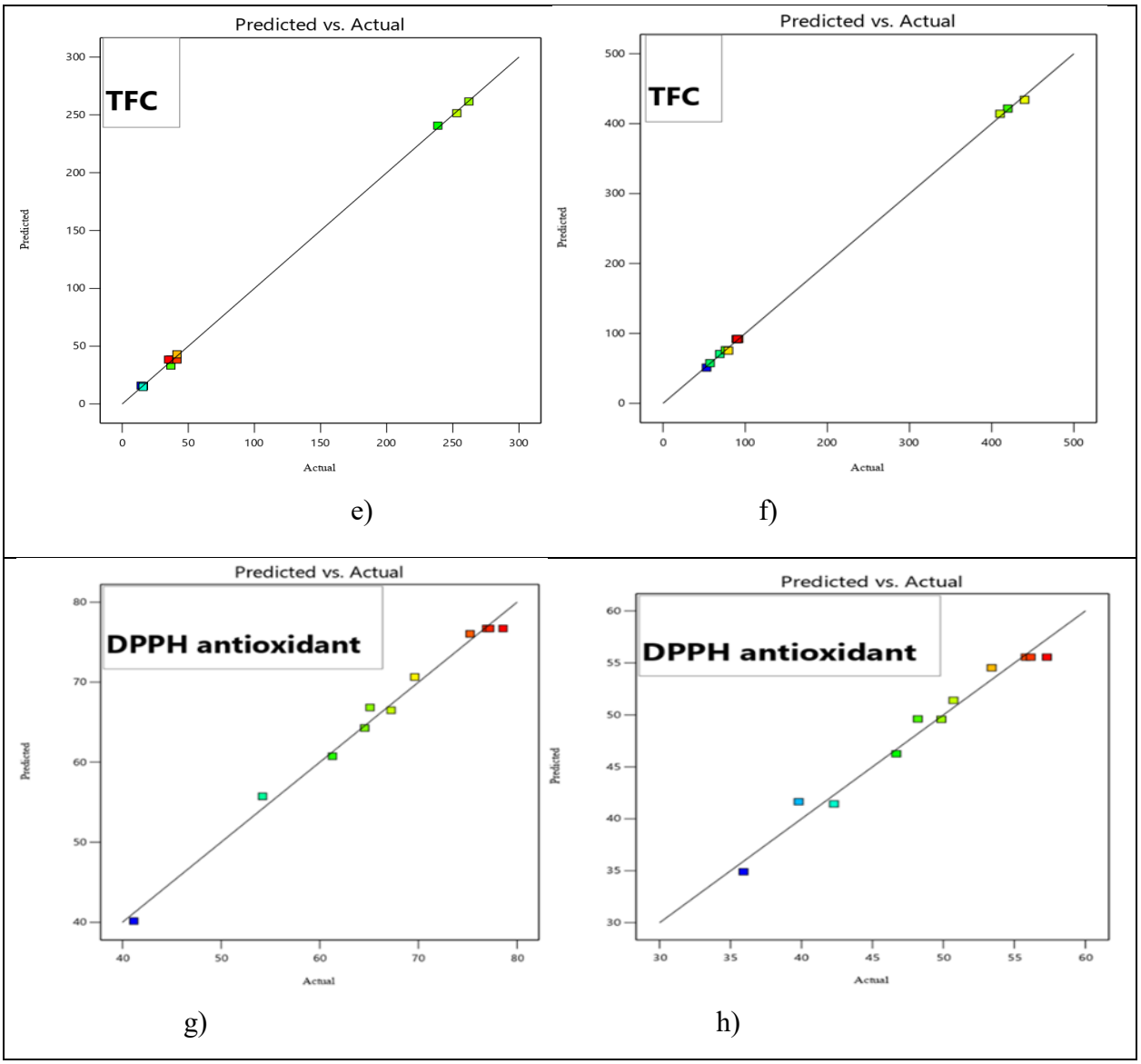
This visualization supports the regression models (Equations 7 - 9), which consistently indicated positive linear contributions from both factors, particularly methanol concentration, alongside negative quadratic terms that highlight diminishing returns at higher levels. The plot therefore provides a clear graphical representation of the nonlinear relationship between extraction conditions and antioxidant response, emphasizing the importance of identifying optimal ranges rather than simply maximizing individual parameters.

Overall, the results from the 3D plots suggested that antioxidant capacities of extracts strongly depend on the solvent concentration and extraction time. This may be associated with the chemical structure of phenolic compounds and the availability of phenolic hydroxyl groups, which can donate electrons or hydrogen, thereby forming stable end products (El Mannoubi 2023).

Agreement Between Experimental and Predicted Values

The experimental TPC, TFC, and antioxidant activity values agreed with the predicted values across the three *Usnea* species samples, as shown in **Figure 4.3**. The diagonal alignment of data points demonstrates a strong correlation between experimental and predicted values. This indicates the predictive ability and the fitting of experimental data with the selected model. Nevertheless, the slightly lower and higher TPC, TFC, and antioxidant activity of experimental results might be due to minor experimental error and to the adjustment of optimal variable values generated by RSM.


