DEMOCRATIC AND POPULAR REPUBLIC OF ALGERIA
Ministry of Higher Education and Scientific Research
UNIVERSITY AMAR TELIDJI LAGHOUAT
FACULTY OF SCIENCES
Department of Material Sciences

MASTER THESIS

Field: Material Sciences

Specialty: Chemistry

Option: Organic Chemistry

Prepared by**: ZIDELMEL Imene**

Title:

Optimization of the Extraction of Phenolic Compounds from *Globularia alypum L*

BOARD OF EXAMINERS

Mr. HAMDI Ahmed	*Pr*	***Chairman***
Mr. *BEN AHMED Ziyad*	*Pr*	***Examiner***
Mr. KORIBA Bakhti	*MCB*	***Supervisor***

Academic Year: 2024 -2025

Acknowledgements

First and foremost, I thank God Almighty for granting me the strength and patience to complete this work.

I would like to express my sincere gratitude to **Mr. KORIBA Bakhti**, Faculty of Science, Amar Telidji University of Laghouat, for agreeing to supervise this work. I am deeply thankful for his guidance, availability, and support throughout the course of this modest study. Please accept my deepest appreciation.

My gratitude also goes to **Mr. HAMDI Ahmed** for kindly agreeing to chair the defense jury of this thesis and I also thank **Mr. BEN AHMED Ziyad** for accepting to serve as jury members and to evaluate this work.

This research was carried out at the **Laboratory of Fundamental Sciences Research** at Amar Telidji University – Laghouat. I am grateful to its director, **Professor YOUSFI Mohammed**, as well as the PhD students in the lab, for their warm welcome and helpful collaboration.

- Finally, I would like to thank everyone who contributed, directly or indirectly, to the completion of this research work.

Dedication

I dedicate this work to:

First of all, to myself

for not giving up. For the long nights, the hard days, and the quiet determination that brought me here.

My dear father

For your strength, guidance, and constant support throughout my academic journey. Your belief in me has been a driving force behind my success.

My loving mother

For your unconditional love, endless encouragement, and unwavering faith in me. Your presence has been my greatest source of comfort and motivation.

To **my siblings and my entire family**

For always being there with love and encouragement.

To **my teachers**

For their guidance, patience, and the valuable knowledge you shared with me.

To **my classmates**

With whom I shared challenges, growth, and unforgettable moments along this journey.

To **all my friends**,

And to everyone who supported me in any way

This journey would not have been the same without you,

Thank you from the bottom of my heart

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List of Abbreviations

- **AAE**: Ascorbic Acid Equivalents
- **ABTS**: 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
- **Adjusted R2**: The adjusted coefficient of determination
- **BBD**: Box–Behnken Design
- **DPPH**: 1,1-Diphenyl-2-picrylhydrazyl
- **DW**: Dry Weight
- **GAE**: Gallic Acid Equivalents
- **$K_2S_2O_8$**: Potassium Persulfate
- **OFAT**: One Factor at a Time
- **PM**: Plant Material
- **R^2**: Coefficient of Determination
- **RSM**: Response Surface Methodology
- **TPC**: Total Phenolic Compounds
- **UAE**: Ultrasound-Assisted Extraction

General introduction

Introduction:

- The use of medicinal plants for the prevention and treatment of various common and chronic conditions has attracted growing interest, particularly due to their natural bioactivity and low toxicity ***(Hennebelle et al. 2004)***. Among these, *Globularia alypum L*. holds a prominent place in the traditional medicine of the Mediterranean region, where it is valued for its anti-inflammatory, antioxidant, and antidiabetic properties. Investigating its bioactive compounds, especially phenolic compounds, requires efficient extraction techniques to maximize both yield and quality ***(Khlifi et al. 2011)***.
- Extraction processes play a crucial role in the study of medicinal plants. Today, a wide range of techniques is available, from traditional to modern methods. Conventional approaches such as maceration or infusion involve prolonged contact between plant material and solvents, often requiring long extraction times. In contrast, more recent techniques, such as ultrasound extraction, offer greater efficiency. However, both conventional and modern solvent-based extraction methods often rely on elevated temperatures and extended extraction times, which may compromise the stability of thermosensitive compounds like phenolic compounds ***(Rasul 2018)***. These molecules play a key role in the biological activity of *Globularia alypum L*. and are predominantly concentrated in its leaves and flower parts.
- Another major limitation of traditional extraction approaches is that they typically vary only one factor at a time, without accounting for interactions between variables. This restricts the ability to accurately identify optimal extraction conditions. To address this issue, multivariate statistical tools such as Response Surface Methodology (RSM) are employed. RSM enables the simultaneous assessment of multiple parameters (e.g., temperature, time, solvent volume) and their interactions to optimize a specific response, such as total phenolic content or antioxidant activity ***(Ahmed et al. 2016)***.
- In this study, we aimed to optimize the ultrasound-assisted extraction conditions for phenolic compounds from *Globularia alypum L*. Using response surface methodology. The effects of different experimental parameters, including extraction temperature, duration, and solvent volume, were evaluated in order to maximize both the yield of phenolic compounds and the antioxidant activity of the resulting extract.

Literature research

II. **Literature research**

I.3. Introduction :

Optimization applied to the extraction of phenolic compounds from *Globularia alypum L* ,aims to improve process performance in order to maximize the yield of bioactive compounds. This concept is used to identify the most effective experimental conditions to achieve reliable, reproducible, and cost-effective results.

Traditionally, optimization was done using a one-factor-at-a-time (OFAT) approach. However, this method does not account for interactions between variables and often leads to suboptimal results and increased resource consumption.

In this study, ultrasound-assisted extraction (UAE) was optimized using a Box-Behnken Design (BBD) and Response Surface Methodology (RSM). Three key factors "extraction time, temperature, and solvent volume"were evaluated for their effects on total phenolic content and antioxidant activity (DPPH, ABTS).

his multivariate approach allowed for efficient optimization while reducing the number of experiments, providing a better understanding of the process and significantly improving extraction outcomes.

I.4 Phenolic compounds:

Phenolic compounds are secondary metabolites in plants, primarily known for their antioxidant properties. They are characterized by the presence of one or more phenol groups in their structure. These compounds are classified into various groups based on the composition of their carbon framework. Phenolic acids (C_6–C_1 or C_6–C_3), particularly flavonoids (C_6–C_3–C_6), are among the most common phenolic compounds found in plant-based foods, along with tannins. They are predominantly stored in plant vacuoles and are typically extracted using organic solvents. The composition of phenolic compounds in an extract depends on the plant's chemical makeup and the extraction method used ***(Kulbat et al. 2016)*.**

There are two pathways leading to aromatic compounds:

"the shikimic acid pathway" and "the polyketide pathway".

Shikimic Acid Pathway

- This is the primary biosynthetic route for the production of aromatic amino acids

- These amino acids serve as precursors for a wide range of phenolic compounds, including phenolic acids, flavonoids, tannins, and lignin ***(Wilson et al. 1998)***.

Polyketide Pathway

- This pathway involves the condensation of acetyl-CoA (acetyl coenzyme A) and malonyl-CoA (malonyl coenzyme A) units.
- It leads to the formation of compounds such as xanthones, stilbenes, and certain flavonoids.
- It plays a crucial role in secondary metabolism and the synthesis of specific aromatic polyketides ***(Wilson et al. 1998)***.

