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Topic

Characterization of Some Primary and Secondary Metabolites of Blueberry (*Vaccinium angustifolium*) and the Study of Its Antioxidant Activity

Presented by:

Mahdjoub Ismail

Ikhlef Ishak

Before the jury composed of

Ammara N.	**Blida 1 University**	**MCA**	**President**
Boukerma L.	**Blida 1 University**	**MCB**	**Examiner**
Kabir W.	**Blida 1 University**	**MAA**	**Co-Supervisor**
Belfarhi L.	**CRAPC**	**MRB**	**Supervisor**

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Abstract

This study investigated the nutritional and antioxidant properties of blueberry leaves by analyzing their primary and secondary metabolites. The leaves were found to have high fiber content (32.67+-0.52g/100g), moderate protein (7.46+-0.48g/100g), and sugar levels (13.2+-1.04g/100g), with low lipid content of (0.455+-0.007g/100g),the moisture and ash contents were measured at (56.73+-0.34%) and (5.81+-0.60g/100g) . Secondary metabolites, including total polyphenols and flavonoids, were extracted using ethanol, chloroform, ethyl acetate, and butanol. The ethyl acetate extract yielded the highest concentrations of both compounds and showed the strongest antioxidant activity in ABTS and DPPH assays. HPLC analysis revealed several bioactive phenolic compounds such as myricetin and p-coumaric acid. These findings suggest that blueberry leaves are a promising source of natural antioxidants for use in nutraceutical and pharmaceutical products.

Keywords: Blueberry (*Vaccinium angustifolium*), Primary metabolites, Secondary metabolites, antioxidant activity, HPLC.

Résumé

Cette étude a examiné les propriétés nutritionnelles et antioxydantes des feuilles de myrtille en analysant leurs métabolites primaires et secondaires. Les feuilles ont montré une teneur élevée en fibres (32,67 ± 0,52 g/100 g), une quantité modérée de protéines (7,46 ± 0,48 g/100 g) et de sucres (13,2 ± 1,04 g/100 g), avec une faible teneur en lipides (0,455 ± 0,007 g/100 g). Les teneurs en humidité et en cendres étaient respectivement de 56,73 ± 0,34 % et 5,81 ± 0,60 g/100 g.

Les métabolites secondaires, notamment les polyphénols et flavonoïdes totaux, ont été extraits à l'aide de l'éthanol, du chloroforme, de l'acétate d'éthyle et du butanol. L'extrait à l'acétate d'éthyle a présenté les concentrations les plus élevées en ces composés, ainsi que la plus forte activité antioxydante dans les tests ABTS et DPPH. L'analyse HPLC a révélé plusieurs composés phénoliques bioactifs tels que la myricétine et l'acide p-coumarique. Ces résultats suggèrent que les feuilles de bleuet constituent une source prometteuse d'antioxydants naturels, utiles dans les produits nutraceutiques et pharmaceutiques.

Mots-clés: Myrtille (*Vaccinium angustifolium*), métabolites primaires, métabolites secondaires, activité antioxydante, HPLC.

الملخص

درست هذه الدراسة الخصائص الغذائية ومضادات الأكسدة في أوراق التوت البري من خلال تحليل المركبات الأولية ، ونسبة معتدلة من (غ/100غ 0.52 ± 32.67) والثانوية. أظهرت النتائج أن الأوراق تحتوي على نسبة عالية من الألياف ، مع محتوى منخفض من الدهون (غ/100غ 1.04 ± 13.2) والسكريات (غ/100غ 0.48 ± 7.46) البروتينات غ/100غ 0.60 ± 5.81و ٪0.34 ± 56.73 وقد تم قياس محتوى الرطوبة والرماد عند .(غ/100غ 0.007 ± 0.455) .على التوالي

تم استخلاص المركبات الثانوية، بما في ذلك إجمالي البوليفينولات والفلافونويدات، باستخدام الإيثانول، والكلوروفورم، وأسيتات الإيثيل، والبيوتانول. وقد أظهر مستخلص أسيتات الإيثيل أعلى تركيز من كلا المركبين وأقوى نشاط مضاد عن وجود عدة مركبات فينولية نشطة مثل الميريسيتين HPLC كشفت تحاليل .DPPH و ABTS للأكسدة في اختبارات وتشير هذه النتائج إلى أن أوراق التوت البري تُعدّ مصدرًا واعدًا لمضادات الأكسدة الطبيعية التي .كوماريك-p وحمض .يمكن استخدامها في المنتجات الغذائية العلاجية والدوائية

الكلمات المفتاحية

، المركبات الأولية، المركبات الثانوية، النشاط المضاد للأكسدة، (*Vaccinium angustifolium*) التوت البري .(HPLC) كروماتوغرافيا السائل عالية الأداء

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List of Abbreviations

ABTS	(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid))
$Al(NO_3)_3$	Aluminum nitrate
BHT	Butylated hydroxytoluene
°C	Celsius
CH_3COOK	Potassium acetate
DPPH	2,2-diphenyl-1-picrylhydrazyl
FCR	Folin-Ciocalteu reagent
G	Gram
GAE	Gallic acid equivalent
$K_2S_2O_8$	Potassium persulfate
Mg	Miligram
Na_2CO_3	Sodium carbonate
nm	Nanometer
QE	Quercetin equivalent
%	Percentage

Introduction

Wild blueberry, which is also known as lowbush blueberry is a fruit that is native to North America, it has not received any genetic improvement breeding programs like other species such as highbush blueberry (*Vaccinium corymbosum* L.), they make up the understory vegetation of forests in Eastern North America (Drummond, 2019). After the year 1906, blueberry began its commercial cultivation, it has been planted worldwide ever since due to its unique characteristics, including its distinctive flavor and rich nutritional content, such as sugar, organic acid, various amino acids and minerals. It is also a valuable source of active substances namely anthocyanin, ascorbic acid and pterostilbene that is useful in improving eyesight, inhibiting the growth of cancer cells, and resisting oxidation. But what truly makes blueberries well-known is their high antioxidant capacity, largely due to flavonoids (Li Y. , et al., 2023)

Many researches have focused on blueberries due to their positive effects on health. But their leaves remain underexplored despite being rich in beneficial compounds. Blueberry leaves are often considered a byproduct of cultivation but have shown great potential due to their content of phenolic acids and flavonols. These bioactive molecules play important roles in both plant defense and human health, especially antioxidative effects. (Wang, et al., 2025)

The objective of this study is to characterize some of both primary and secondary metabolites in wild blueberry leaves. The primary metabolite analysis will include moisture sugar, ash, lipid, proteins and fiber content. Whereas the secondary metabolite profile will focus on the quantification of polyphenols of different extracts (fractions) and flavonoids, HPLC, as well as the evaluation of antioxidant activity using DPPH and ABTS assays.

The present document will be divided into three parts as:

1. Bibliographic section: in which we will describe blueberry tree part and their primary and secondary metabolites composition
2. Experimental section: structured in:

- Material and methods: will be found in this section all protocols adopted for the assessment of primary and secondary metabolites as well as the evaluation of antioxidant activity
- Results and discussion: we will provide all results found and discuss them with others studies.

3. Conclusion and perspectives

Bibliographic Research

Blueberry Shrubs Overview

1. Origins

Lowbush blueberries are small understory shrubs, one of the many members of *Ericaceae* or the Heath family, genus *Vaccinium*, and also the most known (Padmanabhan *et al*., 2016). They thrive on barren landscapes, mainly in the temperate and boreal zones, perennial plants that are native to North America, specifically the northeastern areas of the United States as well as central and eastern Canada (Ratnaparkhe, 2007).

2. Botanic description

A low spreading small deciduous bushes that vary between 5 and 60 centimeters (between 2 and 24 inches) in height. Although the roots are shallow and fibrous, they may have a taproot that reaches a depth of 1 meter (3 feet) (Tirmenstein, 1991). Woody rhizomes spreading to form colonies, it has an average diameter of 4.5 mm (0.18 inches) and a depth of 6 cm (2.4 inches) (Vander Kloet, 1988).

Leaves that are of color green with a blue hue in summer, turning reddish in fall, they are ovate in shape, either stalkless (sessile) or on very short petioles sometimes have slightly serrated edges (Smith, 2016).

Wild blueberry plants grow clusters of usually white or pinkish-white flowers in the period between April and June, bell or urn-shaped 4 to 6 mm (0.12 to 0.25 inches) long, with each flower made up of 5 fused petals (Gardener, 2022).

The fruit is a small round berry averaging 4 to 11mm (0.12 to 0.4 inches) in diameter, usually dark blue to black color but often colored with a waxy coating that gives it its powdery blue appearance, with a five-pointed calyx (the crown-shaped thing) on one end (Yarborough, 2015).

3. Taxonomy

Kingdom: *Plantae* (Plants)

Phylum: *Tracheophyta* (Vascular Plants)

Subphylum: *Angiospermae* (Flowering Plants)

Class: *Magnoliopsida* (Dicotyledons)

Order: *Ericales* (Heathers and Allies)

Family: *Ericaceae* (Heath Family)

Tribe: *Vaccinieae*

Genus: *Vaccinium* (Blueberries, Cranberries and Allies)

Species: *Vaccinium angustifolium* (Lowbush Blueberry)

(U.S Department of Agriculture, U.S Department of Agriculture, Animal and Plant Health Inspection Service, 2010)

4. Nomenclature

Etymology: The species epithet *angustifolium* comes from Latin words *angustum* meaning 'narrow', and *folium* meaning 'leaf'.

Scientific Name: *Vaccinium angustifolium*

Common English Names:

- Lowbush blueberry
- Early low-bush blueberry
- Low sweet blueberry

Arabic Name:

بالفصحى: التوت الأزرق (الفاكهة)

باللغة العامية: الحلموش (الفاكهة), الريحان (الفاكهة / الأوراق)

بالامازيغية: الشلمون (الفاكهة)

French Names:

- Le Myrtille (fruit)
- Myrtillier (plante)

5. Metabolic Composition of Blueberry

5.1.Fruits

5.1.1. Primary Metabolites

Over the past recent years, blueberries garnered significant attention for their rich and high-value micronutrients (Zhang, et al., 2023). Due to their immense nutritional value, blueberries have acquired many labels such as “superfood”, “natural health package”, “functional foods” and “nutrient powerhouse” (Sivapragasam, *et al*., 2023). It comprises amino acids, organic acids, sugar, carbohydrates, proteins, lipids and many more (Li Y. , et al., 2023).

5.1.1.1.Carbohydrates

While blueberry fruits are surprisingly low in carbohydrates compared to other fruits they still contain a decent amount (Alina Sharon, 2024).

Sugars in blueberries contain simple sugars like glucose, fructose, and sucrose, which provide a source of energy containing around 13.26 grams of sugar per 100 grams of blueberries (one serving).

5.1.1.2.Proteins

Despite the fact that blueberries are not the finest source of proteins, they do contain a respectable quantity of amino acids, which are the fundamental components of proteins. Amino acids are regarded to be the building blocks of proteins (Alina Sharon, 2024). Some of these Amino acides may include:

Arginine, glutamine, lysine, glutamic acid as well as tryptophan (Lachowicz-Wiśniewska, et al., 2024).

5.1.1.3.Lipids

As far as lipids are concerned, blueberries contain a respectable amount of various lipid fats. These fats include monounsaturated fats, which account for around 0.02%polyunsaturated fats, which account for approximately 0.09%, and saturated fats, which account for approximately 0.03% while the total fats in blueberries make up to 0.16% (Magrane, 2009).

Table 1: Nutrition facts of blueberries per 100g (Magrane, 2009)

Nutrition Facts	
Typical value per 100g	
Energy	64.96 kcal
Protein	1.24 g
Total Fat	Nil
Mono Unsaturated Fatty Acid	BLQ (0.1) g
Poly Unsaturated Fatty Acid	BLQ (0.1) g
Saturated Fatty Acid	BLQ (0.1) g
Trans Fat	BLQ (0.1) g
Total Carbohydrates	15.10 g
Sugar	13.26 g
Vitamin C	10.11 mg
Vitamin A	1.66 μg
Vitamin B1	0.21 mg
Vitamin B2	0.12 mg
Vitamin B6	0.11 mg
Vitamin B12	0.17 mg
Potassium	80.2 mg
Magnesium	6.14 mg
Sodium	1.14 mg
Calcium	6.32 mg
Iron	0.35 mg
Cholesterol	BLQ (1.0) mg

Table 2: Nutritional Composition of Wild Blueberries (per 100g) (Magrane, 2009).

Component	Amount	Units	Component	Amount	Unit
Calories	45	Calories/100g	Calcium	17.4	mg/100
Calories from Fat	1	Calories/100g	Iron	0.577	mg/100
Total Fat	0.16	%	Vitamin E	0.386	IU/100g
Saturated Fat	0.03	%	Vitamin B1	0.030	mg/100g
Monounsaturated Fat	0.02	%	Vitamin B2	<0.010	mg/100g
Polyunsaturated Fat	0.09	%	Vitamin B6	0.020	mg/100g
Sodium	2.57	mg/100g	Phosphorus	12.9	mg/100g
Potassium	67.6	mg/100g	Magnesium	6.5	mg/100g
Total Carbohydrates	13.2	%	Zinc	0.667	mg/100g
Total Dietary Fiber	4.4	%	Moisture	85.8	%
Insoluble Fiber	3.0	%	Ash	0.187	%
Soluble Fiber	1.4	%	Folic Acid	26.6	mg/100g
Total Sugar	7.04	%	Niacin	0.610	mg/100g
Protein	0.00	%	Manganese	2.87	mg/100g
Total Beta Carotene	57.6	IU/100g	Vitamin C	2.01	mg/100g

5.1.1.4.Fibers

One of the many important components of a healthy diet is fiber, and blueberries can provide a significant amount of it. Dietary fiber can be divided into 2 types, soluble and insoluble fiber, studies show that every 100g of blueberries contains between 2.3 and 2.8g of fiber, approximately 29% being soluble and 71% insoluble. Soluble fiber slows digestion and enhances nutrient absorption meanwhile insoluble fiber helps prevent constipation (USDA, 2019).