Predicted vs. Actual
TPC
Predicted
Actual
a)
Predicted vs. Actual
TPC
Predicted
Actual
b)
Predicted vs. Actual
TPC
Predicted
Actual
c)
Predicted vs. Actual
TFC
Predicted
Actual
d)


Predicted vs. Actual
TFC
Predicted
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0
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e)
Predicted vs. Actual
TFC
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0
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400
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f)
Predicted vs. Actual
DPPH antioxidant
Predicted
Actual
40
50
60
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80
g)
Predicted vs. Actual
DPPH antioxidant
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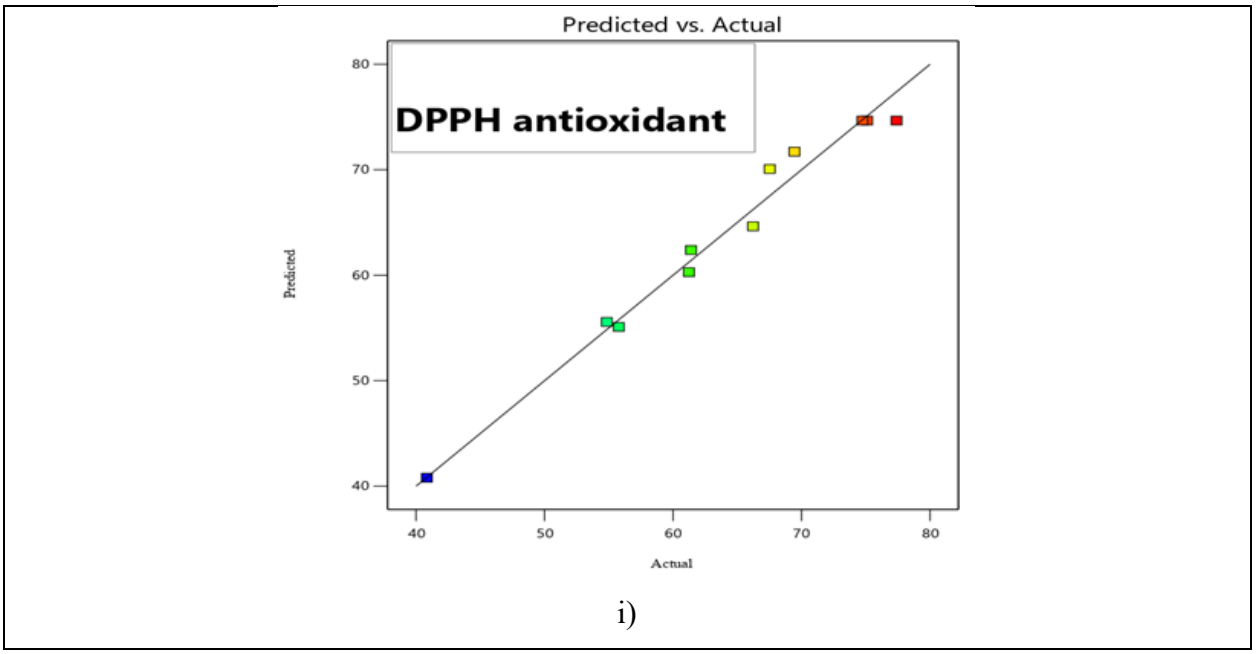

i)

Figure 4.4. Predicted versus actual values for total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity of *Usnea* species collected from different host plants: (a, d, g) *Hagenia abyssinica*, (b, e, h) *Hypericum revolutum*, and (c, f, i) *Erica arborea*.

4.2 Determination and Experimental Verification of Optimal Conditions

The optimization process was carried out to determine the most effective extraction parameters to maximize phenolic and flavonoid content and DPPH radical scavenging activity (% inhibition). In this study, response surface methodology determined that a methanol concentration of **100%** and an extraction time of **115** minutes for *Usnea* species collected from *Hagenia abyssinica*, **130** minutes for *Usnea* species collected from *Hypericum revolutum*, and **120** minutes for *Usnea* collected from *Erica arborea* would provide the highest recovery of bioactive compounds, as shown in **Table 4.5**. This selection was based on the empirical second-order polynomial model and the maximum desirability test, which yielded a value closest to 1 for the phenolic content and was used for an extraction test (Khalafyan et al. 2019). The highest recovery of metabolites with pure methanol may be due to the presence of depsides, depsidones, dibenzofurans, and usnic acid in *Usnea* species (Salgado et al. 2018). Although these compounds exhibit varying polarity, they are generally more soluble in organic solvents. Pure methanol provides a broader solubilizing capacity than mixed methanol/water systems, as it effectively dissolves both moderately polar metabolites (depsides and depsidones) and highly hydrophobic compounds (dibenzofurans). Consequently, extraction with 100% methanol yields a more

complete recovery of phenolic constituents, thereby optimizing the antioxidant potential of *Usnea* species compared to mixed-solvent systems (Oran et al. 2016; Popovici et al. 2021). Organic solvents had a high efficiency in extracting the phenolic compounds from lichens (Odabasoglu et al. 2004). From this, we suggest that the pure methanol extract was found to be a good solvent for the extraction of polyphenolic compounds of the *Usnea* lichen species.