I.2.3. The main classes of phenolic compounds:

I.2.1.6. Phenolic acids and simple phenols :

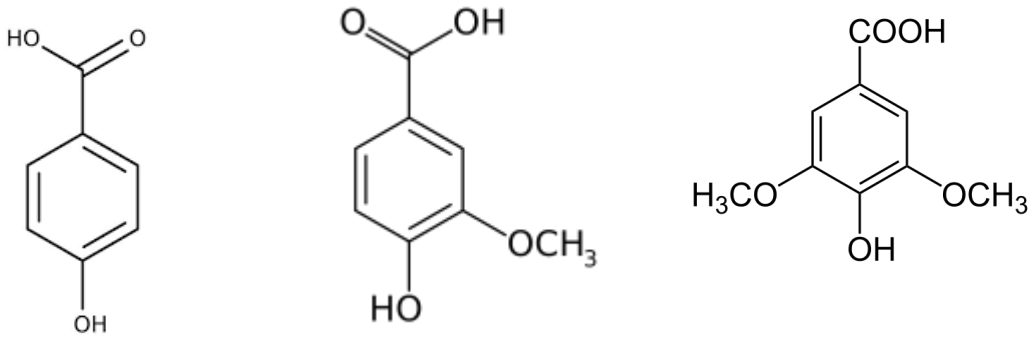
Phenolic acids are organic compounds that contain at least one carboxylic acid group and one phenolic hydroxyl group. Although they are less abundant than other phenolic compounds, they play significant roles in plant metabolism and human health. Phenolic acids are generally classified into two main groups ***(Natella et al. 1999)***:

- Benzoic acid derivatives (C6–C1): These include compounds such as gallic acid and protocatechuic acid, formed through mono- or polyhydroxylation of benzoic acid.
- Cinnamic acid derivatives (C6–C3): These arise from cinnamic acid and include p-coumaric acid, caffeic acid, and ferulic acid .

I.2.1.1.a Hydroxybenzoic acids :

Hydroxybenzoic acids are a subclass of phenolic acids characterized by one or more hydroxyl (-OH) groups attached to a benzoic acid core, which consists of a benzene ring substituted with a carboxylic acid group (-COOH). These compounds are widely distributed in the plant kingdom and are typically categorized based on the number of hydroxyl substitutions on the aromatic ring ***(Da Silva et al. 2023)***:

- **Monohydroxybenzoic acids** – e.g., *p*-hydroxybenzoic acid
- **Dihydroxybenzoic acids** – e.g., *vanillic acid*
- **Trihydroxybenzoic acids** – e.g., *syringic acid*



p-Hydroxybenzoic Acid **Vanillic acid** **Syringic acid**

Figure 01: Structures of certain benzoic acids ***(Da Silva et al. 2023)***

I.2.1.1.b Hydroxycinnamic Acids

Hydroxycinnamic acids are characterized by a three-carbon side chain (–CH=CH–COOH) attached to a benzene ring with one or more hydroxyl groups ***(Da Silva et al. 2023)***

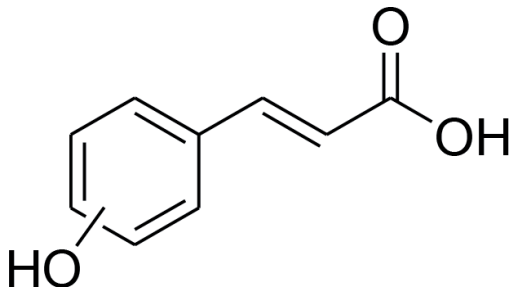


Figure 02. General structures of hydroxyl-substituted cinnamic acids. ***(Da Silva et al. 2023)***

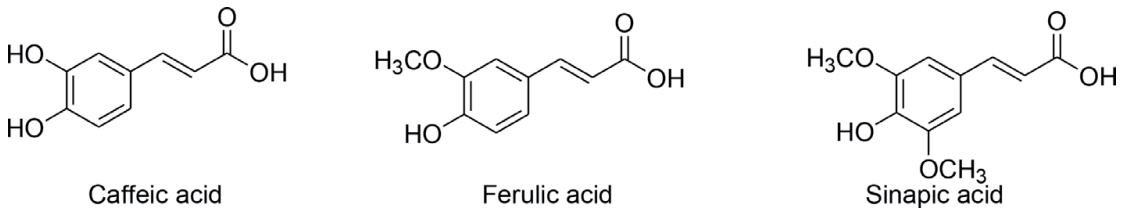


Figure 03. Common examples of hydroxycinnamic acids ***(Da Silva et al. 2023)***

I.2.1.7 Flavonoids :

Flavonoids are a large class of polyphenolic compounds characterized by a common structure consisting of two aromatic rings (designated as A and B) connected by a three-carbon bridge that typically forms a heterocyclic ring (C ring). Variations in the degree of hydroxylation, the pattern of substitution, and the position of attachment of the B ring lead to the classification of flavonoids into several subclasses, including **flavones**, **flavonols**, **flavanones**, and **anthocyanidins (*Panche et al. 2016)***.

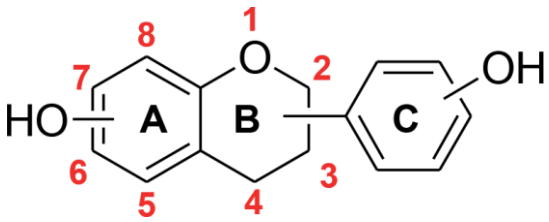


Figure 04. General structure of flavonoids **(*Panche et al. 2016)*.**

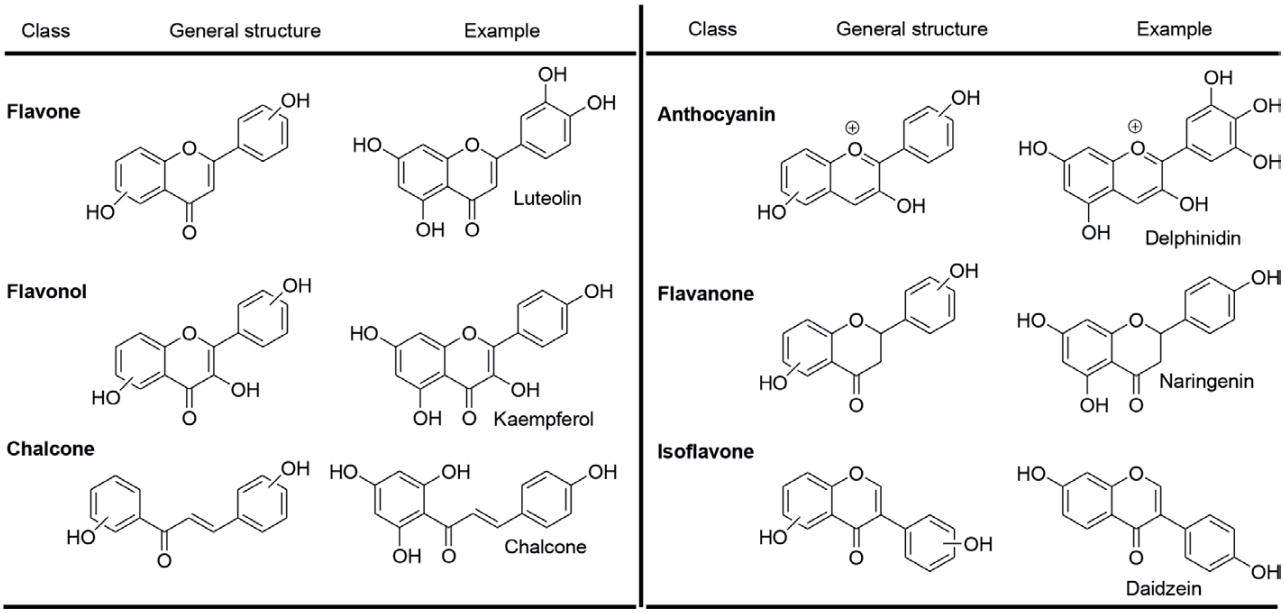


Figure 05. Classification of flavonoids **(*Panche et al. 2016)*.**

I.2.1.8 Stilbenes :

Stilbenes are a class of phenolic compounds consisting of two aromatic rings connected by a two-carbon ethylene bridge (–CH=CH–). Their general structure is based on the 1,2-diphenylethylene backbone, with various hydroxyl substitutions on the rings ***(Chong et al. 2009)***.