5.1.1.5.Vitamins and Minerals

Blueberries are a good source of various vitamins such as:

- Vitamin K: helps the building of bones and inhibits the growth of cancer cell lines (DiNicolantonio, *et al*., 2015),
- Vitamin C: supports the immune system and reduced risk of cardiovascular disease (National Institutes of Health, 2021).
- Each of vitamin E, A, and all B-group are found in moderate amounts in blueberries, except for vitamin D and B12, which are entirely absent (Mazmanyan, 2022)

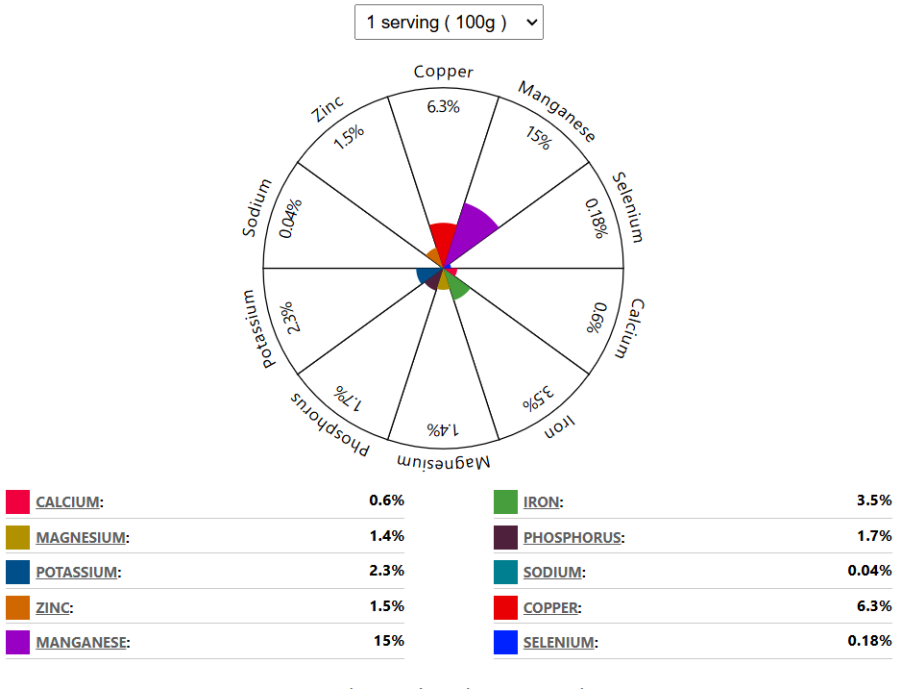


Figure 1: Vitamin coverage chart (per 100g of blueberries) (Mazmanyan, 2022)

As for minerals, they too are an excellent source, especially for manganese (Mn) which is crucial for many aspects, such as regulation of blood sugar and proper immune function (Aschner & Erikson, 2017). The detailed composition of minerals is presented in the figure below

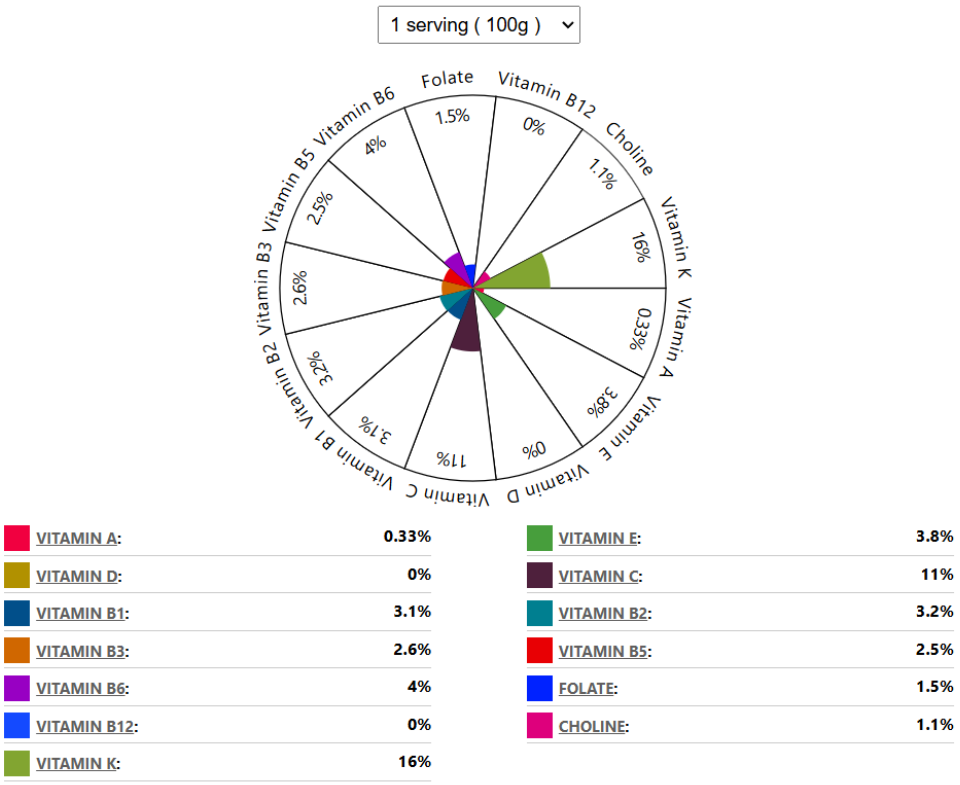


Figure 2: Mineral coverage chart (per 100g of blueberries) (Mazmanyan, 2022)

5.1.2. Secondary Metabolites

Secondary metabolites play vital roles in plant defense and have major impact on the quality of fruits during growth. Among the fleshy berry fruits, blueberries are known for having a high concentration of bioactive substances, especially anthocyanin pigments. In addition to anthocyanins, these berries also contain other valuable metabolites like carotenoid derivatives. Several studies have highlighted the health-promoting effects of blueberries These benefits are largely attributed to their rich composition of phenolic compounds. *Vaccinium* berries are especially abundant in flavonoids such as anthocyanins and flavonols are linked to a variety of biological activities, such as anti-inflammatory and antioxidant effects (Karppinen *et al*, 2016).

5.1.2.1.Flavonoids

Blueberry fruits are a rich source of flavones, a type of flavonoid. Flavonoids are a broad class of natural chemical compounds found in many fruits including blueberries. These compounds possess potent antioxidant properties.

The high concentration of flavones in blueberries contributes significantly to their overall antioxidant capacity (Li D. , et al., 2017)

5.1.2.2.Phenolic Compounds

Polyphenols have drawn a lot of attention over the years because of their ability to lessen stress from oxidation and inflammation. The wild blueberry species (*Vaccinium angustifolium*) is a very well-known rich source of polyphenols, having one of the highest concentrations of antioxidants. Additionally, numerous sources indicate that wild blueberries have higher antioxidant capacity and phenolic compounds compared to those found in the cultivated berries (Debnath-Canning, et al., 2020).

5.1.2.3.Tannins

Blueberries are rich in tannins, including proanthocyanidins, which are condensed tannins with antioxidant properties. These tannins protect cells from free radical damage. Blueberries also contain ellagitannins, hydrolyzable tannins that can break down when exposed to acids or enzymes

The total tannins in blueberries is around 160 milligrams per 100 grams, providing vitamins, fiber, and these powerful plant compounds. Tannins give blueberries their characteristic color and texture. (Diaconeasa, et al., 2015)

5.1.2.4.Anthocyanins

Blueberry anthocyanins are natural pigments that give blueberries their bright blue color. These compounds are part of a larger group called flavonoids, which are plant chemicals known for their health benefits. The vibrant blue color you see in blueberries mainly comes from these anthocyanins. But they do more than just make the fruit look appealing. They act as strong antioxidants, which help protect your body from damage caused by harmful molecules called free radicals (Yang, et al., 2022).

Many studies suggest that regular consumption of blueberries, particularly in moderate amounts, can lower the risk of various health problems. The natural compounds, particularly

anthocyanins, are linked to a lower risk of heart disease, longer life, and lower mortality rates. Blueberries may also protect against type 2 diabetes, a disease that affects sugar usage. Human clinical trials provide biochemical proof of the benefits of blueberries, including lower inflammation and oxidative stress levels, and improved weight control, reducing the likelihood of obesity-related issues (Kalt , et al., 2020)

5.2.Leaves

5.2.1. Primary Metabolites

While blueberry fruit is well-documented and extensively studied in many worldwide articles, information on blueberry leaves remains limited and less explored (Ștefănescu, et al., 2020). Blueberry leaves are considered a byproduct of blueberry cultivation, and despite their potential value, are often overlooked. They are rich sources of nutrients and bioactive compounds, including minerals, free sugars, amino acids, fatty acids and vitamin C (Wang, et al., 2025).

5.2.1.1.Carbohydrates

Sugars: blueberry leaves, like the leaves of many other plants, contain carbohydrates. These carbohydrates serve as essential parts of the plant's structure and energy storage system. The main forms of these carbohydrates are polysaccharides, which are large molecules made from many simple sugar units linked together. giving the leaves strength and rigidity.

The types of carbs found in blueberry leaves include glucose, Fructose, Galactose as well as Sucrose. (Akšić, et al., 2019).

5.2.1.2.Proteins

Blueberry leaves are rich in different types of proteins that serve various functions in the plant. Among these proteins are structural proteins such as actin(0.2mg) and tubulin(0.3mg), which form the cell's skeleton and help maintain its shape. These proteins are essential for cell division, movement, and stability. In addition to structural proteins, blueberry leaves contain enzymes that are key players in the plant's metabolic processes (Cvetkova, *et al*., 2024).

5.2.1.3.Lipids

Blueberry leaves contain a variety of lipids which are also called fatty acids, which are fats or fat-like substances found in plants. These lipids are important because they play a huge

role in the plant's health as it was shown that these fatty acids play a role in keeping the plant leaves healthy and in good conditions

In addition to fatty acids, the plant leaves also contain reasonable amounts of bioactive compounds like fatty alcohols as well as triterpenes which have an anti-inflammatory effect on the plant leaves and help reduce any inflammation (Vrancheva, *et al*., 2021).

- Fatty Acids: Ranged from 45.64% to 60.70%, with a significant portion being polyunsaturated fatty acids
- Fatty Alcohols: from 11.60% to 22.15%.
- Phytosterols: from 8.92% to 15.96%.
- Triterpenes: from 13.85% to 37.16% (Vrancheva, *et al*., 2021).

5.2.1.4. Fibers

Due to the limited availability of targeted studies on *Vaccinium angustifolium*, this review incorporates data from closely related species within the *Vaccinium* genus, particularly *Vaccinium myrtillus*, the anatomical observations of *V. myrtillus* leaves revealed the presence of lignified tissues such as sclerenchyma fibers and secondary walls in xylem vessels, which are associated with insoluble dietary fiber. These structural components, integral to leaf architecture, suggest a potential contribution of blueberry leaves to dietary fiber content. (Mercado *et al*., 2024).

5.2.1.5. Vitamins and Minerals

Bilberry (*Vaccinium myrtillus*) leaves are a source of vitamin C (ascorbic acid), which increases to their antioxidant capacity and enhances their potential health benefits. (Šaponjac *et al*, 2015) Additionally, leaves of other *Vaccinium* species, such as lingonberry (*Vaccinium vitis-idaea*), are recognized for their rich content of vitamins A, B, and C, as well as tocopherols (vitamin E compounds), improving their nutritional and pharmacological value (Yang, et al., 2023).

Bilberry (*Vaccinium myrtillus L.*) leaves are known to contain a variety of essential minerals which contribute to their nutritional and therapeutic value. These components are important for plants' metabolic functions and stress responses in plants. The presence and

concentration of macro- and microelements in bilberry leaves were determined and results are shown in the tables below. (Stanoeva *et al*, 2017).

Table 3: Content of macro- and microelements in leaves of bilberry (Stanoeva, *et al*, 2017) (mean ± SD).

Elements (mg/100g)	Dry Leaves
Macro elements	
Ca (Calcium)	1331 ± 5
K (Potassium)	657 ± 5
Mg (Magnesium)	266 ± 3
P (Phosphorus)	63 ± 1

Elements (mg/100g)	Dry Leaves	Elements	Dry Leaves
Micro elements			
Al (Aluminum)	24.1 ± 0.91	Cu (Copper)	3.39 ± 0.21
Fe (Iron)	9.05 ± 0.05	Mn (Manganese)	240.9 ± 3.11
Na (Sodium)	3.71 ± 0.03	Ni (Nickel)	0.244 ± 0.01
Ag (Silver)	0.012 ± 0.00	Sr (Strontium)	1.392 ± 0.25
Ba (Barium)	7.72 ± 0.95	Zn (Zinc)	1.46 ± 0.11
Cr (Chromium)	0.10 ± 0.00	Li (Lithium)	0.14 0.02

5.2.2. **Secondary Metabolites**

Similar primary metabolites, studies on secondary metabolites leaves are somewhat scarce compared to fruits. However, the available articles indicate that the leaves of blueberries are a rich source of bioactive natural products, such as chlorogenic acid, several flavonols and glycosides (Ștefănescu, et al., 2020). They also show antioxidant and anti-inflammatory properties (Ferlemi & Lamari, 2016). According to research, blueberry leaves contain anthocyanins, flavonols, hydroxycinnamic acids, and proanthocyanidins, with chlorogenic acids being the most abundant. (Ștefănescu, *et al.*, 2019).

Table 4: Function of some secondary metabolites (Kalt , et al., 2020) (Skrovankova, *et al*., 2015).