Table 4.5. Optimum values of methanol concentration and extraction time for TPC, TFC, and DPPH scavenging activity

Samples	Methanol concentration (%)	Extraction time	TPC	TFC	DPPH assay	desirability	
Usnea (*H. a.*)	100.00	115	157.25	410.82	70.71	0.895	Selected
Usnea (*H. r.*)	100.00	130	108.9	253.26	51.59	0.877	Selected
Usnea (*E. a.*)	100.00	120	126.05	434.17	70.05	0.900	Selected

Extraction time in minutes, TPC in mg GAE/100 g of DW, TFC in mg CE/100 g of DW, DPPH antioxidant in % of inhibition

To validate these model predictions, experiments were conducted under the suggested optimum conditions of methanol concentration and extraction time. The results are summarized in **Table 4.6**, which compares the predicted maximum value with the experimentally obtained values.

Table 4.6: Predicted and experimental values of TPC, TFC, and DPPH radical scavenging activity at the optimal conditions

Samples	Responses	Optimum extraction conditions		Maximum value		% difference	p-value
		Methanol (%)	Extraction time	Predicted value	Experimental value		
Usnea (H. abyssinica)	TPC	100.00	115	157.25	161.06 ± 0.73	2.42	0.102
	TFC			410.82	413.68 ±1.82	0.73	0.184
	DPPH assay			70.71	73.39 ± 0.98	3.79	0.218
Usnea (*H. revolutum*)	TPC	100.00	130	108.90	111.14 ±1.24	2.06	0.114
	TFC			253.26	256.14 ± 1.61	1.14	0.077
	DPPH assay			51.59	53.52 ± 1.1	3.75	0.243
Usnea (*E.arborea*)	TPC	100.00	120	126.05	123.44 ± 1.07	-2.07	0.282
	TFC			434.17	429.12 ± 2.19	-1.38	0.051
	DPPH assay			70.05	71.1 ± 0.96	1.56	0.362

Experimental values are means ± standard deviation. methanol concentration in %, extraction time in minutes, TPC in mg GAE/100 g of DW, TFC in mg CE/100 g of DW, DPPH antioxidant in % of inhibition.

The percentage differences and p-values between the predicted and experimental values for TPC, TFC, and DPPH scavenging activity were also reported in **Table 4.6.** Those percentage differences are less than 10%, and all p-values were greater than 0.05 ($p > 0.05$) in the three samples of their response parameters, respectively. These findings were a good indication that the response model accurately reflects the expected optimum extraction. Optimization was

achieved with a difference of less than 10% and $p > 0.05$, indicating no significant difference between the experimental and predicted values (Jaafar, Ramli, and Salleh 2020). Overall, the experimental results closely matched the model predictions, confirming the validity of the optimum process and demonstrating that the selection conditions are optimal for extraction efficiency. This minimal difference from the predicted value also indicates that the established model was effective.

To the best of our knowledge, the determination of phenolic and flavonoid contents and antioxidant activity in *Usnea* species extracts using expert design optimization methods has not been investigated previously. While existing studies have analyzed phenolic contents and metabolite profiles of *Usnea* species using conventional and modern extraction and chromatographic techniques, none have applied statistical optimization approaches to maximize TPC, TFC, and antioxidant activity.

The present study revealed total phenolic content (TPC) values of **161.06 ± 0.77**, **111.14 ± 1.26**, and **123.44 ± 1.07** mg GAE/100 g DW in the optimized pure methanolic extracts at **115, 130,** and **120** minutes of extraction time of the investigated *Usnea* species collected from three host plants: *Hagenia abyssinica*, *Hypericum revolutum*, and *Erica arborea*, respectively. This indicates higher phenolic content in *Usnea* collected from *Hagenia abyssinica*, followed by *Erica arborea.* Higher TPC values observed in samples from *Hagenia abyssinica* compared to *Hypericum revolutum and Erica arborea* may suggest that this host plant provides more favorable chemical and microenvironmental conditions for metabolite accumulation. In the previous study, for instance, Naama et al. (2023) documented TPC values of 7.73 ± 0.25 mg GAE/g DW in *Evernia prunastri* and 5.22 ± 0.2 mg GAE/g DW in *Ramalina lacera*. These values are notably higher than those obtained in the present study, suggesting that *Evernia* and *Ramalina* species may accumulate phenolics more efficiently under the conditions examined. Conversely, Oran et al. (2016) reported substantially higher TPC values for *Usnea filipendula*, *Usnea fulvoreagens*, and *Usnea intermedia* (291.5 ± 12.1, 229.9 ± 14.0, and 239.7 ± 0.3 mg GAE/100 g DW, respectively). These values exceed those of the present study but remain lower than those reported for *Evernia* and *Ramalina* species by Naama et al. (2023). However, Nguyen et al. (2019) reported TPC values of 101.2, 50.14 ± 0.69, and 19.36 ± 0.70 µg GAE/g, extracted from the methanol extracts of *Usnea baileyi*, *Usnea flammea Stirt*, and *Usnea ceratina* Ach,

respectively, which are much lower than those in the present study. Pradhan et al. (2023) reported methanol extracts of *Trypethellium virens* and *Phaeographis dendritica* yielding 0.63 ± 0.007 and 0.46 ± 0.0024 mg GAE/g of dry extract, respectively, lower than the present study. The observed variation in TPC values between this study and previous reports on other species can be attributed to species-specific phenolic profiles, plant part analyzed, and extraction parameters such as solvent type, extraction method, and duration.