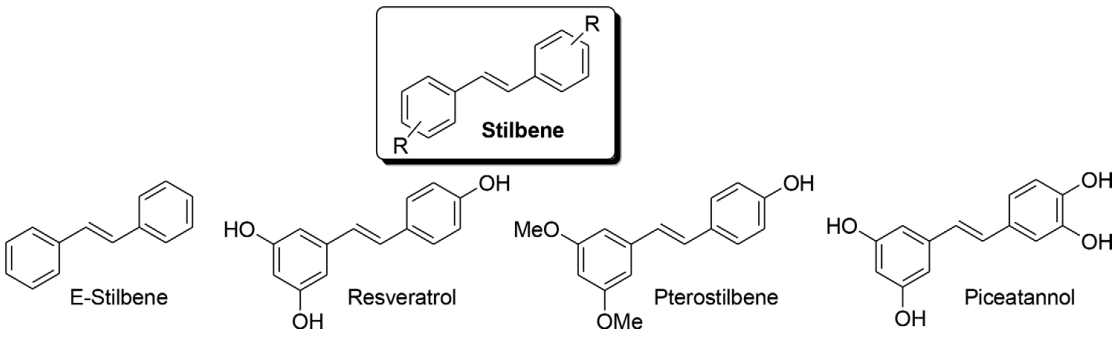


Figure 06. Examples of stilbenes ***(Chong et al. 2009)***.

I.2.1.9 Lignans

Lignans are a class of phenolic compounds composed of two phenylpropanoid (C6–C3) units linked by a central four-carbon bridge, forming a C6–C3–C3–C6 structure. They are commonly found in seeds, whole grains, and vegetables. For exemple matairesinol, secoisolariciresinol, and pinoresinol ***(Cassidy et al. 2000)***.

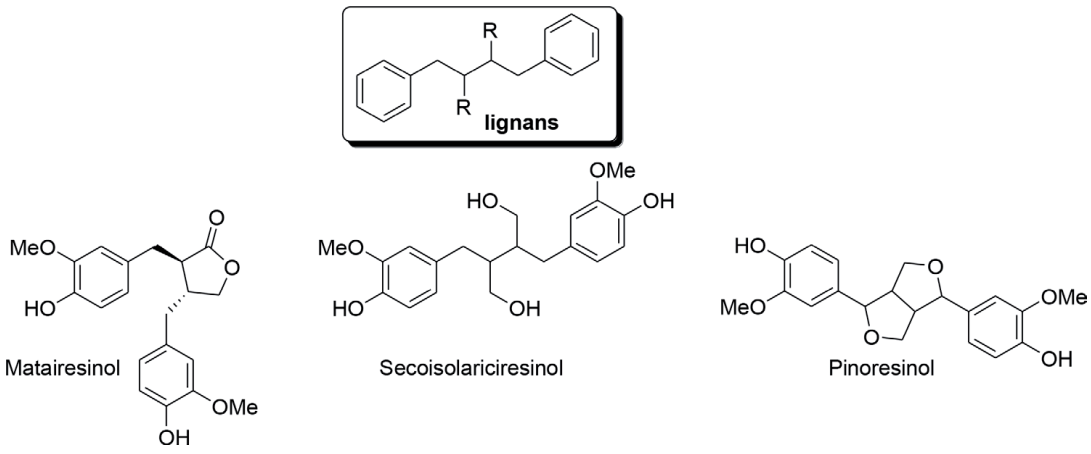


Figure 07 . General structure of lignans and examples ***(Cassidy et al. 2000)***.

I.2.1.10 Lignins :

Lignins are complex, high-molecular-weight polymers composed of phenolic units linked through various types of carbon–carbon and ether bonds. These phenolic units are primarily derived from three monolignols: p-coumaryl alcohol**,** coniferyl alcohol**,** and sinapyl alcohol ***(Cassidy et al. 2000)*.**

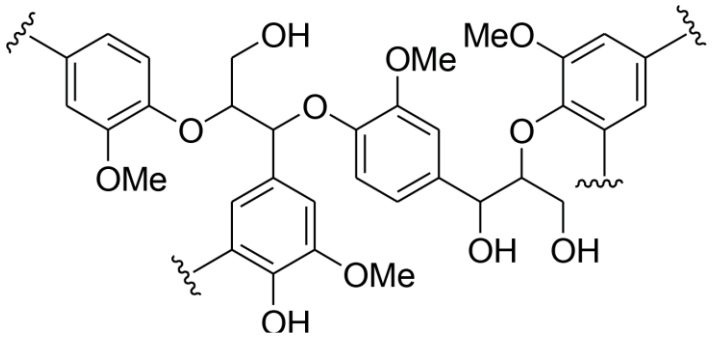


Figure 08. A segment of lignins ***(Cassidy et al. 2000)***

I.2.4 Extraction of phenolic compounds:

I.2.2.6 **Extraction definition:**

Extraction is a separation process used to isolate specific compounds from a complex mixture, such as plant material. In phytochemistry, it involves transferring bioactive compounds from solid plant matter into a liquid solvent ***(Handa et al. 2008)***.

I.2.2.7 **Organic Extraction Solvents:**

Organic solvents are commonly used in extraction due to their ability to dissolve a wide range of chemical compounds. Examples include:

- Methanol, ethanol, acetone, chloroform, and hexane these solvents are selected based on the polarity of the target compounds.

I.2.2.8 **Traditional Extraction Methods :**

a. Cold Maceration

- This method consists of soaking the powdered plant material in a solvent at room temperature for an extended period, typically 24 to 72 hours.
- It is particularly well-suited for the extraction of thermolabile (heat-sensitive) compounds.
- However, cold maceration is often associated with lower extraction efficiency and longer processing times ***(Luque-Rodríguez et al. 2006)*.**

b. Hot Maceration:

- In hot maceration, the plant material is immersed in a solvent and exposed to moderate heating, usually below the solvent's boiling point.
- The application of heat enhances solvent penetration**,** mass transfer**,** and compound solubility, leading to improved extraction yields.
- Nonetheless, the use of heat may result in the degradation of sensitive bioactive compounds ***(Luque-Rodríguez et al. 2006)***.

Hot Maceration Example : Soxhlet Extraction

- A continuous hot extraction method where the solvent is repeatedly evaporated and condensed over the plant material.
- It ensures efficient and exhaustive extraction, especially for compounds with low solubility.
- However, it requires long durations**,** large solvent volumes, and heat, which may degrade sensitive compounds ***(Luque-Rodríguez et al. 2006)***.

I.2.2.9 **Disadvantages of Traditional Extraction:**

- Long extraction times
- Large volume of solvent required
- Thermal degradation of sensitive compounds during hot processes
- Limited efficiency compared to modern techniques ***(Rasul 2018).***

I.2.2.10 **Limitations in Experimental Design of Traditional Extraction:**

Traditional extraction methods typically involve changing one variable at a time (OVAT), such as temperature or extraction time, while keeping all other conditions constant. This approach not only increases the number of required experiments but also fails to account for interactions between variables**,** which can significantly influence extraction efficiency ***(Jacyna et al. 2019)***.

To overcome these limitations, Response Surface Methodology (RSM) is employed.

I.5 Response Surface Methodology (RSM):

Response Surface Methodology (RSM) is a collection of **statistical and mathematical techniques** used for developing, improving, and optimizing processes. It is particularly effective when multiple input variables influence a response of interest, allowing researchers to evaluate **interactions between variables** and determine optimal conditions with **fewer experimental runs** than traditional methods ***(Bezerra et al. 2008)***.

I.3.1 Factor :

A factor is described as a group of treatments of the same kind that are mutually exclusive and logically related in the context of an experimental design. Stated differently, a factor is an independent variable whose various values utilized in experiments are called levels or modalities.
In general, factors can be divided into two groups:

- Quantitative factors include temperature, time, solvent concentration, pressure, and other measurable variables that are represented by continuous or discontinuous numerical values.
- Qualitative factors: These are variables that are not quantifiable or classified, such color, or material type ***(Alara et al. 2021)***.