Class	**Examples**	**Functions**
Anthocyanins	Delphinidin, cyanidin, malvidin	Pigmentation, antioxidant
Flavonols	Quercetin, myricetin	Antioxidant, anti-inflammatory
Phenolic acids	Chlorogenic acid, caffeic acid	Antioxidant, antimicrobial
Tannins	Proanthocyanidins	Astringency, defense

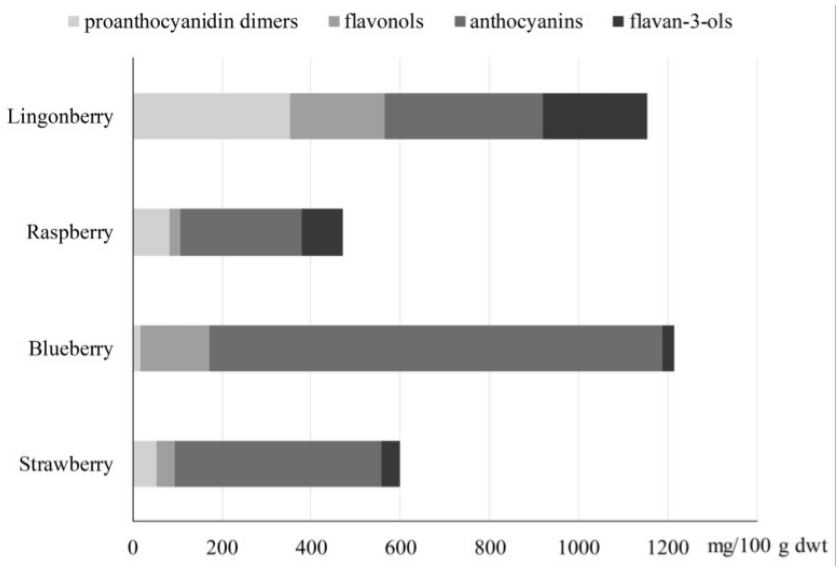


Figure 3: concentrations of secondary metabolites in different varieties of berries including blueberry (Liu, Hefni, & Witthöft, 2020).

5.2.2.1.Flavonoids

Blueberry leaves are packed with different flavonoids, which are natural compounds that give plants their color and protect them from damage. Among these flavonoids are myricetin, rutin, quercetin, and kaempferol. These are part of a larger group called polyphenols. Polyphenols are known for their ability to act as antioxidants. This means they help protect the leaves from harmful molecules called free radicals, which can cause damage to cells (Shi, et al., 2017).

5.2.2.2.Phenolic Compounds

One of the most abundant secondary metabolites in plants are phenolic compounds (Ayad & Akkal, 2019). Because of their many health benefits, natural polyphenols have been growing in popularity, these benefits include antioxidant and anti-inflammatory properties and the inhibition of cancer cell growth. According to studies of Wang *et al*., (2025), blueberry leaves are an abundant source of polyphenols and may contain even more than the fruit, as claimed by some sources. The chemical analysis of the leaves identified three major phenolic compounds: chlorogenic acid, quercetin and arbutin (Ștefănescu, *et al*., 2019)

5.2.2.3.Tannins

Tannins are present in many plant tissues, such as leaves, roots, and stems. Blueberry leaves contain astringent tannins. Proanthocyanidins are the predominant class of tannins found in blueberries, while ellagitannins may also be found (Ferlemi & Lamari, 2016).

Material and Methods

Aim of study

In order to characterize a medicinal plant "blueberry" or "*Vaccinium angustifolium*", the present study was performed to:

- Determine the amount of some primary metabolites including dry matter, ash, sugars, lipids, fibers and proteins
- Profile the ethanolic extract by HPLC.
- Quantify polyphenols and flavonoids as secondary metabolites.
- Assess the antioxidant activity: *in vitro* test (DPPH and ABTS test)

Internship location and period

Duration: March ~ July (4 Months)

1. Scientific and Technical Research Centre in Physico-Chemical Analyses (CRAPC)

Location: BP 384, Zone Industrielle Bou-Ismail RP 42004 Bou Ismaïl, Tipaza, Algeria

2. Pedagogic Laboratory of the Faculty of Natural Sciences and Life. Blida 1 University.
3. Altesse Lab (Quality Control Laboratory)

Location: Zabana street opposite to Frantz Fanon hospital, Blida 09000

1. Material

Biologic: Blueberry leaves.

Non biologic:

The non-biological materials used in this study include chemical products and reagents, equipment, glassware, and consumables, as illustrated in the Appendix 1.

2. Methods

2.1. Harvesting: Blueberry leaves were collected during the morning in January 2025 in Mount Zaccar, Miliana, Ain Defla (Semi-arid mediterranean climate)

2.2. Blueberry leaves powder preparation

The collected leaves were placed in a piece of cloth and left in the dark until thoroughly dried. Once fully dehydrated, they were ground into a fine powder using a Moulinex grinder.

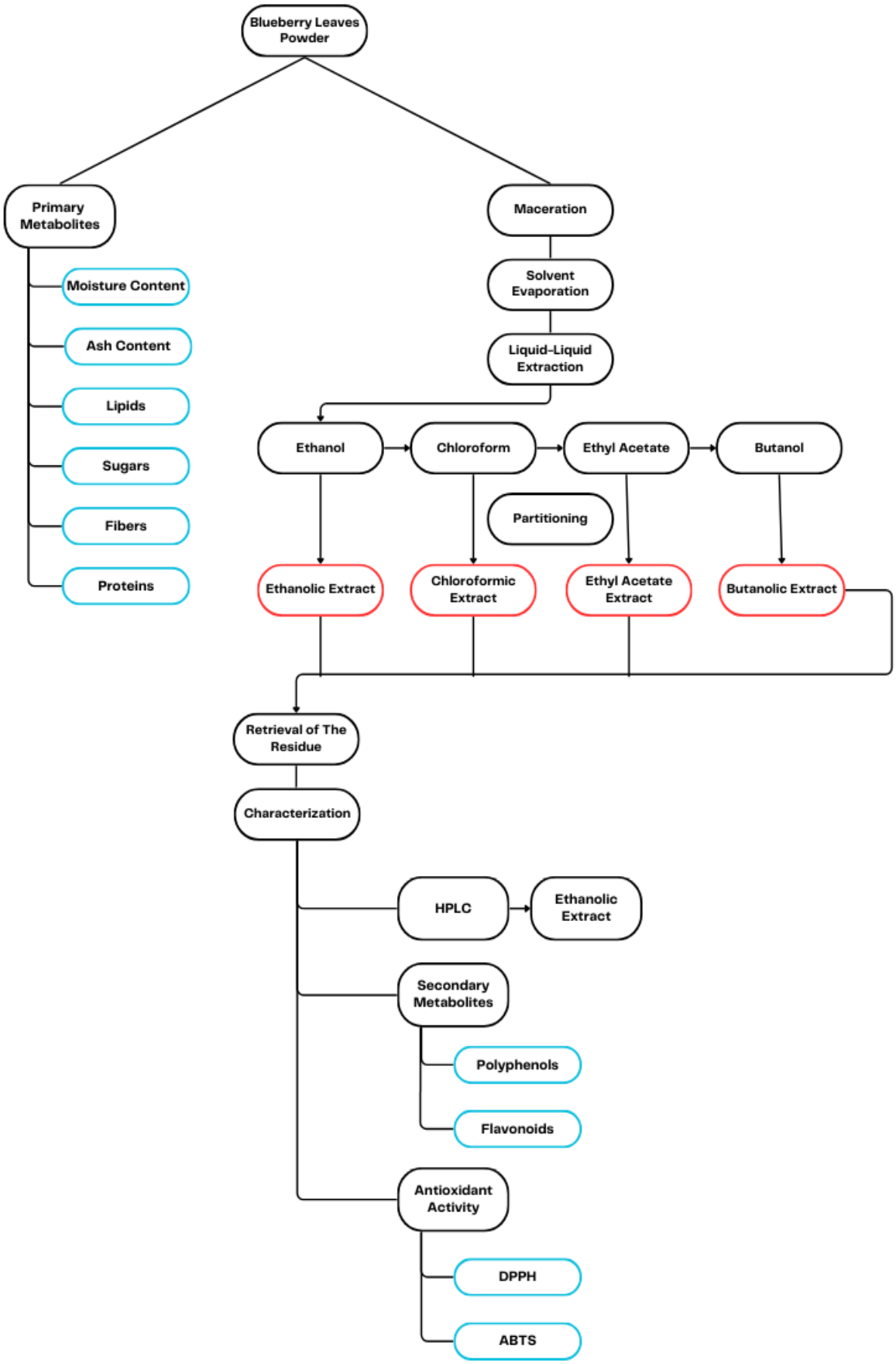


Figure 4: Schematic Representation of the Thesis Workflow

1. Blueberry Leaves Powder

1.1. Primary Metabolites

1.1.1. Moisture Content

Principle

Moisture content is determined by drying a sample at a constant temperature until it reaches a constant weight. The loss in weight represents the water (moisture) originally present in the sample.

Procedure

- Following the (AOAC official method 2005) we:
- Accurately weigh about 1g of the sample into a clean, dry weighing dish or crucible.
- Weigh the dish + sample
- Place the dish with the sample in the oven at 105°C for 3-5 hours, or until constant weight is achieved. This removes moisture content.
- Transfer the dish to a desiccator to cool.
- Weigh the dish with the dried sample

Calculations

$$\boldsymbol{Dry\ matter}\ (\%) = \frac{\boldsymbol{M1 - M2}}{\boldsymbol{M1}} \times \mathbf{100}$$

M1: initial weight (g)

M2: weight after drying (g)

1.1.2. Ash Content

Principle

Ash content represents the total mineral content of a sample. It is determined by incinerating the organic matter at high temperature in a muffle furnace until only inorganic minerals (ash) remain.

Procedure

According to (NF V05-113,01972 method):

- In porcelain crucibles, we weigh 2 g of ground sample.

- Place the crucibles in a furnace set to 550 ± 15 °C for 5 hours until a grey, light, or whitish color is obtained.
- Remove the crucibles from the furnace and allow them to cool in a desiccator, and then weigh them.

Calculations

$$\boldsymbol{Ash\%} = \frac{(\boldsymbol{M1} - \boldsymbol{M2})}{\boldsymbol{P}} \times \mathbf{100}$$

Ash% = Ash percentage

M1 = Mass of the crucible containing the ashes.

M2 = Mass of the crucible empty.

P = Mass of the test portion.

1.1.3. Lipids

Principle

Extraction is carried out using a Soxhlet-type extractor. This technique involves using a non-polar organic solvent.

Procedure

- Using a precision balance, we weighed 50 g of the ground sample and placed it in a thick filter paper cartridge. It is then positioned in the middle part of the apparatus.
- A round-bottom flask with a capacity of 1000 ml is filled with 550 ml of solvent (we used petroleum ether as the solvent).
- This flask is connected to the extractor, and the apparatus is then placed on a heating plate with continuous cold water circulation for the condenser.
- Six hours of extraction are sufficient to extract all the oil from our samples.
- The solvent is removed using a rotary evaporator.

$$\boldsymbol{R\%} = \frac{\boldsymbol{P1} - \boldsymbol{P2}}{\boldsymbol{PE}}$$

P1: Weight of the flask after extraction, containing oil.

P2: Weight of the empty flask.

PE: Sample weight.

1.1.4. Sugars

- According to (Dubois *et al*., 1956)
- We start by weighing 0.5 g of our leaf powder.
- We mix it with 8 mL of sulfuric acid (H_2SO_4), then we place the mixture in a crucible.
- We place it in an oven for 2.5h at 119°C.
- We transfer the solution to a flask of 500 mL and adjust the volume by adding distilled water until it reaches 500 mL.
- We filter the solution and then make three dilutions of 1/3.
- In each tube we add 1 mL of each dilution and add to it 1 mL of phenol at 5% and 5 mL of sulfuric acid at 98%.
- Then the tubes are placed in an oven at 105°C for 5 minutes later placed away from light for 30 minutes.
- Finaly, we use the spectrophotometer to read its absorbance at a 490 nm wavelength.

Then we start preparing our glucose

- We take 1 mg of glucose and pour it in 100 mL of distilled water.
- We prepare phenol at 5% by adding 5 g of phenol to 100 mL of water.
- Returning to the glucose, we prepare solutions with different concentrations (10, 25, 40, 60, 75, 100) (µg/mL).
- We take 1 mL off each of these dilution, 1 mL of phenol at 5% and 5 mL of sulfuric acid at 98%.
- Then we leave them in the dark for 30 minutes.
- Then we measure the wavelength at 490 nm and we plot the calibration curve.

1.1.5. Fibers

Principle

The Weende fiber determination involves a series of steps to estimate the fiber content in samples by removing digestible components and isolating the indigestible fiber fraction.

Procedure

According to the method of Weende:

- 2 g of dried and ground blueberry leaf powder were weighed using a precision balance.

- The sample was boiled for 30 minutes in 200 mL of 1.25% sulfuric acid (H_2SO_4) while stirring sometimes.
- Filter and wash the residue thoroughly with hot distilled water to remove acid and soluble components.
- The residue was then boiled for another 30 minutes in 200 mL of 1.25% sodium hydroxide (NaOH) to dissolve remaining proteins and other digestible materials.
- After the alkaline digestion, the mixture was again filtered and washed thoroughly with hot water.
- This dried residue, which includes crude fiber and ash, was weighed.
- The residue was then incinerated in a muffle furnace at approximately 550°C to remove all organic matter
- The remaining ash weight was subtracted from the dried residue weight to calculate the crude fiber content.

$$\boldsymbol{Fiber}\% = \frac{(\boldsymbol{BA} - \boldsymbol{AA})}{\boldsymbol{S}} \times \mathbf{100}$$

BA= Dry weight of residue before ashing.

AA= Weight of residue after ashing.

S= Weight of sample

1.1.6. Proteins

Principle

The Kjeldahl method measures the total nitrogen content in the sample, which is then converted to protein using a conversion factor, the method includes three main steps.

Procedure: (Kjeldahl method)

1. **Digestion**

- Weigh 0.5–1.0 g of dried blueberry leaf powder into a Kjeldahl digestion flask.
- Add 10–15 mL of concentrated sulfuric acid.
- Add a catalyst mixture to raise the boiling point and speed digestion.
- Heat the flask until the mixture becomes clear and colorless.