In this study, the total flavonoid content (TFC) of the optimized pure methanol extracts at **115, 130,** and **120** minutes of extraction time of the investigated *Usnea* species collected from three host plants: *Hagenia abyssinica*, *Hypericum revolutum*, and *Erica arborea*, was found to be (**413.68 ± 1.82, 256.14 ± 1.61,** and **429.12 ± 2.19** mg CE/100g DW) respectively. This indicates the flavonoid content of this species, collected from *Erica arborea*, was higher; this may be due to its acidic bark and favorable chemistry, which enhance lichen secondary metabolite production. Nguyen et al. (2019) reported the TFC as 66.87 ± 1.72, 86.80 ± 3.73, and 75.60 ± 1.91 µg QE/g for the methanol extracts of *Usnea baileyi, Usnea flammea Stirt*, and *Usnea ceratina Ach*, respectively, which is lower than the present study; this difference may be attributed to the use of RSM optimization in the present study, which enhances extraction efficiency. Kocakaya et al. (2024) reported that extraction with 80% methanol from *Usnea lapponica, Usnea intermedia, and Usnea florida* of total flavonoid content (TFC) was 4.29, 3.19, and 4.98 mg CAE/g DW. These findings are consistent with the present study. Environmental factors such as climate, soil composition, altitude, and sunlight exposure influence the biosynthesis and accumulation of phenolic compounds, with plants collected from higher altitudes generally exhibiting greater phenolic content (Brahmi et al. 2022; Mattera et al. 2024). The content of secondary metabolites in the same species varies with environmental factors such as precipitation, temperature, and altitude (Carrasco et al. 2024).

Aydin et al. (2018) found that the ethanol extract of *Usnea longissima* showed 4.63 ± 0.020 µg AAE/g lichen, and the ethyl acetate extract showed 1.44 ± 0.004 µg AAE/g lichen in antioxidant capacity assays, which exhibits weak scavenging activity compared to the present study. In the present study, the optimized pure methanolic extracts at **115, 130,** and **120** minutes of extraction time of the investigated *Usnea* species collected from three host plants: *Hagenia abyssinica*, *Hypericum revolutum*, and *Erica arborea*, exhibited antioxidant activities of 0.49 ± 0.01, 0.35 ±

0.01 and 0.47 ± 0.01 mg AAE/g DW (73.39 ± 0.98, 53.52 ± 1.1, and 71.1 ± 0.96 % inhibition), respectively. These values indicate a moderate to strong radical-scavenging potential. The higher antioxidant activity of *Usnea* from *Hagenia abyssinica* may be due to its more favorable bark chemistry and microenvironment, which enhance the accumulation of antioxidant secondary metabolites. Differences in light exposure and environmental stress on this host can further stimulate antioxidant compound production in lichens. The stronger inhibition observed in our methanol extracts may suggest that solvent choice plays a critical role in extracting flavonoids and other antioxidant compounds, with methanol being more efficient than a mixed solvent of methanol/water for *Usnea* species. Odabasoglu et al. (2004) found 13.38% inhibition in *Usnea florida* and 82.44% inhibition in *Usnea longissimi*. The observed difference in antioxidant activity results may be attributed to variation in phytochemical composition, climate conditions, plant species, extraction conditions, and solvent polarity. Meeses et al. (2013) further emphasized that mixtures of polar solvents enhance the recovery of phenolic constituents, thereby improving antioxidant potential. Taken together, these results reinforce the importance of solvent polarity and species variability in determining the antioxidant potential of *Usnea* extracts. Their antioxidant potential depends on their hydroxyl group arrangement (Ansari, Ahmad, and Haqqi 2020).

4.3 Correlation Between Total Phenolic, Flavonoid Contents, and Antioxidant Activity

Pearson's correlation coefficients were calculated to evaluate the relationships among TPC, TFC, and the antioxidant activity of *Usnea* lichen species, and the data are presented in **Table 4.7**.

Table 4.7. Pearson's correlation coefficients between total phenol, flavonoid content, and antioxidant activity.

	DPPH (% inhibition)
Total Phenolic Content	0.746^{*} (P =0.021)
Total Flavonoid Content	0.982^{**} (P = 0.0001)

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Pearson's correlation (r) was used to study the relationship between secondary metabolites, such as total phenolic and flavonoid contents, and antioxidant activity of lichen species. Several researchers have found high correlations between the antioxidant activity and phenolic content (Popovici et al. 2021). Lalremruata et al. (2022) suggest that phenol present in the methanol extract of the lichen sample was a major contributor to antioxidant activity by the DPPH method. Our study showed a strong correlation between TPC and antioxidant activity (r = 0.746) and a very strong correlation between TFC and antioxidant activity (r = 0.982). The strong relationships between total flavonoid content and antioxidant activity may suggest that the flavonoids present in the methanol extract of the *Usnea* lichen sample might be the major contributors to antioxidant activity as measured by the DPPH method.

4.4 Crude Lipid Content Determination

The lipid content of the *Usnea* species samples in this study ranged from 15.9% to 29.92% (w/w), as shown in **Table 4.8**. The *Usnea* species collected from the *Hypericum revolutum host plant* had a relatively higher lipid content, followed by those from *Hagenia abyssinica* and *Erica arborea*. This variation may be attributed to more favorable environmental conditions or to specific biochemical interactions between the lichen and the host plant that influence lichen metabolism (Aragón et al. 2025).