I.3.2 Response

A response variable is the outcome of each trial of an experiment. It is always numerical and typically is measured only once for each observation. It represents the effect of changes in experimental factors and is the foundation for process analysis and improvement ***(Hibbert 2012)***.

I.3.3 Experimental space

The variation range of a factor, or factor domain, is the set of all possible values of a factor between the low and the high level. By convention, the low level is generally denoted by (–1) and the high level by (+1) ***(Hibbert 2012)***.

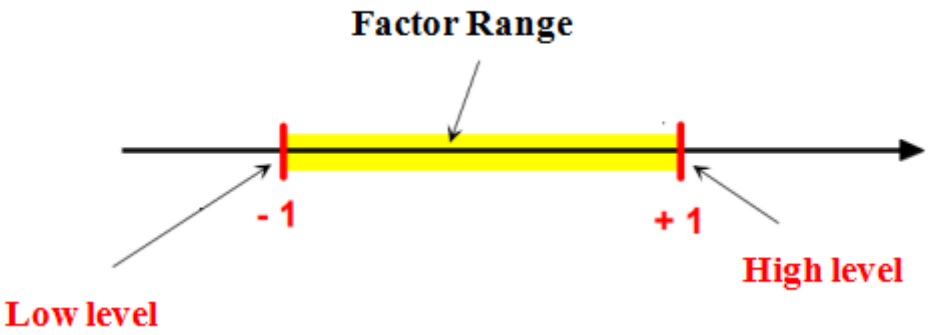


Figure 09. Graphical representation of a factor domain ***(Hibbert 2012)*** .

To simplify the representation of the experimental space, a two-dimensional plot was chosen, where every linked factor is represented by a scaled axis. In the case of two factors, the axes are placed at right angles to each other to form a Cartesian plane. Every point on the plane represents a different experiment, whereas the entire area represents the experimental space.

This concept can be easily extended to more dimensions ***(Hibbert 2012)***.

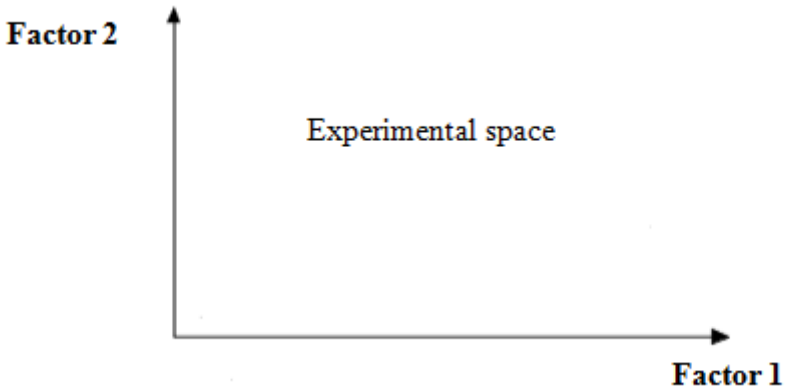


Figure 10. Study domain defined by the combination of the ranges of the different factors ***(Hibbert 2012)***.

I.3.4 Study Domain

The grouping of the factor domains defines the study domain, which is the area of the experimental space chosen by the experimenter for conducting tests. A study is a set of well-defined experiments, represented by points distributed throughout the study area ***(Hibbert 2012)***.

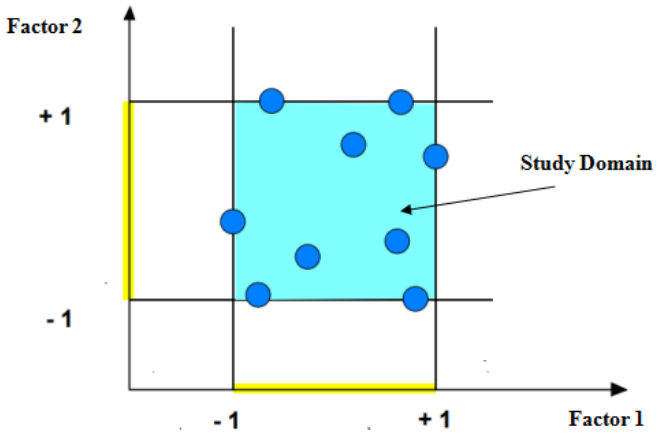


Figure 11. Schematic representation of a study domain ***(Hibbert 2012)***.

I.3.5 Modeling

Finding the mathematical and graphical shape (linear, curved, etc.) that most accurately depicts the impact of the chosen factors on the response variable is the task of this phase. The objective is to depict, to a certain degree of precision, how the phenomena evolves as the experimental parameters change.

Selecting experimental points is necessary for modeling a response; the number of points must at least match the entire number of effects, interactions, and quadratic factors taken into account. In order to estimate the model coefficient, a data matrix with n rows (the number of experiments) and k columns (the number of effects) is created ***(Maran et al. 2013)***.

I.3.6 Optimisation

Once the response has been formulated with a graphical and analytical representation, a search for that condition which stands as the best experimental set-up should be carried out next. Optimisation is very important if a maximization or minimization of the response is

desired and often requires full knowledge of the phenomenon being studied. An optimisation methodology will yield exact results if the model is valid and the response surface involved has been correctly interpreted ***(Maran et al. 2013)***.

I.3.7 Box–Behnken Design with Three Factors:

I.3.7.1 Definition of the Box–Behnken Design:

The Box–Behnken design is a class of symmetric experimental designs in which each factor is studied at three levels. It is constructed by combining two-level factorial designs with balanced incomplete block designs, arranged in a specific pattern. The structure includes several replicates at the center point of the experimental domain, which improves the estimation of experimental error and enhances the robustness of the model ***(Hibbert 2012).***

I.3.7.2 Description of the Design

So, the Box-Behnken design for three factors is constructed on a cube with 12 edges. The experimental points are not placed at the corners of the hypercube, but in the middle of the edges, in the center of the faces (squares), or in the center of the cube ***(Hohe et al. 2018)***.

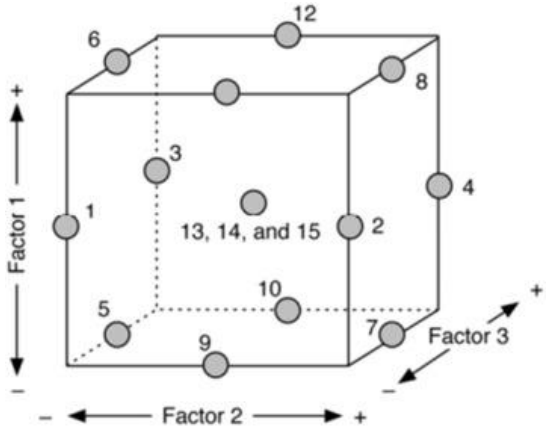


Figure 12. Illustration of the Box–Behnken design for three factors ***(Hohe et al. 2018).***

Therefore, the Box Behnken design for three factors has 15 trials ***(Myers et al. 2004)***:

$$N=2k(k-1)+C_0$$

Where:

- N = total number of experiments (trials)
- k = number of factors
- C_0 = number of center points (replicates at the center of the domain)

We have 3 factors k=3, and 3 central points C=3

That means: N=2×3× (3−1) +3=2×3×2+3=12+3=15 runs

Table 01: The experimental matrix of the Box-Behnken design for 3 factors

(Myers et al. 2004)

Trial No	Factor 1	Factor 2	Factor 3
1	**0**	**0**	**0**
2	**-1**	**0**	**1**
3	**1**	**0**	**-1**
4	**0**	**-1**	**-1**
5	**-1**	**-1**	**0**
6	**1**	**-1**	**0**
7	**0**	**1**	**-1**
8	**0**	**0**	**0**
9	**0**	**1**	**1**
10	**1**	**0**	**0**
11	**-1**	**1**	**0**
12	**0**	**-1**	**1**
13	**-1**	**0**	**-1**
14	**0**	**1**	**1**
15	**0**	**0**	**0**