2. **Distillation**

- Transfer the digested solution to the distillation unit.

- Add sodium hydroxide NaOH solution carefully to make the mixture alkaline.
- Distill the released ammonia into a known volume of boric acid solution.

3. Titration

- Titrate the ammonia absorbed in boric acid with a standard acid HCl to quantify the nitrogen content.

Figure 5: Before and after protein titration (Original)

Calculations

$$\boldsymbol{Nitrogen}\ (\%) = \frac{(\boldsymbol{V} \times \boldsymbol{N} \times \boldsymbol{1.4007})}{\boldsymbol{S}}$$

V= Volume of acid used in titration (ml)

N= Normality of the acid

S= Weight of sample

$$\boldsymbol{Protein}\ (\%) = \boldsymbol{Nitrogen}\ (\%) \times \boldsymbol{6.25}$$

1.2. Extraction technique

According to (Tambun, *et al.*, 2021), the preparation of the blueberry leaves extract was performed with a ratio of 10% of plant material/solvent, we:

- Place 150g of dried ground leaves in a beaker.
- Add 500mL of hydro-ethanol (20/80: V/V).

- Stir to ensure proper contact between the plant material and solvent. Then it is covered and left to macerate for 24 hours at room temperature.

After 24 hours of maceration, the solution was filtered using filter paper and a vacuum pump (figure 6). The filtered extract was collected in an opaque bottle and stored in the refrigerator until further use.

Figure 6: Filtration Procedure Using Paper Filter and a Vacuum (Original)

1.2.1. Solvent Evaporation

The first extract was concentrated using a rotary evaporator to remove water and residual ethanol. The solution was placed in a round-bottom flask and subjected to reduced pressure and gentle heating, allowing the solvent to vaporize. The vapor was then condensed and collected in a receiving flask. After complete removal of the solvent, 250 mL of distilled water was heated and added to the remaining plant residue in the flask. This step aimed to dissolve any remaining water-soluble compounds, including polyphenols and other polar constituents. This mixture is defined as hydroethanolic extract $E_{H.E.}$

Figure 7: Rotary Evaporator (Original)

Another 50 grams of blueberry leaf powder was weighed for another maceration. Then mixed with 300 mL of a hydroethanolic solution (80% V/V). The mixture was left at room temperature for 24 hours, then filtered, and the resulting extract was stored in the refrigerator until further use.

Figure 8: Leaf Powder (Original)

1.2.2. Liquid-Liquid Extraction

1. Chloroform

- We take 250mL of the filtered ethanolic extract and add it to the separatory funnel.
- We also take 250mL of chloroform (a non-polar solvent used to extract non-polar compounds) and add it to the separatory funnel.
- We seal the funnel shake the mixture vigorously to allow thorough mixing.

- We place it on a stand and leave it undisturbed for approximately 20 minutes to allow phase separation.
- We collect carefully the lower organic layer (chloroform phase).
- We repeat this process to ensure maximum recovery of non-polar compounds.

Figure 9: The Collected Lower Organic Layer (Chloroform Phase) (Original)

2. Ethyl Acetate

- After removing the organic layer, we are left with the aqueous layer, we take 250 mL of ethyl acetate (to extract semi-polar compounds) and add it to the separatory funnel.
- We shake the funnel and leave it to separate for 20 minutes.
- After 20 minutes, we recover the upper layer (the ethyl acetate layer).
- This separation was repeated 3 times.

Figure 10: Separation Procedure (Ethyl Acetate Phase) (Original)

3. Butanol:

- Similar to the previous extractions, after the removal of the ethyl acetate layer, we add 250 mL of butanol to the funnel (to recover moderately polar compounds).
- The mixture is shaken thoroughly and then left to separate for 20 minutes.
- We retrieve the upper organic layer.
- This procedure was repeated 3 times.

Figure 11: Butanol Separation Procedure (Original)

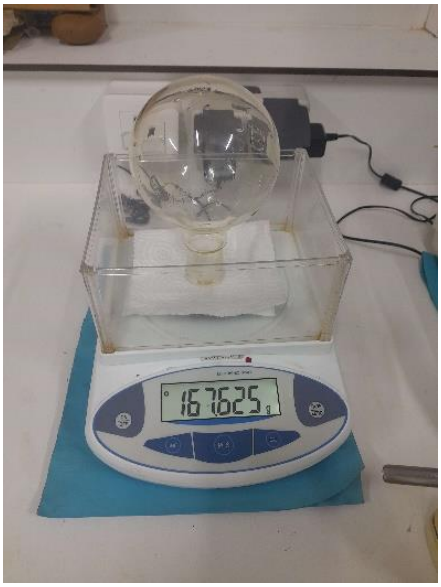
1.2.3. Solvent Removal Using Rotary Evaporator

- We weigh each flask prior to use to determine the exact mass of the recovered compounds.
- The chloroform, ethyl acetate, and butanol extracts were each placed in their respective flasks and subjected to rotary evaporation.
- This process allowed for the removal of the solvents, leaving behind the extracted compounds from the liquid-liquid extraction.

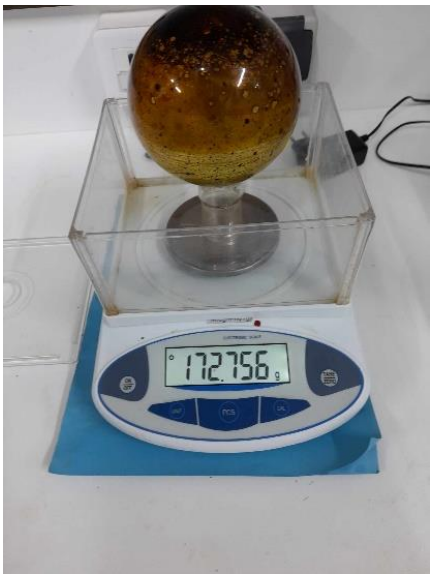
Figure 12: Butanol evaporation (Original)

1.2.4. The Retrieval of the Residue

After solvent evaporation, a solid residue was obtained, adhering to the bottom and inner walls of the flask. The dried extract was carefully scraped off using a spatula and collected in plastic vials for compounds quantification. Before evaporating each solvent extract, the corresponding round-bottom flask was weighed and the weight recorded. After rotary evaporation, the flasks were weighed again to determine the weight of the dried extract by subtracting the initial weight of the empty flask. This allowed for the quantification of the solid residue obtained from each solvent phase.



Ethanol Flask Before



Ethanol Flask After

Figure 13: Flask weighing (Original)

1.2.5. HPLC Analysis

Principle:

High-performance liquid chromatography or commonly known as HPLC is a technique used to separate, identify and quantify components in a mixture based on their different interactions with a stationary phase and a mobile phase.

Equipment: Agilent 1100

Software: CHEMSTATION

Injection loop: 20 µL

Mobile phase: A: Water with 1% acetic acid

B: Methanol

Wavelength: 254 nm

Flow rate: 1 mL/min

Table 5: Gradient Program

Time (min)	A (%)	B (%)
0	95	5
55	5	95
57	95	5
59	95	5

Column Specifications:

Type: C18

Brand: KNAUER

Length: 250 mm

Diameter: 4.6 mm

Particle size: 5 µm

1.2.6. Secondary Metabolites

1.2.6.1. Flavonoids Quantification:

Principle:

Flavonoids form a yellow-colored complex with aluminum chloride, and the intensity of the color reflects the total flavonoid content in the sample.

Procedure:

- According to Zhishen, *et al.*, (1999)
- 1 g of aluminum nitrate ($Al(NO_3)_3$) was dissolved in 10 mL of distilled water.
- 0.98 g of potassium acetate (CH_3COOK) was dissolved in 10 mL of distilled water.

- We weigh 1 mg of each of our extract samples in an Eppendorf tube then we add 1 mL of methanol to dissolve the samples at a final concentration of 1mg/mL.
- Triplicate Eppendorf tubes were prepared for each repetition.
- A volume of 100 µL from the sample solution was transferred into each of the three separate tubes (100 µL for each tube).
- To each of these tubes, 100 µL of potassium acetate and 100 µL of aluminum nitrate were added, followed by 900 µL of distilled water.
- We leave the Eppendorf tubes in a drawer protected from light for 30 minutes to allow the reaction to proceed.
- In the meantime, we prepare a blank (reference) solution for the spectrophotometric analysis. The latter contains all the reagents with replacing the sample with our solvent (methanol, potassium acetate solution, aluminum nitrate solution, and distilled water) in the same volumes as the samples.
- After the 30-minute incubation period, the absorbance of the samples was measured using a spectrophotometer set to 415 nm and the concentration was expressed in mg equivalent quercetin/mg of extract.
- In the same conditions, we prepare different concentration of quercetin as standard.

Spectrophotometer Sample Reading Procedure:

- We place the reference in the appropriate slot.
- The software that was used is “WinASPECT Plus”, we press on “Measure reference” to calibrate the device.
- We place each sample individually into the spectrophotometer and note down the absorbance values accordingly.

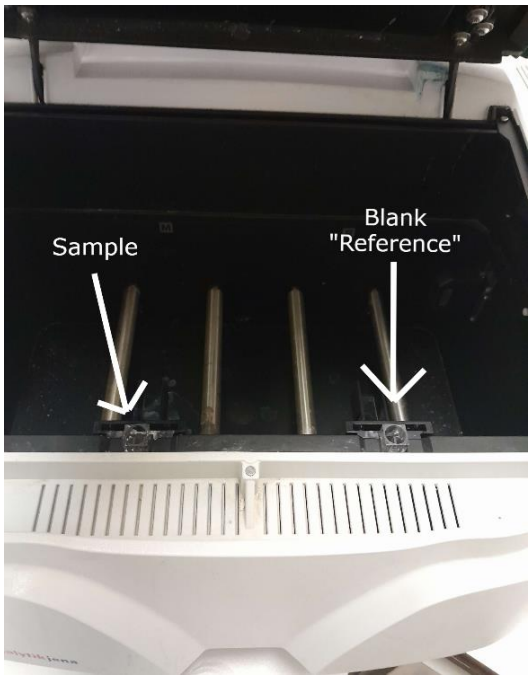


Figure 14: Open View of the Spectrophotometer (Analytik Jena Specord 210 Plus) Showing Sample Slot/Cuvette Holder (Original)

1.2.6.2. Polyphenols Quantification

By following the Folin-Ciocalteu method

- First, 1 mL of Folin-Ciocalteu reagent (FCR) was diluted with 9 mL of water.
- 0.75 g of sodium carbonate (Na_2CO_3) was dissolved in 9 mL of water.
- We weigh 1 mg of each extract and put it in separate Eppendorf tubes.
- 1 mL of methanol was added to each of these tubes.
- We take 100 µL off each sample and add it to 3 other tubes (triplicate).
- Then we add 400 µL of sodium carbonate (Na_2CO_3).
- We wait for 3 minutes, and then we add 500 µL of Folin–Ciocalteu reagent (FCR).
- The mixtures were gently agitated to ensure thorough mixing and then incubated in a water at 40°C for 5 minutes.
- We measure the absorbance later just as the flavonoids procedure except the difference is the reading is set to 760nm.

Figure 15: Folin–Ciocalteu Reagent (FCR) and Sodium Carbonate (Na_2CO_3) (Original)

1.2.7. Antioxidant Activity (DPPH)

Principle:

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay is a widely used method to evaluate the antioxidant capacity of a compound or extract based on its ability to scavenge free radicals.

Procedure:

According to Brand-Williams *et al*., (1995),

- We start by weighing 4 mg of each extract and place them into separate Eppendorf tubes.
- We add 1 mL of methanol to each tube to dissolve the samples.
- We prepare 6 additional tubes for each extract for serial dilution, all filled with 500 µL of methanol.
- We transfer 500 µL from the first tube (containing the dissolved extract) into the second tube, then we vortex to ensure proper mixing.
- We repeat the same procedure with the second tube and transfer it to the third and vortex again, and so on until the seventh dilution (tube 7).

For each dilution tube, three additional Eppendorf tubes were prepared to serve as replicates

- A volume of 150 µL was taken from each dilution and distributed into its corresponding three replicate tubes.
- Before adding the DPPH solution, we measure its absorbance at 517 nm to ensure it fell within the range of 0.5 to 0.7.

- After that, we add 600 µL of DPPH solution to each replicate. Note that the addition of DPPH was performed in low-light conditions to prevent degradation caused by exposure to room light.
- We vortex the tubes to ensure proper mixing, then we place them in a drawer and keep them in the dark for 30 minutes.
- After 30 minutes of incubation, we measure the samples using a spectrophotometer set at 517 nm and note the values.
- In the same conditions, BHT was used as standard in different concentrations.

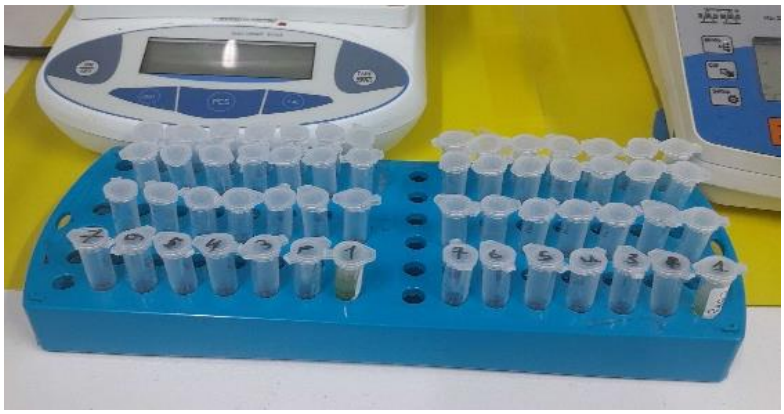


Figure 16: Setup of Eppendorf Tubes with 6 Dilutions, Each in Triplicate (Original)

Equation: inhibition percentages of each dilution were calculated using a negative control or blank:

$$\%\boldsymbol{Inhibition} = \frac{\boldsymbol{WB - WS}}{\boldsymbol{WB}}$$

WB = Blank absorbance

WS = Sample absorbance.