Table 4.8. Lipid weight and calculated lipid content (%) of *Usnea* species

Samples	Sample weight	Lipid weight	Lipid content (%)
Usnea (Hagenia abyssinica)	0.5	0.0817	16.34
Usnea (Hypericum revolutum)	0.5	0.1496	29.92
Usnea (Erica arborea)	0.5	0.0795	15.9

The lipid content observed in this *Usnea* species is novel, as previous studies on *Usnea barbarta, Usnea subfloridana, Usnea Antarctica, Usnea rubicunda*, and *Usnea lethariiformis* have reported fatty acids and other secondary metabolites, including depsides, depsidones, and dibenzofurans, but have not quantified the lipid yield.

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

This study assessed the composition of total phenolic compounds, flavonoids, antioxidant activity, and crude fat contents of *Usnea* species collected from three host plants. Extraction conditions were optimized for phenolic compounds, flavonoids, and antioxidant activity. By varying the methanol concentration and the extraction time, the optimum conditions were identified as 100% methanol and an extraction time of 115, 130, and 120 minutes for *Usnea* species collected from *Hagenia abyssinica*, *Hypericum revolutum*, and *Erica arborea*, respectively. The present study revealed total phenolic content (TPC) values of 161.06 ± 0.73, 111.14 ± 1.24, and 123.44 ± 1.07 mg GAE/100 g DW, while the total flavonoid content (TFC) of the optimized extracts was 413.68 ± 1.82, 256.14 ± 1.61, and 429.12 ± 2.19 mg CE/100 g DW, and demonstrated significant yield antioxidant inhibition, confirming the efficiency of the applied extraction optimization methodology. The findings highlight the *Usnea* lichen species as a valuable source of phenolic compounds and natural antioxidants. In addition to total phenolics, flavonoids, and antioxidants, crude lipid content analysis revealed appreciable oil yields ranging from 15.9% to 29.92% (w/w), with the highest value observed in *Usnea* associated with *Hypericum revolutum.* This study highlights the *Usnea* lichen species as a valuable source of phenolic compounds and natural antioxidants, alongside significant fat composition of *Usnea* species.

5.2 Recommendation

This study shows *Usnea* species are a promising source of natural antioxidants under optimized conditions. Future studies should focus on isolating and identifying specific bioactive compounds. Further optimization using different solvents and extraction parameters is also recommended. In addition, validation of antioxidant activity through in vivo studies is important. These findings can support future applications in nutraceutical and pharmaceutical development.

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APPENDIX

Appendix A: Workflow Diagram

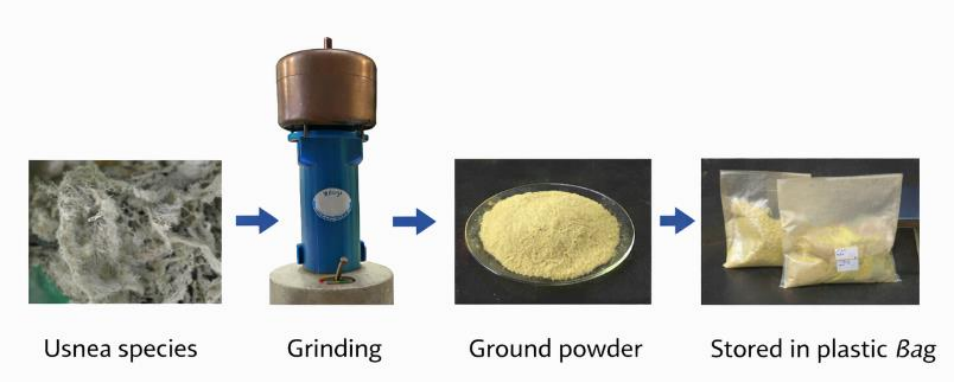

Figure 1: Workflow for Sample Preparation

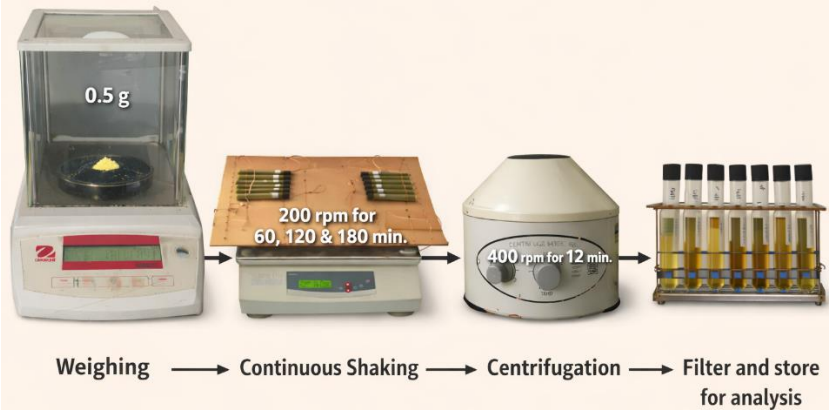

Figure 2: Workflow of the Extraction for Total Phenolics, Flavonoids, and Antioxidants

Appendix B: Raw Data

Table 1: Absorbance data of gallic acid standard for TPC assay

Concentration (µg/ml)	Absorbance (mean)
1	0.01
10	0.0955
20	0.183
40	0.35
60	0.529
80	0.6615
100	0.857