I.3.7.3 Postulated Mathematical Model

In chemical research, Response Surface Methodology (RSM) serves as a valuable tool for modeling and optimizing processes influenced by multiple variables. It employs statistically designed experiments to evaluate how independent input factors affect a measurable response, such as yield, concentration, or activity. A mathematical function is typically defined *a priori* to represent this relationship, allowing the response to be predicted within the studied domain. form of the model is given as follows ***(Dean et al. 1999)***:

$$y = f(X_1,\ X_2,\ X_3 \dots\dots\dots\dots X_k) \qquad \textbf{(Eq.(01))}$$

In this context, the response function f describes how the output depends on a set of independent random variables xi. Assuming a linear relationship between the response and the influencing factors, the model can be formulated as follows:

$$y = a_0 + \sum_{i=1}^{n} a_i X_i + \sum_{j>i} \sum_{i=1}^{n-1} a_{ij} X_i X_j \qquad \textbf{(Eq.(02))}$$

There is the use of other models, for example a second order polynomial ***(Myers et al. 2004).***

$$y = a_0 + \sum_{i=1}^{n} a_i X_i + \sum_{j>i} \sum_{i=1}^{n-1} a_{ij} X_i X_j + \sum_{i=1}^{n} a_{ii} X_i^2 \quad \textbf{(Eq.(03))}$$

where

- y is the response variable, representing the quantity of interest measured during the experiment. It is obtained with a specified degree of precision
- X_i represents the level of factor i assigned by the experimenter. This level is assumed to be known exactly under the classical assumption of no error in the independent variables.
- a_0, a_i, a_{ij}, and a_{ii} are the coefficients of the mathematical model chosen a priori. These coefficients are not known in advance and are estimated from the experimental data.

The advantage of modeling the response with a polynomial is that it allows for the prediction of responses across the entire experimental domain without performing additional experiments. This model is referred to as a "postulated model" or an "a priori model." ***(Vander Heyden et al. 1996)***

I.3.7.4 The Experimenter's Model

Two important refinements must be added to the purely mathematical model:

- Lack of fit: This refers to the difference between the model chosen a priori by the experimenter and the true model that governs the studied phenomenon. There is often a discrepancy between the assumed model and the actual behavior of the system ***(Vander Heyden et al. 2001)***.
- Random error (or pure error): This accounts for the natural variability of the response. When measuring the same response multiple times at the same experimental point, the results will vary slightly. This variability is attributed to experimental (random) error ***(Sridevi 2013)***.

Therefore, the general model (Equation 01) must be revised to include these two components, as follows:

$$y = f(X_1, X_2, X_3 \ldots\ldots\ldots\ldots X_k) + \Delta + \varepsilon \quad \textbf{(Eq.(04))}$$

Where

Δ**:** lack of fit

ε**:** Pure Error**.**

Materials and methods

III. Materials and methods

II.2. Plant studied: *Globularia alypum L*

II.1.1. Botanical description of *Globularia alypum L (Quezel et al. 1963)*

- **Habit:** Small evergreen shrub, approximately 30–60 cm tall.
- **Stems:** Woody at the base, branched, and covered with soft hairs.
- **Leaves:** Oblong to linear, leathery, dark green, with smooth margins.
- **Flowers:**
  - Violet-blue, grouped in round *(globular*) heads.
  - Appear at the ends of stems.
  - Bloom mainly in spring.
- **Fruit:** Small, dry nutlet.

II.1.2. Habitat:

- Grows in dry, rocky, and sunny areas in the Mediterranean region***(Quezel et al. 1963)***

.

II.1.3. Common names *(Chograni et al. 2013)*:

- **English name:** Alypon, Globe Daisy.
- **French name:** Globulaire, Alypon.
- **Botanical name:** *Globularia alypum L.*
- **Arabic name:** Commonly called "Tasselgha," "Zerga," "Zeriga."

Figure 13. Morphological features of *Globularia alypum L* as observed in its natural habitat ***(SELLAOUI Hadjer 2019)***

II.1.4. The botanical classification of: *Globularia alypum L*:

Table 02. Classification of the species *Globularia alypum* L ***(Boussoualim 2018)***

• **Kingdom: Plantae** • **Subkingdom: Tracheobionta** • **Superdivision: Spermatophyta** • **Division: Magnoliophyta (Angiosperms)** • **Class: Magnoliopsida (Dicotyledons)** • **Subclass: Asteridae** • **Order: Lamiales** • **Family: Plantaginaceae (previously Globulariaceae)** • **Genus: Globularia** • **Species: *Globularia alypum* L.**	

II.1.5. Geographical Distribution :

This plant is native to southern Europe, particularly around the Mediterranean basin, extending to Greece, North Africa (including Algeria and Morocco, reaching into the Sahara), and parts of the Middle East (such as Egypt and the Arabian Peninsula). It typically grows in forests and rocky terrains and is known for its heliophilic (sun-loving) and thermophilic (heat-loving) nature ***(AMEUR 2006)***.

II.1.6. Sample preparation :

The leaves of *Globularia alypum L* w*as* used in this study. The preparation process involved the following key steps:

- **Harvesting:** The aerial parts of *Globularia alypum* were harvested in the Djebel Amour region (Wilaya Aflou), during the month of October 2024

- **Drying:** The plant material was air-dried in the shade at a cool, controlled ambient temperature to preserve heat-sensitive compounds.
- **Grinding and Sieving:** The floral parts were manually separated from the stems, then ground into a fine powder using a mortar and pestle. The resulting powder was sieved to obtain a uniform particle size, ensuring consistency during subsequent extraction and analysis.

The following figure shows the ground and powdered flowers and leaves of *Globularia alypum* L:

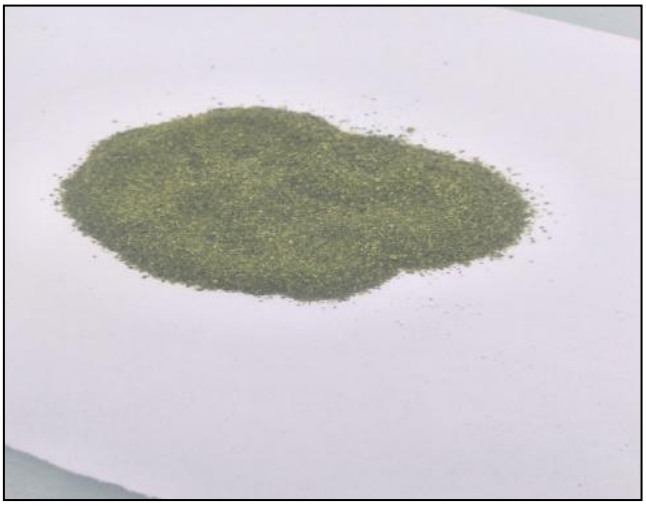


Figure 14. Ground leaves of *Globularia alypum* L. in powdered form.

II.2. Optimization of CPT extraction parameters by ultrasound:

The extraction of phenolic compounds is influenced by several parameters, such as extraction method, choice of solvent, solvent volume, particle size, extraction time, acidity, temperature,.....

II.2.1. Preliminary Studies :

The objective of the preliminary study on extraction conditions was to determine the optimal value of each parameter within a defined range in order to maximize the yield of total phenolic compounds. This was accomplished by varying one of the three studied parameters at a time while keeping the other two constant. Each experimental condition was repeated three times to ensure reproducibility and reliability of the results.

II.2.1.1. Extraction Time:

An amount of (0,5) g of *Globularia alypum* L leaves powder was mixed with 10 ml of pure methanol and placed in ultrasonic extraction for a extraction period ranging from 5 to 60 minutes. The resulting extract was filtered.