1.2.8. Antioxidant Activity (ABTS)

Principle

By reacting with potassium persulfate ($K_2S_2O_8$), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) forms the $ABTS^+$ radical, which is blue to green in color. The addition of antioxidants will reduce this radical and cause the decolorization of the mixture.

Procedure

The ABTS procedure follows the same steps as the DPPH, we start by weighing 4 mg of each extract and place them in tubes, then add 1 mL of methanol to them. We prepare 6 other tubes for serial dilution, we take 500 µL from the first tube onto the second, then vortex and from the second to the third and so on until the last tube. For each dilution we have 3 other tubes (triplicates) using the protocol of Re *et al*. (1999).

We transfer 150 µL from the diluted tube to the triplicates. Then, we have to make sure that the ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) absorbance falls between 0.702 and 0.72 at a wavelength of 734 nm. After that we add 600 µL of ABTS to each tube of the triplicates, all performed while in darkness to prevent degradation caused by light. We vortex the tubes and place them in a drawer away from light for 10 minutes. After 10 minutes we measure the samples in a spectrophotometer set at 734 nm.

Results and Discussion

1. Primary Metabolites

1.1. Moisture Content result

The result of dry matter and moisture content are represented in the diagram below (Figure 17)

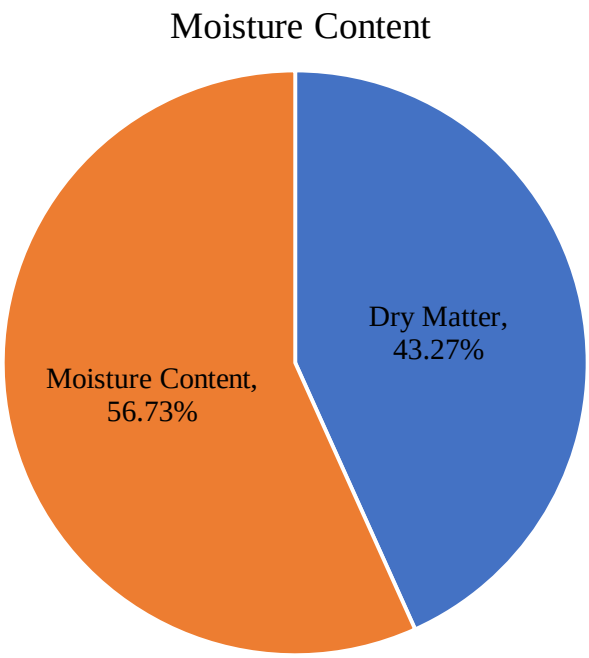


Figure 17: Moisture and Dry Matter Composition of Blueberry Leaves

The analysis was carried out to measure the moisture content in blueberry leaves. The results showed that the leaves contain approximately 56.73% water by weight, with a corresponding dry matter content of 43.27%. This indicates that more than half of the leaf's weight consists of water, emphasizing the essential role moisture plays in the leaf's structure and the plant's overall growth.

In contrast, *Vaccinium parvifolium* (huckleberry) leaves were found to contain significantly less moisture. According to (Sodamade, et al., 2025), their moisture content was $9.89 \pm 0.051\%$, suggesting they are much drier than blueberry leaves. showing that they are much drier than blueberry leaves.

These findings offer valuable insight for growers. Plants with higher moisture content, like blueberry, may be more sensitive to water stress or drought, potentially requiring more consistent irrigation. Whereas, the drier huckleberry leaves suggest better resilience in arid

conditions. Understanding these variations can aid in crop selection and optimizing cultivation practices based on environmental conditions.

Standard deviation : 56.73 ± 0.34%

1.2. Ash Content

The results from this test showed that approximately 5.81% of the blueberry leaves' initial weight was mineral ash, indicating a relatively high mineral presence in the leaves.

When compared to other species, such as *Vaccinium myrtillus* (bilberry), which contains 3.05% ash (Saracila, *et al.*, 2023), and *Vaccinium parvifolium* (huckleberry) with 3.23 ± 0.12% (Sodamade, et al., 2025), blueberry leaves appear to accumulate more minerals. These differences suggest variability in mineral uptake and storage among *Vaccinium* species, although all remain within a comparable range.

Standard deviation: 5.81 ± 0.60g/100g

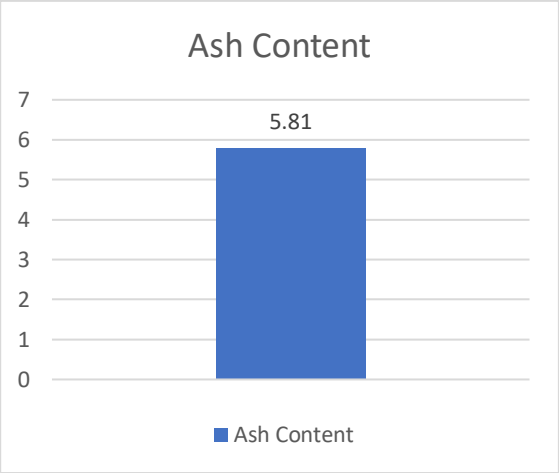


Figure 18: Ash content in blueberry leaves

1.3. Lipids

The primary goal of this step was to determine the lipid content in blueberry leaves. The findings from our study show that, on average, blueberry leaves contain about 0.455g/100g indicating a relatively low fat content.

When comparing these results to other blueberry species, such as *Vaccinium virgatum* Aiton, the differences are minimal but noteworthy. Research by (Yamasaki , *et al*., 2015) reported lipid values around 0.5g/100g. This indicates that different blueberry species tend to

have comparable lipid levels in their leaves, supporting the idea that leaves generally do not store much fat. These small differences could be due to variations in environmental conditions, genetic factors, or specific adaptations among species.

This low lipid content is typical because leaves mostly focus on performing vital functions such as capturing sunlight to produce energy, transporting nutrients within the plant, and supporting overall growth. They are not meant to store energy like seeds or fruits, which usually contain higher fat levels.

Standard deviation: 0.455 ± 0.007g/100g

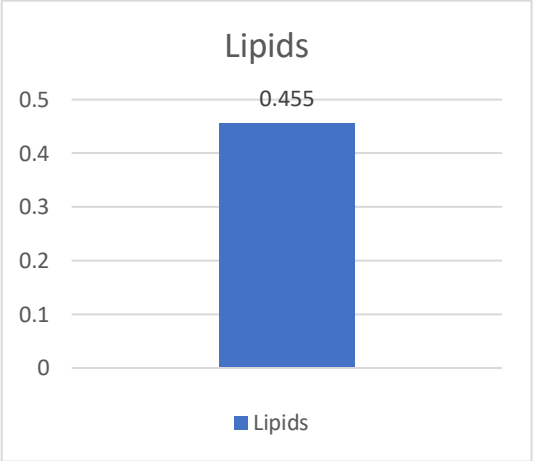


Figure 19: Lipids content in blueberry leaves

1.4. Sugars

The main goal of this step was to measure the amount of sugar present in a standard amount of blueberry leaves. The test results revealed that on average there are about 12.2g/100g.

The amount of sugar present in blueberry leaves in this study is quite significant when you compare it to other research in the field. In fact, it stands out as a noteworthy point of interest. A study conducted by (Akšić, et al., 2019) investigated the sugar content in *Vaccinium corymbosum* (blueberry) leaves, and the results showed an average of approximately 16.693g/100g of leaves. This figure provides a clear benchmark for what can be expected in terms of natural sugar levels in similar plant material

This data point is especially important because it hints at the potential for blueberries' leaves to serve as a natural source of sugar, perhaps for further processing or nutritional studies. Even if the sugar content seems high at first glance

The fact that our results slightly mirror the findings by (Akšić, et al., 2019) adds a layer of validation, indicating that these levels of sugar may be characteristic of blueberry leaves regardless of minor environmental differences.

Standard deviation: 13.2 ± 1.04g/100g

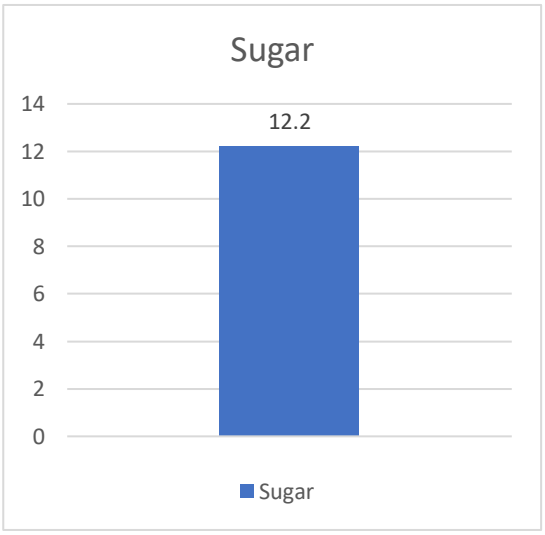


Figure 20: Sugar content in blueberry leaves

1.5. Fibers

To identify the amount fiber in blueberry leaves, a test was conducted, the purpose was to measure the fiber content accurately and gather reliable data. After completing the test, the results revealed that on average, blueberry leaves contain about 16.82g/100g.

These findings highlight that blueberry leaves are a valuable source of fiber. When compared with other plant leaves, they rank favorably. For example, the leaves of *Vaccinium parvifolium* known as red huckleberry, were reported to contain 5.93 ± 0.11 g/100 g of fiber as reported by (Sodamade, et al., 2025), a value significantly lower than that of blueberry leaves. This suggests that blueberry leaves can make a more substantial contribution to daily fiber intake.

Similarly, blackberry leaves (*Rubus fruticosus*) have been shown to contain 18.98 g/100 g of fiber according to findings reported by (Biel & Jaroszewska, 2017) a value comparable to that of blueberry leaves. These results confirm that both blueberry and blackberry leaves are rich in fiber and may serve as excellent ingredients in functional foods.

Standard deviation: 32.67 ± 0.52g/100g

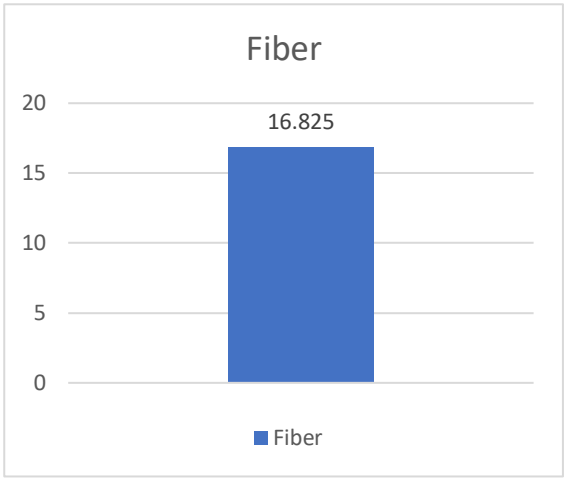


Figure 21: Crude fiber in blueberry leaves

1.6. Proteins

The results of this study showed that the protein content in blueberry leaves ranged from 7.12 to 7.80 g/100 g, with an average of 7.46 ± 0.48 g/100 g. This suggests that blueberry leaves have a moderate level of protein when compared to other types of edible or medicinal leaves. While not exceptionally high, blueberry leaves still offer a nutritionally relevant amount of protein. Many other studies on other *Vaccinium* species have found similar results. For instance, a study by (Saracila, *et al.*, 2023) reported that *Vaccinium myrtillus* (bilberry) leaves contain approximately 5.91 g/100 g of protein, In contrast, *Vaccinium parvifolium* (red huckleberry), was found to contain significantly more, with 15.71 ± 0.30 g/100 g, according to a study conducted by (Sodamade, et al., 2025). These comparisons show that while blueberry leaves are a decent source of protein, they are not among the highest within the *Vaccinium* genus.

Standard deviation: 7.46 ± 0.48g/100g

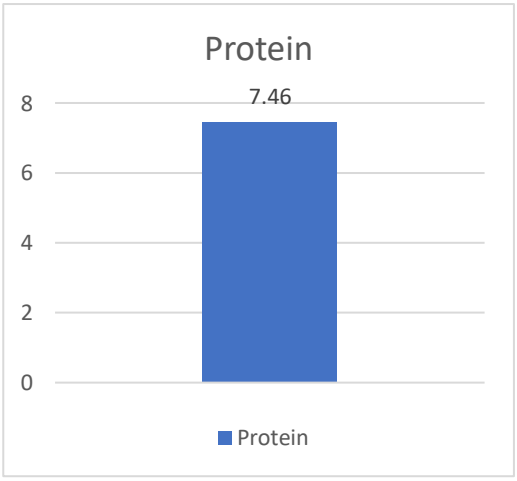


Figure 22: Protein content in blueberry leaves

2. HPLC Analysis

Table 6: Identified HPLC compounds and their Area%

Retention time	Compound	Type	Area%
19.734	Morin hydrate	Flavonoid	4.1745
23.044	p-Coumaric acid	Polyphenol	0.3286
28.009	Varinigene	Flavonoid	0.0766
31.084	Myricetin	Flavonoid	0.1539
36.222	Hesperetin	Flavonoid	0.1959
44.970	Galangin	Flavonoid	0.6544
46.917	Methoxyflavone	Flavonoid	0.5984

Table of standards will be found in appendix 1

Total sum of area% is 6.1823%

The purpose of this step was to reveal the compound in blueberry leaves *Vaccinium angustifolium*

The HPLC test conducted on the leaves uncovered a variety of important phenolic and flavonoid compounds. The specific substances identified include morin hydrate, p-coumaric acid, varinigene, myricetin, hesperetin, galangin, and methoxyflavone. Each of these compounds has unique structures and roles in plant health, but they all share common features related to their antioxidant, anti-inflammatory, and health-promoting effects.