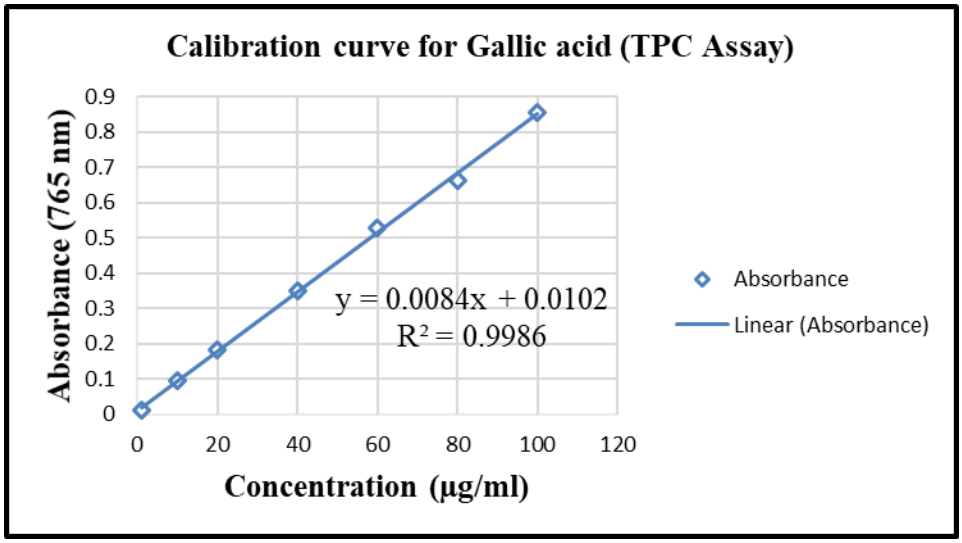

Figure 1: Calibration curve of gallic acid

Table 2: Absorbance data of catechin standard for TFC assay

Concentration (µg/ml)	Absorbance (mean)
4.71	0.013
18.84	0.039
37.68	0.071
75.36	0.135
113.04	0.209
150.72	0.293
188.40	0.362
226.08	0.405
282.60	0.527

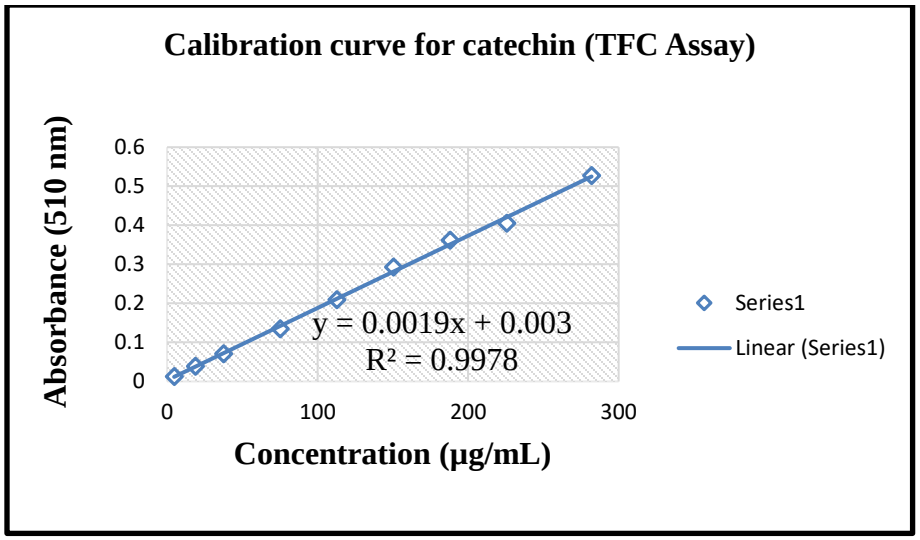

Figure 2: Calibration curve of catechin

Table 3: Absorbance data of ascorbic acid standard for DPPH antioxidant activity assay

Concentration (µg/ml)	**Absorbance (trial 1)**	**Absorbance (trial 2)**	**Control (trial 1)**	**Control (trial 2)**
1 ppm	0.505	0.487	0.52	0.508
5 ppm	0.445	0.422	0.52	0.508
10 ppm	0.365	0.331	0.52	0.508
15 ppm	0.285	0.261	0.52	0.508
20 ppm	0.21	0.199	0.52	0.508
25 ppm	0.135	0.121	0.52	0.508
30 ppm	0.06	0.05	0.52	0.508