II.2.1.2. Extraction Temperature :

0.5 g of *Globularia alypum L* leaves powder was combined with 10 ml of pure methanol and placed in an ultrasonic device for an extraction temperature ranging from 30 to 60°C for 20 minutes. The resulting extract was then filtered.

II.2.1.3. Solvent Volume:

The extraction was performed in several series of methanol volumes from 5 to 55 ml. While the time and mass are fixed at 20 min and 0.5 g, respectively. The extract was finally filtered.

II.2.2. Total Polyphenol Determination:

To quantify the total polyphenol content in *Globularia alypum* L. extracts, a UV-Visible spectrophotometric method using the **Folin–Ciocalteu reagent (FCR)** was employed. This colorimetric assay is based on the reduction of the Folin–Ciocalteu reagent by phenolic compounds, which leads to the formation of a blue complex measurable at a specific wavelength (760 nm). The intensity of the blue coloration is proportional to the concentration of hydroxyl groups in the sample, reflecting the total phenolic content (TPC) ***(Yang et al. 2010)***.

II.2.2.1. Experimental Procedure :

A volume of 100 µL of extract was mixed with 500 µL of Folin–Ciocalteu reagent. After two minutes of reaction, 2000 µL of 2% sodium carbonate (Na_2CO_3) solution was added. The mixture was incubated at 25°C for 30 minutes, and the absorbance was measured at 760 nm using a blank solution as the reference ***(Yang et al. 2010)***.

II.2.2.2. Results Presentation :

The total phenolic content was determined by reference to a linear calibration curve established using gallic acid at various concentrations. Results were expressed as milligrams of gallic acid equivalents per gram of dry weight of plant material (mg GAE/g DW).

II.2.3. Antiradical activity (DPPH assay) :

DPPH (2,2-diphenyl-1-picrylhydrazyl) is a stable free radical, distinguished by its intense violet color and high solubility in methanol. It was one of the first free radicals employed to study the structure–activity relationships of phenolic antioxidants. The reduction of the DPPH radical by antioxidant compounds can be quantitatively evaluated using UV-Visible spectrophotometry by monitoring the decrease in absorbance at **517 nm**. In the presence of antioxidant compounds, the violet-colored DPPH radical is reduced to the yellow-colored **2,2-diphenyl-1-picrylhydrazine**, indicating antioxidant activity ***(Marinova et al. 2011)***.

II.2.3.1. Experimental Procedure :

The experimental protocol involved mixing 1 mL of a methanolic DPPH solution (250 µM) with 1 mL of the test solution , prepared by combining 80 µL of plant extract with 920 µL of methanol. The mixture was stirred and incubated for 30 minutes in the dark at room temperature. The decrease in absorbance was measured at **517 nm** using UV-visible spectrophotometry. The antioxidant capacity was expressed in **milligrams of ascorbic acid equivalents per gram of dry matter (AAEAC)**. Each experiment was performed in triplicate to calculate the average. Ascorbic Acid Equivalent Antioxidant Capacity (AAEAC) was determined using the following equation ***(Bouafia et al. 2021)***:

$$\mathbf{AAEAC} = \frac{(\mathbf{Abs_C} - \mathbf{Abs_E})}{\mathbf{A}} \mathbf{\ x\ D\ x\ } \frac{\mathbf{V}}{\mathbf{1000}} \mathbf{\ x\ } \frac{\mathbf{1}}{\mathbf{Q_E}} \qquad \textbf{(Eq.(05))}$$

Where:

Abs $_C$: Absorbance of the control (without the extract)

Abs $_E$: Absorbance in the presence of the extract (residual DPPH after reaction with the extract)

A: Slope of the ascorbic acid (vitamin C) calibration curve

D: Dilution factor of the extract

V: Total volume of the extract (mL)

Q $_E$: Mass of sample used in grams (0.5 g)

II.2.4. ABTS Test:

The test is based on the ability of the extracts to stabilize the cationic radical $ABTS^{\cdot+}$, which has a green-blue color and can be converted into a colorless form. The radical [2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)] or $ABTS^{\cdot+}$ is generated in the presence of potassium persulfate, and the reaction is initiated by the addition of potassium persulfate solution ($K_2S_2O_8$). The decrease in green-blue color can be measured spectrophotometrically at a wavelength of 734 nm ***(Chan et al. 2016)***.

II.2.4.1. Experimental Protocol

The ABTS assay was conducted to assess the antioxidant activity of plant extracts. A phosphate-buffered saline (PBS) solution was prepared by dissolving 0.4476 g of Na_2HPO_4 (5 mM), 0.195 g of Na_2HPO_4 (5 mM), and 2.25 g of NaCl (153.84 mM) in 250 mL of distilled water. This buffer was used to dilute the $ABTS^{+\bullet}$ working solution. The ABTS stock solution (20 mM) was prepared by dissolving 0.1097 g of ABTS in 10 mL of distilled water, and the potassium persulfate solution (70 mM) was prepared by dissolving 0.1892 g of $K_2S_2O_8$ in 10 mL of distilled water. To generate the $ABTS^{+\bullet}$ radical cation, 100 µL of the potassium persulfate solution was added to 10 mL of the ABTS solution, and the mixture was incubated in the dark at room temperature for 24 hours. After incubation, 1 mL of the resulting $ABTS^{+\bullet}$ radical solution was diluted to 25 mL using the previously prepared PBS buffer to obtain the working solution used for antioxidant testing.

To perform the antioxidant activity measurement, 10 µL of the plant extract was diluted in 450 µL of methanol**,** resulting in a final volume of 500 µL. From this solution, 100 µL was taken and mixed with 900 µL of the $ABTS^{+\bullet}$ working solution. The mixture was incubated in the dark for 15 minutes at room temperature, after which the absorbance was measured at 734 nm using a UV-Visible spectrophotometer ***(Chan et al. 2016)***. The calculation of AAEAC was performed using equation **(Eq.(05))**.

II.2.5. Experimental Design Matrix

The study used a Box–Behnken design for its experiments, which looked at three factors: extraction time (X_1), solvent volume (X_2), and temperature (X_3), each tested at three different levels (−1, 0, and 1). This design generated a total of 45 experimental runs, including 15 factorial points and 3 replicates at the center to estimate the pure error.

Based on the experimental data, a second-order polynomial model derived from the Box–Behnken design was employed to establish the relationship between the independent variables and the response and to predict the optimal extraction conditions. The general form of the quadratic model is expressed as follows ***(Ragonese et al. 2000)***:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 \quad \textbf{(eq.(06))}$$

Y: represents the predicted response

b_0: is the intercept (theoretical mean value of the response)

b_1, b_2, b_3 : are the coefficients of the linear terms

b_{11}, b_{22}, b_{33} : are the coefficients of the quadratic (squared) terms

b_{12}, b_{13}, b_{23} : are the coefficients of the interaction terms

II.2.6. Statistical Analysis

Statistical analysis of the experimental results of the single-factor preliminary study was performed using Excel 2016 software. All data were obtained from three replicates to calculate the mean. Design-Expert software (Version 13) was used to estimate the coefficients of the polynomials corresponding to each response.

An analysis of variance (ANOVA) was used to assess the significance of the model coefficients, with a significance threshold set at $p < 0.05$. The p-value was used to determine the significance of each regression coefficient. Model adequacy was assessed by examining the absence of lack of fit.

Results and discussion

III. Results and discussion:

III.1 Ultrasonic Extraction of Phenolic Compounds

III.1.1 Ultrasonic Extraction

Before proceeding with extraction using this method, it is essential to identify the initial conditions. This preliminary step allows for the assessment of the optimal conditions required for successful extraction. Understanding the initial parameters allows for the optimization of the extraction process and the achievement of high-quality results.