Comparing these results with a similar study by (Zamljen Aljaz, 2023), which focused on bilberry leaves, also called *Vaccinium myrtillus*, reveals interesting similarities and differences. The bilberry leaves tested showed the presence of a different set of compounds, including Peonidin-3-O-galactoside, two luteolin derivatives, (+) catechin, p-coumaric acid hexoside, feruloylquinic acid derivative, and myricetin-3-O-galactoside. These compounds also have powerful antioxidant properties, and many are common to both plant types, like myricetin and p-coumaric acid. The luteolin derivatives and catechin are known for their anti-inflammatory effects and abilities to protect cells from oxidative damage.

The comparison suggests that different plants contain specific blends of these health-promoting compounds. While both sets show strong antioxidant potential, the unique combinations may influence their effects on health

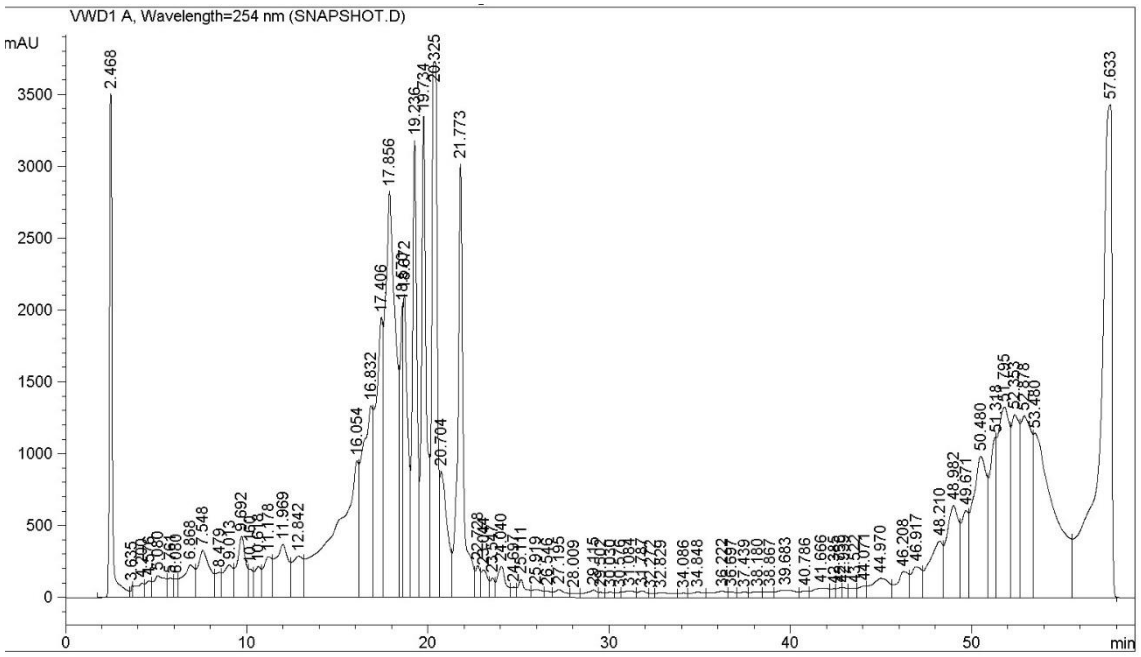


Figure 23: HPLC Chromatogram of Phenolic Compounds Detected in Blueberry Leaf Extract

3. Secondary metabolites

3.1.Polyphenols and flavonoids

Ethanolic extract: 94.213

Chloroformic extract: 173.836

Ethyl acetate extract: 1109.843

Butanolic extract: 505.403

Table 7: Polyphenol and Flavonoid content of each extract

Extract	Polyphenols (mg GAE/g)	Flavonoids (mg QE/g)
Ethanol	94.213	1036.845
Chloroform	173.836	161.964
Ethyl Acetate	1109.843	2452.738
Butanol	505.403	263.273

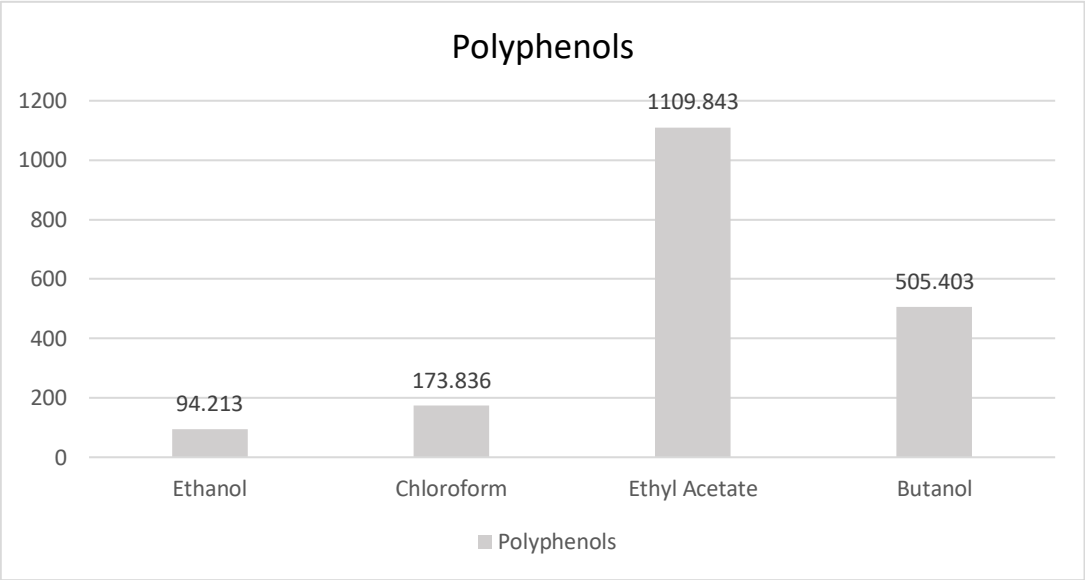


Figure 24: Chart showing each extract with its corresponding polyphenols

The total polyphenol content (TPC) of *Vaccinium angustifolium* leaf extracts, expressed in mg gallic acid equivalent per gram of extract (mg GAE/g), showed clear differences depending on the solvent used. The ethyl acetate extract had the highest TPC (1109.84 mg GAE/g), followed by butanol (505.40 mg GAE/g), chloroform (173.84 mg GAE/g), and ethanol (94.21 mg GAE/g). These values suggest that ethyl acetate is the most effective solvent for extracting polyphenolic compounds from the leaves, likely due to its moderate polarity, which favors the solubility of many phenolic compounds. Butanol also performed reasonably well, while chloroform and ethanol were less efficient, possibly due to their lower ability to solubilize certain polyphenols.

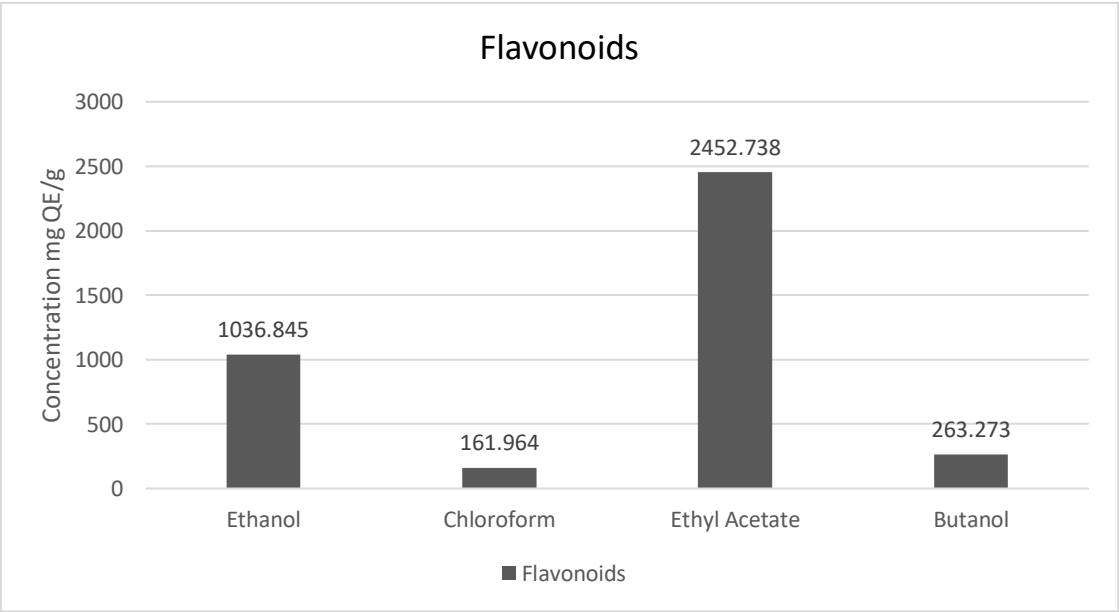


Figure 25: Chart showing each extract with its corresponding polyphenols

The total flavonoid content (TFC) varied significantly across the four solvent extracts of *Vaccinium angustifolium* leaves. Ethyl acetate showed the highest TFC (2452.738 mg QE/g), followed by ethanol (1036.845 mg QE/g), butanol (263.273mg QE/g), and chloroform (161.964 mg QE/g). These values are expressed in quercetin equivalents, meaning that 1 gram of the ethyl acetate extract has the same flavonoid-reactive capacity as over 2 grams of pure quercetin. This indicates a high concentration of flavonoid compounds or a strong interaction with the colorimetric reagent. The results highlight the importance of solvent polarity: ethyl acetate and ethanol, being polar or moderately polar, extracted more flavonoids than butanol or chloroform. This suggests high antioxidant potential and makes ethyl acetate an excellent solvent for extracting flavonoids from *Vaccinium angustifolium* leaves.

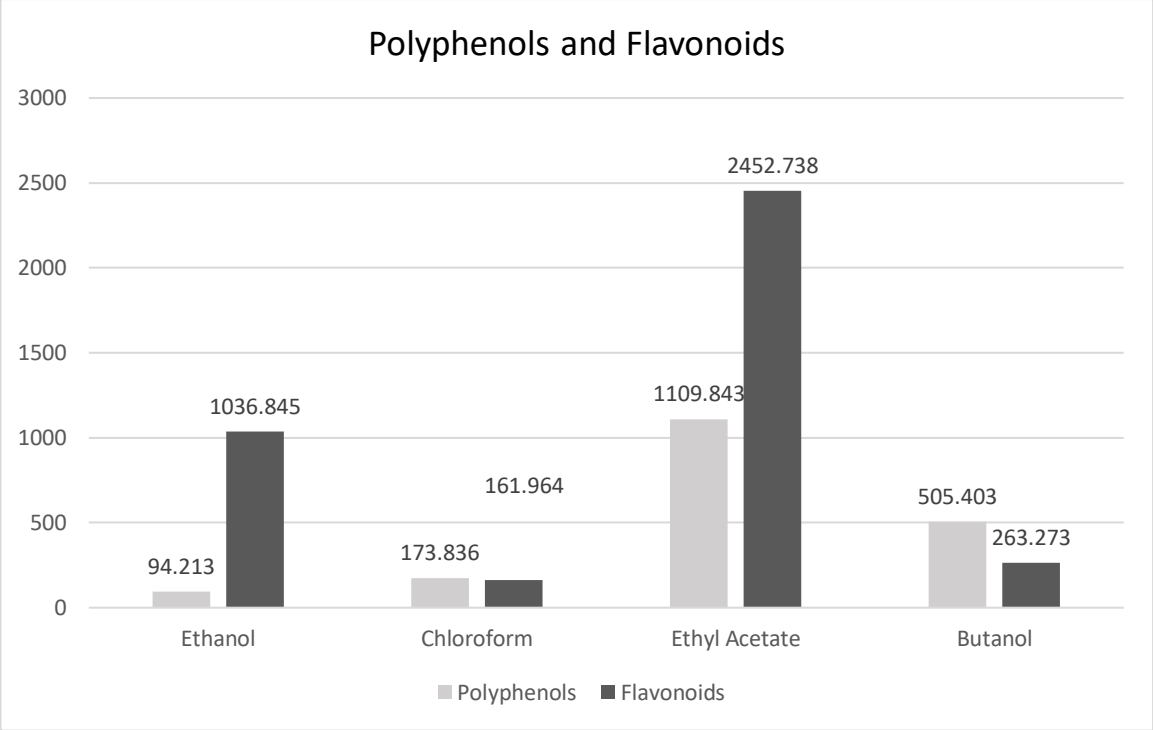


Figure 26: Comparison between polyphenols and flavonoids content in each extract

The results show that both polyphenol and flavonoid contents varied significantly depending on the solvent used. Ethyl acetate was the most effective extractant for both groups of compounds, yielding the highest values for polyphenols (1109.84 mg GAE/g) and flavonoids (2452.74 mg QE/g).

This confirms its strong ability to extract a wide range of phenolic compounds. Ethanol, while less effective for polyphenols (94.21 mg GAE/g), showed high flavonoid content (1036.85 mg QE/g), suggesting it favors certain flavonoid classes. Butanol extracted a moderate amount of both polyphenols (505.40 mg GAE/g) and flavonoids (263.27 mg QE/g), while chloroform was the least efficient for flavonoids (161.96 mg QE/g) and relatively low for polyphenols (173.84 mg GAE/g).

Overall, ethyl acetate stands out as the best solvent for extracting both total polyphenols and flavonoids from *Vaccinium angustifolium* leaves, followed by butanol and ethanol, with chloroform being the least effective.