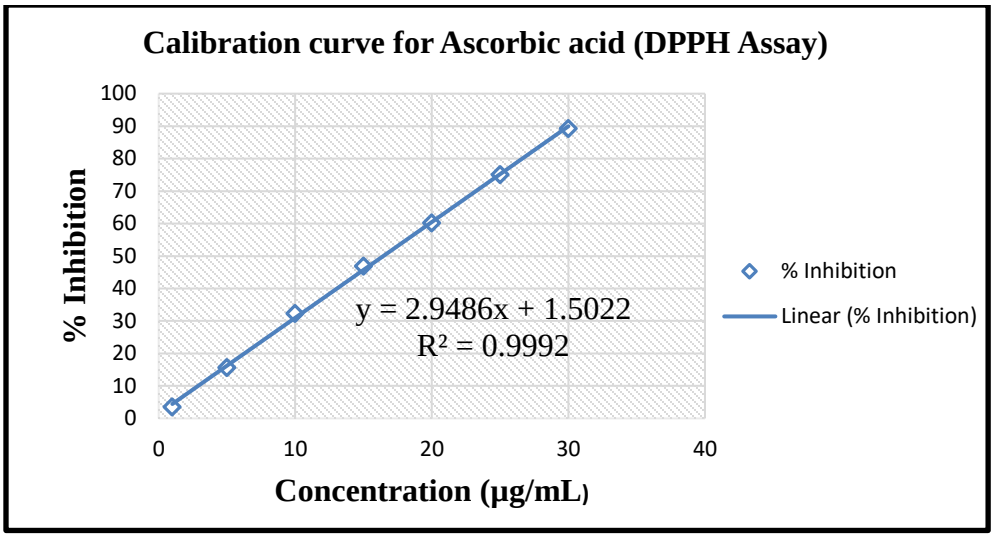

Figure 3: Calibration curve of ascorbic acid

Table 4: Absorbance data of *Usnea* species (*Hagenia abyssinica*) from TPC assay

Run	Methanol concentration (%)	Extraction Time (min.)	Absorbance (trial 1)	Absorbance (trial2)
1[a]	70	120	0.385	0.387
2	100	180	0.589	0.591
3	100	60	0.608	0.61
4[a]	70	120	0.4	0.399
5[a]	70	120	0.385	0.387
6	70	60	0.3	0.303
7	70	180	0.367	0.366
8	40	60	0.252	0.255
9	40	180	0.309	0.305
10	40	120	0.294	0.3
11	100	120	0.697	0.702

Table 5: Absorbance data of *Usnea* species (*Hypericum revolutum*) from TPC assay

Run	Methanol concentration (%)	Extraction Time (min.)	Absorbance (trial 1)	Absorbance (trial 2)

1[a]	70	120	0.278	0.274
2	100	180	0.421	0.428
3	40	60	0.1	0.106
4	100	60	0.461	0.457
5	70	60	0.254	0.259
6	40	180	0.183	0.179
7[a]	70	120	0.285	0.295
8	100	120	0.488	0.481
9[a]	70	120	0.289	0.281
10	40	120	0.152	0.155
11	70	180	0.278	0.27

Table 6: Absorbance data of *Usnea* species (*Erica arborea*) from TPC assay

Run	**Methanol concentration (%)**	**Extraction Time (min.)**	**Absorbance (trial 1)**	**Absorbance (trial 2)**
1[a]	70	120	0.313	0.322
2	100	60	0.526	0.533
3	100	180	0.473	0.483
4	70	180	0.301	0.312
5[a]	70	120	0.31	0.317
6	70	60	0.295	0.306
7	40	180	0.259	0.266
8	40	120	0.257	0.26
9	100	120	0.561	0.565
10[a]	70	120	0.32	0.333
11	40	60	0.242	0.255

Table 7: Absorbance data of *Usnea* species (*Hagenia abyssinica*) from TFC assay

Run	**Methanol concentration (%)**	**Extraction Time (min.)**	**Absorbance (trial 1)**	**Absorbance (trial2)**	**Absorbance (trial 3)**
1[a]	70	120	0.097	0.095	0.094

2	100	180	0.377	0.373	0.37
3	100	60	0.391	0.386	0.38
4[a]	70	120	0.1	0.095	0.098
5[a]	70	120	0.088	0.091	0.094
6	70	60	0.096	0.098	0.098
7	70	180	0.097	0.099	0.09
8	40	60	0.043	0.051	0.041
9	40	180	0.066	0.064	0.069
10	40	120	0.064	0.063	0.061
11	100	120	0.403	0.4	0.409

Table 8: Absorbance data of *Usnea (Hypericum revolutum)* from TFC assay

Run	**Methanol concentration (%)**	**Extraction Time (min.)**	**Absorbance (trial 1)**	**Absorbance (trial2)**	**Absorbance (trial 3)**
1[a]	70	120	0.037	0.037	0.04
2	100	180	0.25	0.247	0.259
3	40	60	0.016	0.019	0.015
4	100	60	0.224	0.234	0.231
5	70	60	0.033	0.039	0.042
6	40	180	0.016	0.02	0.018
7[a]	70	120	0.036	0.035	0.038
8	100	120	0.246	0.246	0.238
9[a]	70	120	0.038	0.045	0.044
10	40	120	0.018	0.02	0.017
11	70	180	0.039	0.044	0.044

Table 9: Absorbance data of *Usnea (Erica arborea)* from TFC assay

Run	**Methanol concentration (%)**	**Extraction Time (min.)**	**Absorbance (trial 1)**	**Absorbance (trial2)**	**Absorbance (trial 3)**
1[a]	70	120	0.086	0.09	0.087
2	100	60	0.407	0.4	0.398
3	100	180	0.389	0.389	0.4
4	70	180	0.078	0.078	0.08

5[a]	70	120	0.09	0.089	0.092
6	70	60	0.078	0.08	0.068
7	40	180	0.06	0.058	0.054
8	40	120	0.068	0.071	0.067
9	100	120	0.418	0.424	0.421
10[a]	70	120	0.09	0.089	0.088
11	40	60	0.047	0.045	0.043