IV.1.2. **Determination of the experimental ranges for the relevant factors:**

Before applying the RSM, preliminary tests were conducted to define the appropriate experimental ranges for the key factors influencing phenolic extraction yield—namely, extraction temperature, liquid-to-solid ratio, and extraction time. Although particle size is another potentially relevant variable, we opted to use the smallest particle size available (0.60–0.90 mm) to avoid complications during the filtration stage of the extraction process. The total phenolic content (TPC) was quantified using the Folin-Ciocalteu method and expressed in gallic acid equivalents (GAE). However, it is important to note that this method does not provide a comprehensive or specific analysis of phenolic compounds. The Folin-Ciocalteu reagent reacts not only with simple phenols but also with other reducing substances such as sugars, leading to possible overestimation. Despite its lack of specificity, the method remains widely used for estimating the total phenolic content in plant extracts.

IV.1.2.1 **Extraction Time**

The extraction kinetics of phenolic compounds were assessed to determine the rate of extraction and define a suitable experimental range for the extraction time variable in the RSM design. As illustrated in **Figure 15**, the effect of extraction time on the total phenolic content (TPC), expressed in mg GAE/g of plant material, exhibited a clear trend. Initially, the TPC increased steadily, reaching a peak at 20 minutes with a value of 26.71 mg GAE/g. This indicates that the extraction process was most efficient during the first 20 minutes, likely due to the rapid diffusion of phenolic compounds from the plant matrix into the solvent.

However, after 20 minutes, a gradual decline in TPC was observed, with a notable decrease between 25 and 40 minutes. This reduction may be attributed to thermal or oxidative degradation of sensitive phenolic compounds, solvent saturation, or the co-extraction of interfering substances that diminish the effective concentration of phenolics. Beyond 40 minutes, TPC values appeared to stabilize, fluctuating slightly but remaining around 16 mg GAE/g, suggesting a dynamic equilibrium where the extraction rate is balanced by degradation or loss mechanisms.

Based on these findings, 20 minutes was identified as the optimal extraction time, beyond which no further gain in phenolic yield was achieved—and longer durations may, in fact, negatively impact extract quality. Consequently, an optimal time range between 15 and 25 minutes was selected for further experimental design and optimization.

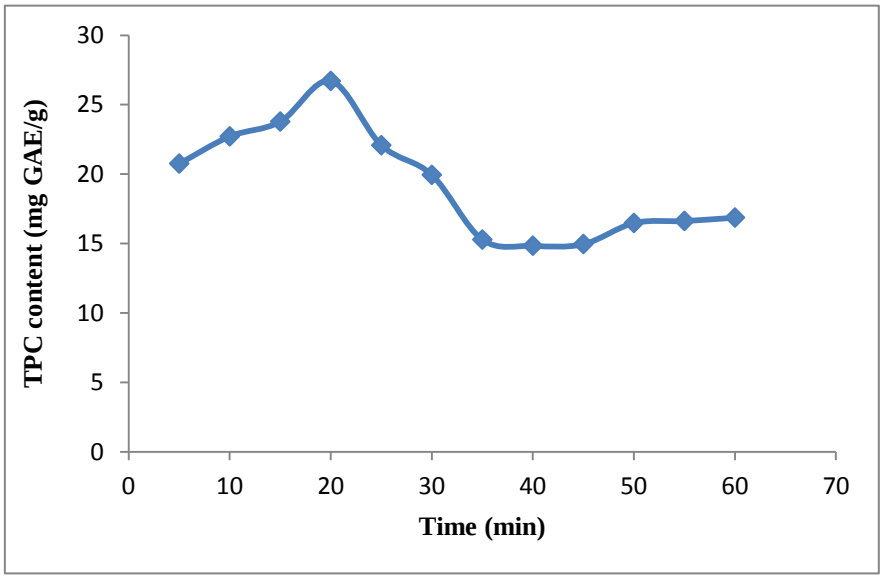


Figure. 15 . Effect Extraction Time on TPC

IV.1.2.2. **Liquid-to-Solid Ratio**

The influence of the liquid-to-solid ratio on the extraction efficiency of phenolic compounds from *Globularia alypum L*. was investigated using twelve different ratios: 5:0,5, 10:0,5, 15:0,5, 20: 0,5, 25: 0,5, 30: 0,5, 35:0,5, 40: 0,5, 40: 0,5, 50:0,5, 55:0,5 and 60:0,5 mL g^{-1}, under fixed conditions of 20 minutes extraction time at 25 °C

(Figure. 16). These ratios correspond to methanol volumes ranging from 5 to 60 mL, with a constant plant mass of 0.5 g.

The effect of solvent volume on the total phenolic content (TPC) exhibited a distinct pattern. Initially, increasing the solvent volume from 5 to 35 mL resulted in a progressive rise in TPC, indicating enhanced extraction efficiency. The phenolic content increased from 16.72 mg GAE/g at 5 mL to a maximum of 34.73 mg GAE/g at 35 mL. This trend suggests that larger solvent volumes improve the solubility and diffusion of phenolic compounds, facilitating their transfer from the plant matrix into the solvent.

However, beyond 35 mL, a decline in TPC was observed. At 40 mL, a slight decrease occurred (33.60 mg GAE/g), followed by a more pronounced drop to 28.11 mg GAE/g at 45 mL and further to approximately 25.57 mg GAE/g at 55 mL. This reduction may be due to dilution effects, where larger volumes decrease the concentration gradient, slowing extraction kinetics. Additionally, excessive solvent may extract non-phenolic or interfering substances, reducing the overall specificity of the TPC measurement.

Therefore, based on the results, the optimal liquid-to-solid ratio is considered to be between 30:0,5 and 40:0,5 mL g^{-1}.

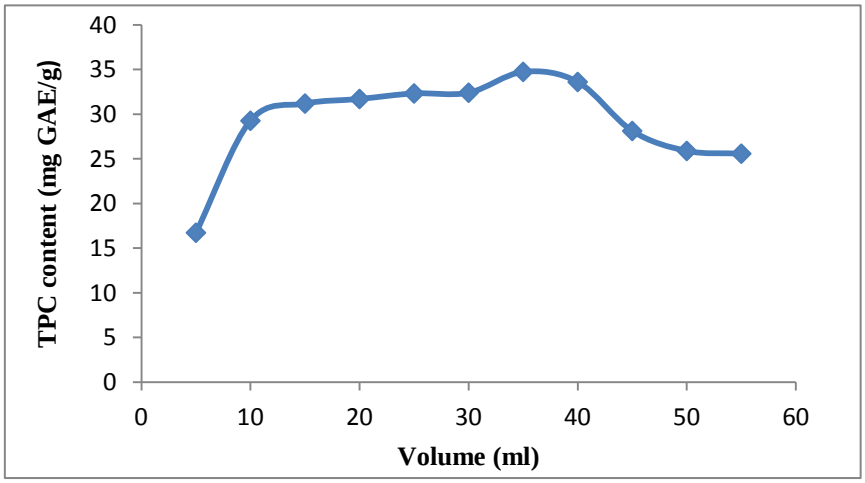


Figure 16. Effect liquid to solid ratio on TPC

IV.1.2.3. Extraction Temperature

Figure 17 illustrates the effect of extraction temperature (30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, and 60 °C) on the yield of total phenolic compounds (TPC). Extractions were performed for 20 minutes at a fixed liquid-to-solid ratio of 35:1 mL g^{-1}.

The results revealed a clear trend: as the temperature increased from 30 °C to 45 °C, the TPC rose significantly, reaching a maximum value of 33.43 mg GAE/g at 45 °C. This suggests that moderate heating enhances extraction efficiency by increasing the solubility of phenolic compounds and improving solvent penetration into plant tissues. Elevated temperatures likely accelerate mass transfer and facilitate the release of bound phenolics, contributing to higher yields.