3.2. Antioxidant activity (DPPH assay)

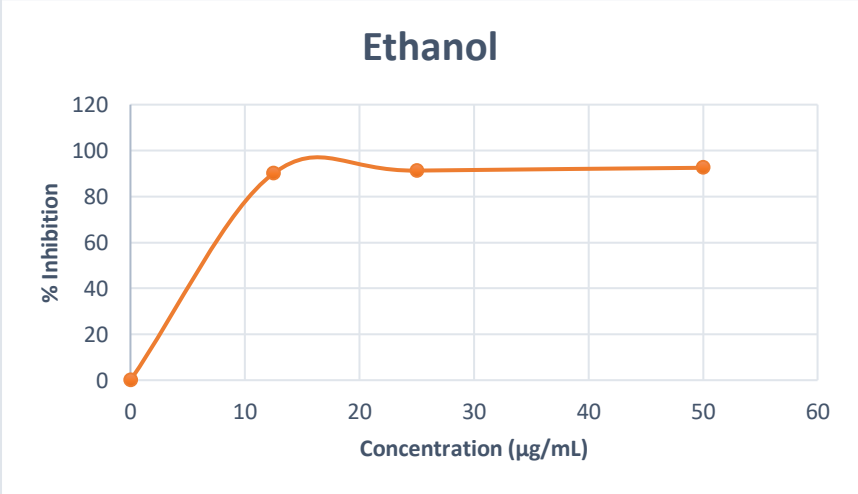


Figure 27: Ethanol curve (DPPH assay)

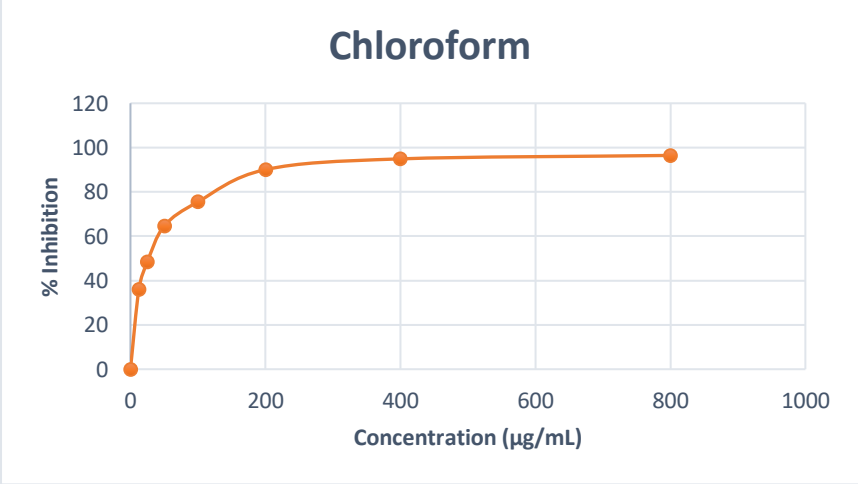


Figure 28: Chloroform curve (DPPH assay)

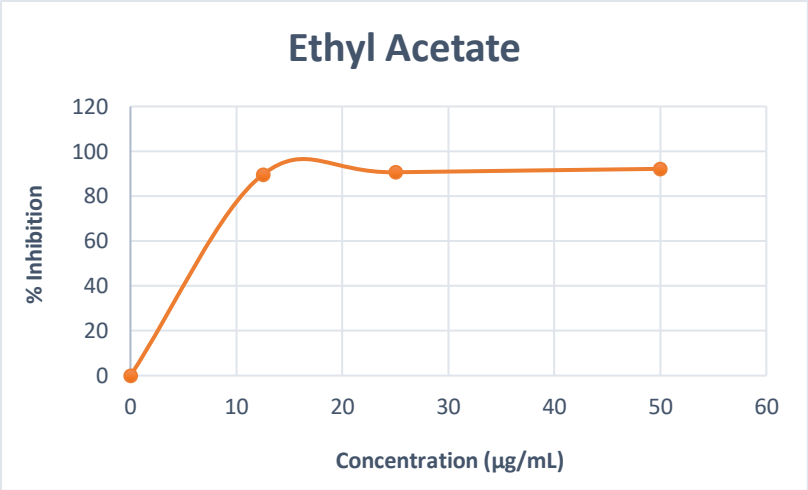


Figure 29: Ethyl acetate curve (DPPH assay)

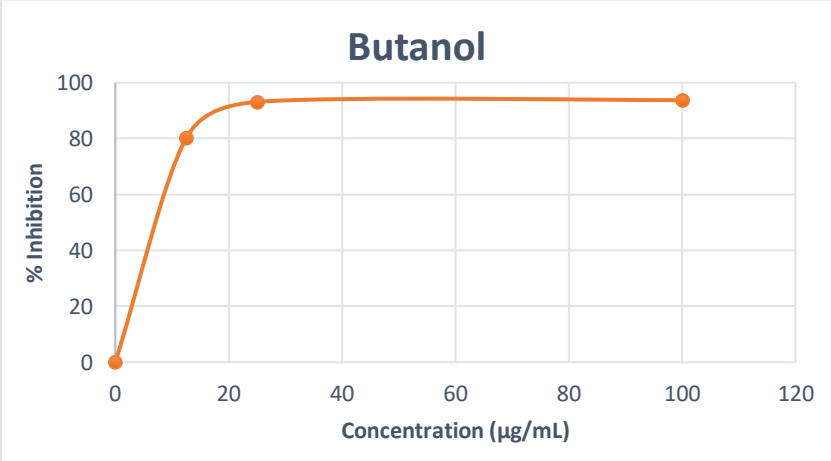


Figure 30: Butanol curve (DPPH assay)

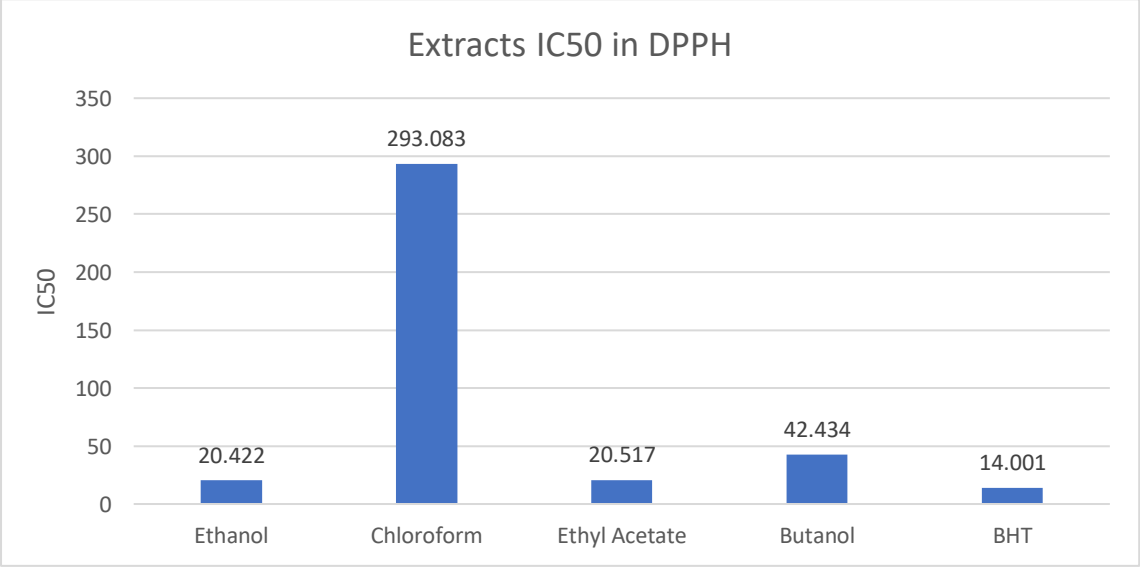


Figure 31: Comparison between the IC50 of extracts and BHT in DPPH assay

Among the natural extracts, both ethanol (IC_{50} = 20.42 µg/mL, PA = 0.04897) and ethyl acetate (IC_{50} = 20.52 µg/mL, AC = 0.04874) exhibited high antioxidant power, with AC (antioxidant capacity) values approaching that of BHT. This indicates that these extracts contain significant levels of active antioxidant compounds, likely polyphenols or flavonoids, which effectively scavenge free radicals. The butanol extract showed moderate antioxidant activity (IC_{50} = 42.43 µg/mL, AC = 0.02357), requiring approximately double the concentration to reach similar inhibition as the ethanol extract. In contrast, the chloroform extract had the lowest antioxidant power, with a much higher IC_{50} of 293.08 µg/mL and an AC of only 0.00341, indicating that it contains fewer or less effective antioxidant compounds.

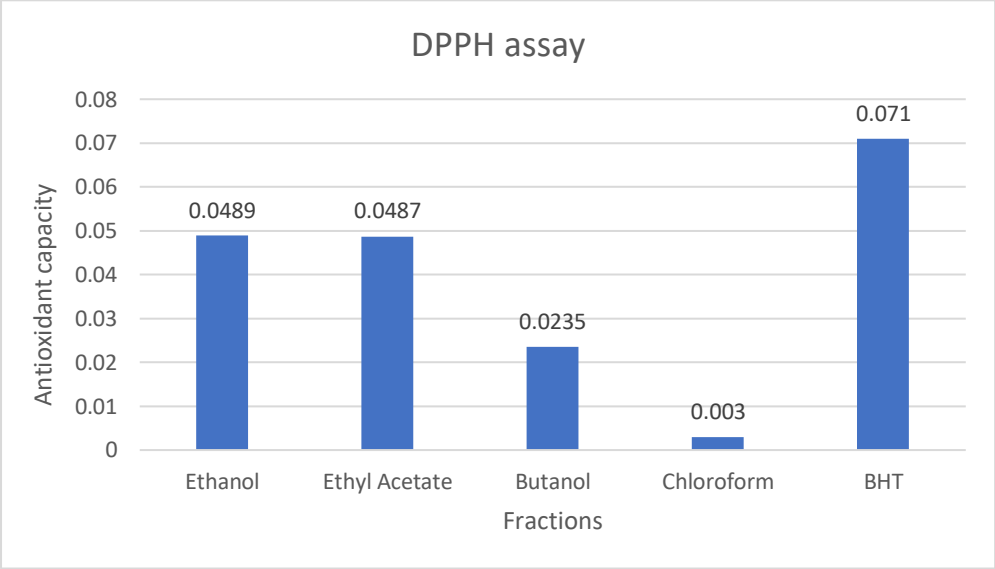


Figure 32: Antioxidant capacity of blueberry leaves fractions compared to BHT

3.3. Antioxidant activity (ABTS assay)

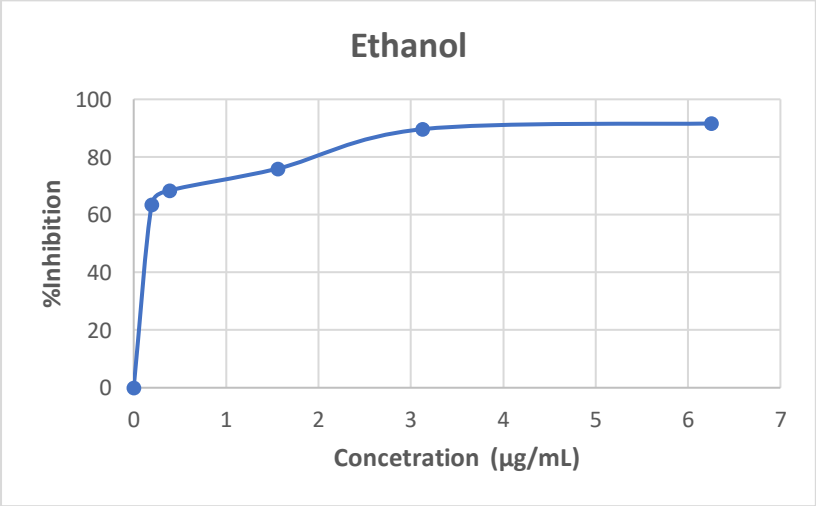


Figure 33: Ethanol curve (ABTS assay)

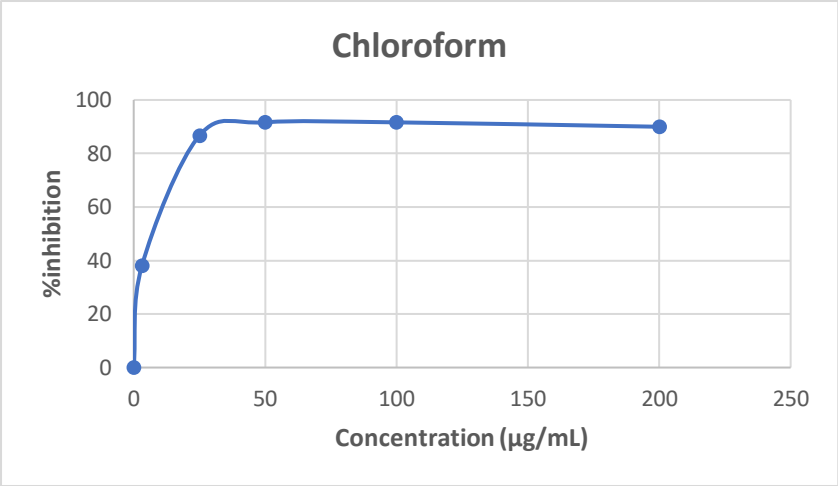


Figure 34: Chloroform curve (ABTS assay)

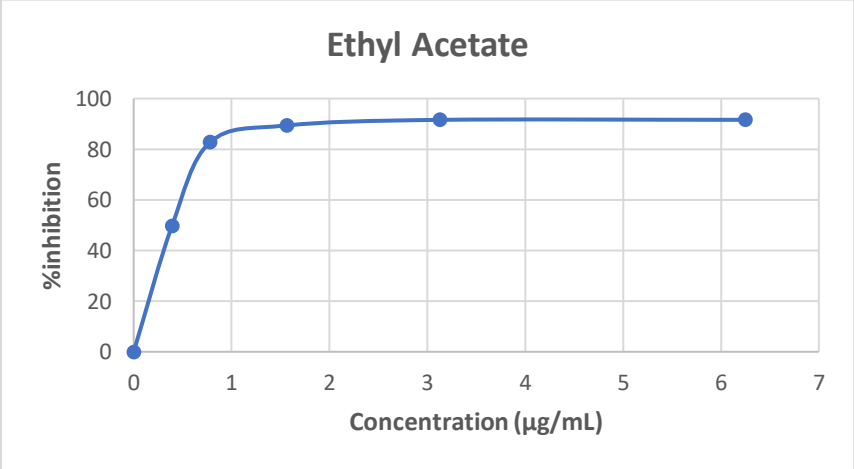


Figure 35: Ethyl acetate curve (ABTS assay)