Table 10: Absorbance data for DPPH antioxidant activity of *Usnea* species (Hagenia abyssinica)

Run	Methanol concentration (%)	Extraction time (min)	Control Absorbance	Sample Absorbance (trial 1)	Control Absorbance	Sample Absorbance (trial 2)	Control Absorbance	Sample Absorbance (trial 3)
1[a]	70	120	0.402	0.091	0.399	0.092	0.388	0.091
2	100	180	0.406	0.141	0.368	0.13	0.409	0.148
3	100	60	0.406	0.128	0.368	0.119	0.409	0.141
4[a]	70	120	0.402	0.095	0.399	0.09	0.388	0.086
5[a]	70	120	0.402	0.09	0.399	0.084	0.388	0.081
6	70	60	0.402	0.141	0.399	0.139	0.388	0.135
7	70	180	0.402	0.103	0.399	0.098	0.388	0.094
8	40	60	0.409	0.24	0.399	0.229	0.412	0.249
9	40	180	0.409	0.158	0.399	0.151	0.412	0.163
10	40	120	0.409	0.196	0.399	0.167	0.41	0.195
11	100	120	0.406	0.124	0.368	0.11	0.409	0.125

Table 11: Absorbance data for DPPH antioxidant activity of *Usnea (Hypericum revolutum)*

Run	Methanol concentration (%)	Extraction time (min)	Control Absorbance	Sample Absorbance (trial 1)	Control Absorbance	Sample Absorbance (trial 2)	Control Absorbance	Sample Absorbance (trial 3)
1[a]	70	120	0.402	0.18	0.399	0.174	0.388	0.171
2	100	180	0.406	0.208	0.368	0.168	0.409	0.219
3	40	60	0.409	0.26	0.399	0.243	0.412	0.267
4	100	60	0.406	0.221	0.368	0.185	0.409	0.226
5	70	60	0.402	0.208	0.399	0.207	0.388	0.201

6	40	180	0.409	0.23	0.399	0.213	0.412	0.237
7[a]	70	120	0.402	0.173	0.399	0.171	0.388	0.164
8	100	120	0.406	0.204	0.368	0.165	0.409	0.216
9[a]	70	120	0.402	0.191	0.399	0.19	0.388	0.188
10	40	120	0.409	0.254	0.399	0.222	0.412	0.259
11	70	180	0.402	0.193	0.399	0.19	0.388	0.183

Table 12: Absorbance data for DPPH antioxidant activity of *Usnea (Erica arborea)*

Run	Methanol concentration (%)	Extraction time (min)	Control Absorbance	Sample Absorbance (trial 1)	Control Absorbance	Sample Absorbance (trial 2)	Control Absorbance	Sample Absorbance (trial 3)
1[a]	70	120	0.402	0.106	0.399	0.102	0.388	0.093
2	100	60	0.406	0.159	0.368	0.138	0.409	0.162
3	100	180	0.406	0.137	0.368	0.12	0.409	0.143
4	70	180	0.402	0.126	0.399	0.123	0.388	0.114
5[a]	70	120	0.402	0.108	0.399	0.096	0.388	0.092
6	70	60	0.402	0.156	0.399	0.153	0.388	0.15
7	40	180	0.409	0.182	0.399	0.173	0.412	0.185
8	40	120	0.409	0.188	0.399	0.175	0.412	0.188
9	100	120	0.406	0.131	0.368	0.112	0.409	0.142
10[a]	70	120	0.402	0.095	0.399	0.09	0.388	0.084
11	40	60	0.409	0.246	0.399	0.229	0.412	0.247

Table 13: Absorbance data for TPC assay of the optimum extraction condition

Sample Name	Absorbance (trial 1)	Absorbance (trial2)	Absorbance (trial 3)
Usnea (Hagenia abyssinica)	0.686	0.69	0.684
Usnea (Hypericum revolutum)	0.483	0.474	0.474
Usnea (Erica arborea)	0.529	0.533	0.524

Table 14: Absorbance data for TFC assay of the optimum extraction condition

Sample Name	**Absorbance (trial 1)**	**Absorbance (trial2)**	**Absorbance (trial 3)**
Usnea (Hagenia abyssinica)	0.397	0.394	0.397
Usnea (Hypericum revolutum)	0.245	0.246	0.248
Usnea (Erica arborea)	0.409	0.41	0.413

Table 15: Absorbance data for the control and sample of the DPPH antioxidant activity of the optimum extraction condition

Sample Name	**Control (trial 1)**	**Absorbance (trial 1)**	**Control (trial 2)**	**Absorbance (trial 2)**
Usnea (Hagenia abyssinica)	0.326	0.089	0.328	0.085
Usnea (Hypericum revolutum)	0.326	0.149	0.328	0.155
Usnea (Erica arborea)	0.326	0.092	0.328	0.097

Table 16: Lipid yield of *Usnea* species samples

Sample	**Mass of the beaker**	**Mass of sample**	**Mass of Beaker + lipid mass**
Usnea (Hagenia abyssinica)	9.2662	0.5	9.3479
Usnea (Hypericum revolutum)	8.6930	0.5	8.8426
Usnea (Erica arborea)	8.421	0.5	8.5005