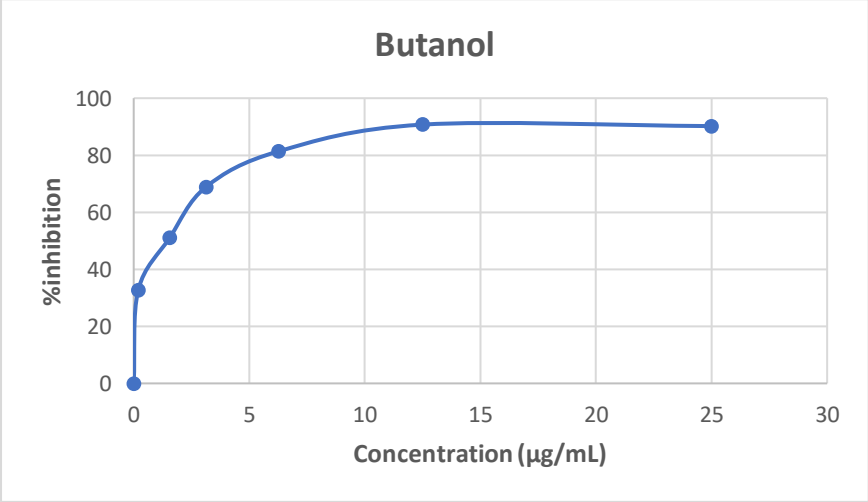


Figure 36: Butanol curve (ABTS assay)

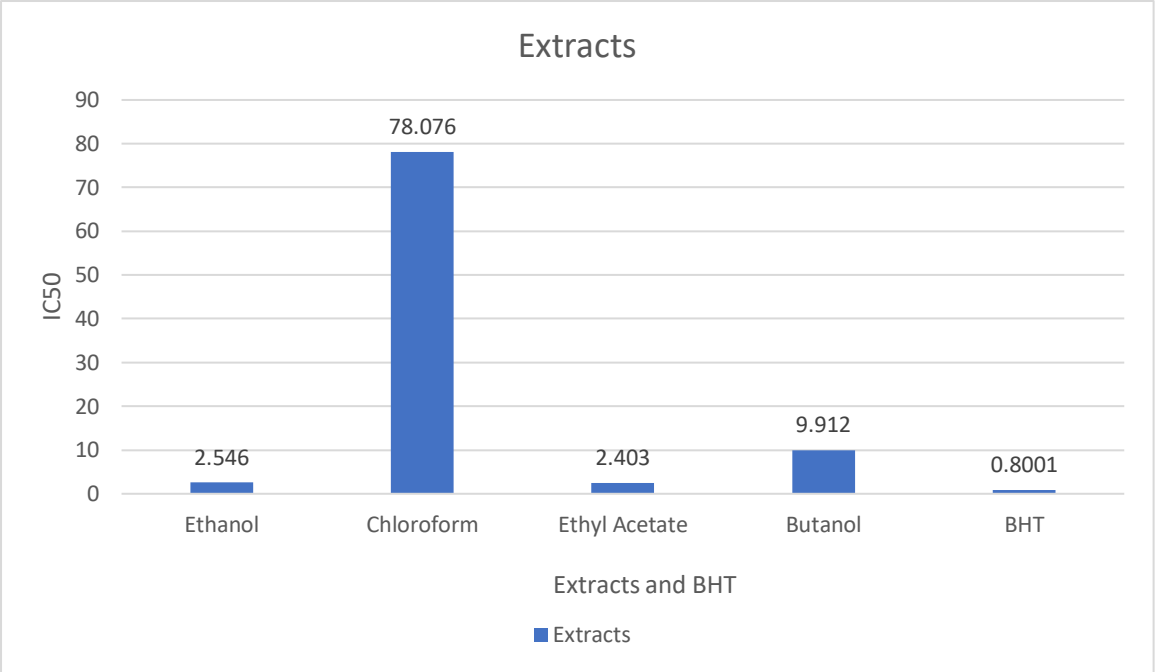


Figure 37: Comparison between the inhibition percentage of extracts and BHT in ABTS assay

Among the tested extracts, the ethyl acetate extract demonstrated the highest antioxidant activity, with an IC_{50} of 2.403 µg/mL and an antioxidant power of 0.4161. This result suggests that ethyl acetate was most efficient at neutralizing free radicals, likely due to its ability to extract medium-polarity compounds such as polyphenols and flavonoids.

The ethanol extract followed closely with an IC_{50} of 2.546 µg/mL, showing similar potency. Ethanol is known for extracting a wide range of antioxidant compounds, including both polar and moderately nonpolar phytochemicals, which likely contributed to its effectiveness.

The butanol extract demonstrated moderate antioxidant activity in the ABTS assay, with an IC_{50} value of 9.912 µg/mL and an antioxidant power ($1/IC_{50}$) of 0.1009. Its IC_{50} was significantly higher than those of the ethyl acetate and ethanol extracts, indicating a weaker overall radical scavenging efficiency

In contrast, the chloroform extract showed the lowest antioxidant activity, with a significantly higher IC_{50} value of 78.08 µg/mL and the lowest antioxidant power (0.01281). Chloroform is more efficient at extracting nonpolar compounds, which may not contribute significantly to ABTS radical scavenging.

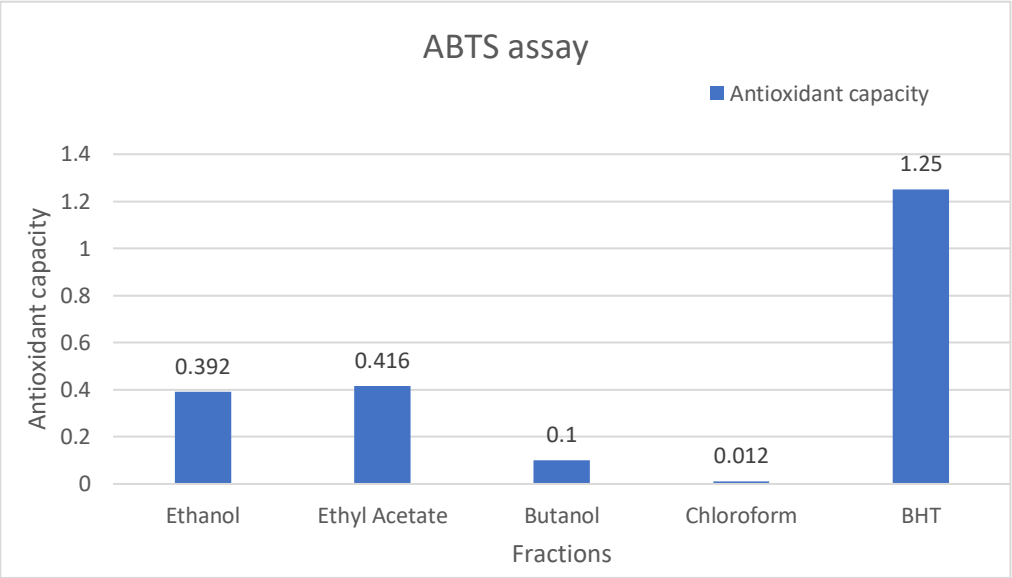


Figure 38: Antioxidant capacity of blueberry leaves fractions compared to BHT

DPPH and ABTS assays results

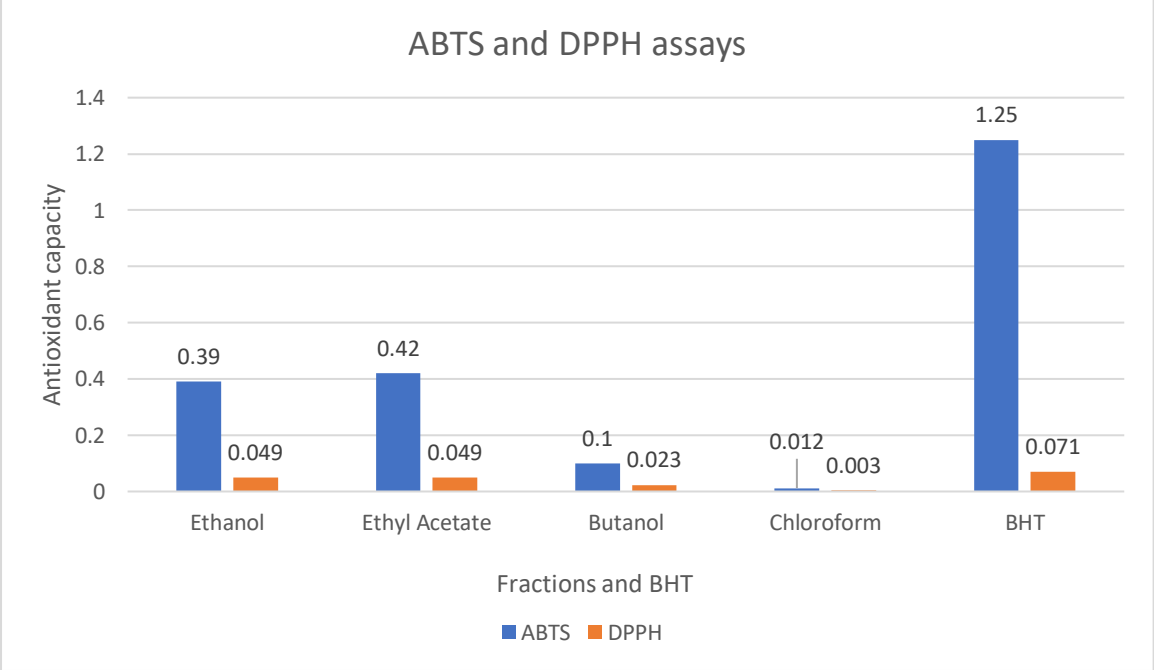


Figure 39: Comparison between fractions and BHT antioxidant capacity

The synthetic antioxidant BHT remains the most potent reference compound, with an IC_{50} of 14.00 µg/mL in DPPH and an even stronger value of 0.8001 µg/mL in ABTS, confirming its high efficiency in both radical scavenging systems. Compared to BHT:

Ethanol and ethyl acetate extracts showed excellent natural antioxidant capacity, with IC_{50} values only slightly higher than BHT's, especially in the ABTS assay where they reached 2.40–2.55 µg/mL.

The butanol and chloroform extracts demonstrated significantly lower activity, particularly in ABTS, with IC_{50} values near 78 µg/mL

These findings suggest that ethyl acetate and ethanol are the best solvents for extracting potent antioxidant compounds from your plant material. They closely mimic the performance of BHT and could be valuable in natural antioxidant formulations.

Conclusion

This study focused primarily on discovering and understanding both primary metabolites which are the vital for basic of life and secondary metabolites which often have special functions like defending the plant in blueberry leaves as well as their antioxidant activity

With that study we were able to successfully pinpoint primary metabolites such as sugars lipids proteins as well as secondary metabolites such as flavonoids and phenolic anthocyanins compounds secondary metabolites caught a particular attention for their ability to work as antioxidants these compounds help protect cells from damage caused by free radicals which are molecules that can harm DNA and other vital cell components by neutralizing these free radicals these metabolites could reduce oxidative stress

The strong antioxidant capacity observed in blueberries suggest that these natural compounds might be useful in preventing or slowing down the damage caused by oxidative stress

In summary this research underscores the importance of detailed metabolic profiling such profiling helps identify specific compounds with potential health benefits and it pushes forward the goal of discovering new and natural ways to protect health and fight disease using the power of nature's chemical diversity.

Perspectives

- Comparative study of leaves and fruits:

Future studies should explore a comparative analysis between blueberry leaves and fruits focusing on both primary and secondary metabolites comparing both plants could help determine their synergistic value

- Industrial and functional applications:

Given their richness in fiber and sugars blueberry leaves could be explored for use in functional foods

- Toxicological and regulatory assessment:

Before recommending blueberry leaves for widespread human consumption toxicity studies and regulatory approval processes should be considered to ensure safety.

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Appendix 1

Non-biological material:

Rotary evaporation

Vortex

Balance

Pipette

Eppendorfs

Pipette tips

Reagents:

aluminum nitrate

CH_3COOK

Na_2CO_3 sodium carbonate

Pump

separatory funnel

spectrophotometer

Standards	Retention time
3-Hydroxyflavone	47.130
Ascorbic acid	3.101
Galic acid	8.145
Caffeic acid	19.922
p-Coumaric acid	23.219
Salicylic acid	27.527
Acacetin	44.901

Apigenin	39.263
Apinin	28.276
3-tert-butyl-4-hydroxyanisole (BHA)	42.012
BHTL D. test butyl	55.016
Catechin hydrate	15.793
Curcumin	45.575
Dihydroxyflavone	35.688
Galangin	44.911
Gospine	29.815
Hesperetin	36.027
HP ChemSpool0169	34.940
Kaempferol	45.706
Methoxyflavone	47.014
Morin hydrate	19.737
Myricetin	31.254
Tangeritin	44.808 47.351
Varinigene	27.970

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UNIVERSITY SAAD DAHLEB – BLIDA 1

FACULTY OF SCIENCE OF NATURE AND LIFE

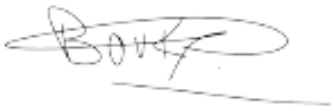
Department of Biotechnology

Specialization: Biotechnology and Plants Genomics

FINAL YEAR DISSERTATION

Topic

Characterization of Some Primary and Secondary Metabolites of Blueberry (*Vaccinium angustifolium*) and the Study of Its Antioxidant Activity



Presented by:

Mahdjoub Ismail

Ikhlef Ishak

Before the jury composed of

Ammara N.	Blida 1 University	MCA	President
Boukerma L.	Blida 1 University	MCB	Examiner
Kabir W.	Blida 1 University	MAA	Co-Supervisor
Belfarhi L.	CRAPC	MRB	Supervisor

ACADEMIC YEAR 2024/2025