However, beyond 45 °C, a gradual decline in TPC was observed, with values decreasing to 32.14 mg GAE/g at 50 °C, 29.99 mg GAE/g at 55 °C, and 28.76 mg GAE/g at 60 °C. This reduction is likely due to thermal degradation or oxidation of heat-sensitive phenolic compounds at higher temperatures. Prolonged exposure to excessive heat may lead to the structural breakdown or transformation of these bioactive molecules, ultimately reducing their measurable concentration.

Therefore, based on these findings, the optimal extraction temperature range is considered to be between 40°C and 50°C.

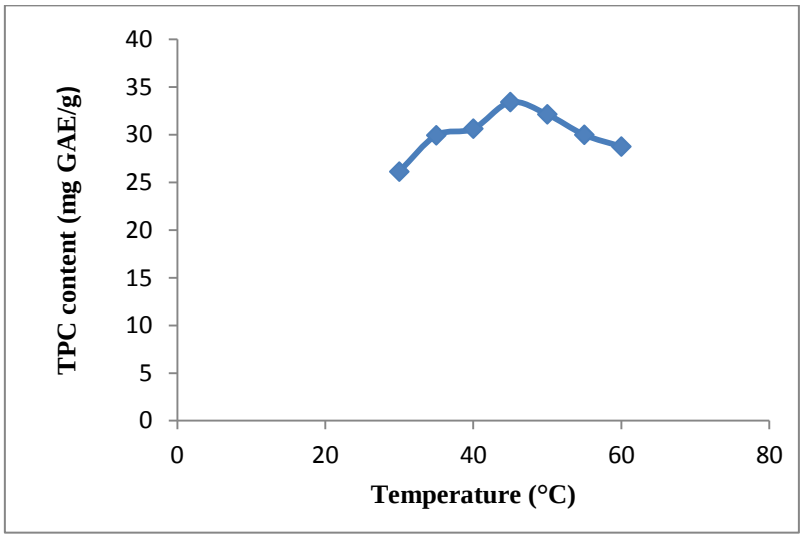


Figure 17. Effect extrcation temperature on TPC

III.2. Optimization of Ultrasonic Extraction Conditions for TPC

Three factors, namely extraction time (X1), extraction volume (X2), and extraction temperature (X3), were used to optimize the ultrasonic extraction of total polyphenols from *Globularia alypum L*.

The analysis presented above highlights the influence of all the variables mentioned on extraction efficiency and yield. Although they were studied separately, it should be noted that they interact with each other within the system. The results obtained show that the interaction of these variables is reflected in the phenolic content of the extracts obtained. The key factors to be considered during optimization, as well as their respective optimal conditions determined from the study carried out, are presented. The three factors (X_1, X_2 and X_3) were tested at three levels (-1, 0 and +1) to optimize the extraction of the compounds, as shown in **Table 3**.

Table 3: Experimental areas of factors studied in the optimization of phenolic compounds of *Globularia alypum L*

Variables		**Levels**		
		-1	**0**	**+1**
Time (min)	**X_1**	15	20	25
Volume (ml)	**X_2**	30	35	40
Temperature (°C)	**X_3**	40	45	50

IV.2.1. **RSM experiments**

Based on the findings from the single-factor experiments, the experimental ranges for the key variables extraction time (X_1), liquid-to-solid ratio (X_2), and extraction temperature (X_3) were defined for further optimization. To optimize the extraction of total phenolic content (TPC) from *Globularia alypum L*., the following central values were selected: an extraction temperature of 45 °C, an extraction time of 20 minutes, and a liquid-to-solid ratio of 35:1 (mL g^{-1}). These values formed the experimental domain for constructing the Box–Behnken Design (BBD) within the response surface methodology (RSM), which was used to identify the optimal extraction conditions.

Table 4 presents the experimental design matrix based on the BBD, along with the corresponding TPC values obtained from the extractions. The maximum TPC was recorded under the conditions of 50 °C, 20 minutes, and a 40:0,5 mL g^{-1} ratio.

Table 4: The experimental Box-Behnken matrix for three factors (X_1, X_2 and X_3) and their TPC, DPPH and ABTS responses expressed in (mg/g of plant matter).

	Factors			**Response**		
Experimental	**X1 (min)**	**X2 (ml)**	**X3 (°C)**	**TPC**	**DPPH**	**ABTS**
1	**20 (0)**	**35 (0)**	**45 (0)**	28,848	0,02432	0,04581
2	**15 (-1)**	**35 (0)**	**50 (+1)**	24,585	0,02373	0,04193
3	**25 (+1)**	**30 (-1)**	**45 (0)**	23,987	0,02088	0,04221
4	**20 (0)**	**30 (-1)**	**40 (-1)**	20,807	0,01739	0,03734
5	**15 (-1)**	**30 (-1)**	**45 (0)**	22,538	0,02127	0,04092
6	**25 (+1)**	**35 (0)**	**50 (+1)**	29,192	0,02616	0,05947
7	**20 (0)**	**40 (+1)**	**40 (-1)**	22,976	0,01957	0,04054
8	**20 (0)**	**35 (0)**	**45 (0)**	27,120	0,02611	0,05476
9	**25 (+1)**	**40 (+1)**	**45 (0)**	27,153	0,02604	0,04285
10	**25 (+1)**	**35 (0)**	**40 (-1)**	19,671	0,01895	0,03407
11	**20 (0)**	**40 (+1)**	**50 (+1)**	33,249	0,02742	0,06150
12	**20 (0)**	**30 (-1)**	**50 (+1)**	28,482	0,02581	0,04898
13	**15 (-1)**	**35 (0)**	**40 (-1)**	21,922	0,01952	0,03678
14	**15 (-1)**	**40 (+1)**	**45 (0)**	24,201	0,02489	0,04905
15	**20 (0)**	**35 (0)**	**45 (0)**	29,328	0,02763	0,05714

By applying multiple regression analysis (**Equation .(07)**) to the experimental data in **Table 4**, the following mathematical models for the response variable (TPC) were obtained:

$$\mathbf{TPC} = -101{,}505 + 1{,}75967\ X_1 + 0{,}816667\ X_2 + 3{,}301\ X_3 + 0{,}015\ X_1 X_2 + 0{,}0686\ X_1 X_3 + 0{,}026\ X_2 X_3 - 0{,}130067\ X_1^2 - 0{,}028467\ X_2^2 - 0{,}053667\ X_3^2 \quad \textbf{(Eq.(07))}$$

The results of the response surface methodology **(Table 5)** for the extraction of total phenolic compounds (TPC) indicate that the quadratic model provides the best fit to the data. The model was statistically significant, with a p-value of 0.0175, a high adjusted R^2 of 0.8903, and a non-significant lack of fit (p = 0.4647), suggesting that the model accurately represents the experimental results.

Additionally, the high coefficient of determination (R^2 = 0.9608) and adequate precision value of 12.70 confirm the model's reliability and a strong signal-to-noise ratio. The low coefficient of variation (CV = 4.92%) also reflects good experimental precision and repeatability.

Table 5. Analysis of the response surface quadratic model

Statistic	Value	Interpretation
R^2	**0.9608**	**Excellent correlation between predicted and actual CPT values**
Adjusted R^2	**0.8903**	**High model fit after accounting for predictors**
Predicted R^2	**0.5565**	**Acceptable but not excellent prediction capability**
Adequate Precision	**12.70**	**> 4 → model has a strong signal-to-noise ratio**
C.V. (%)	**4.92%**	**Low variation; reliable data**
Std. Dev.	**1.26**	**Small standard error**
Mean	**25.60**	**Average CPT value**

Among the studied factors, extraction temperature was identified as the most influential variable (p = 0.0004), exhibiting a strong positive effect on total phenolic content. The liquid-to-solid ratio also had a statistically significant impact (p= 0.0214), while extraction time showed a comparatively weaker effect.

In addition, the interaction between extraction time and temperature (AC) was significant (p = 0.0416), indicating a synergistic effect between these two variables. The presence of significant quadratic terms, particularly for extraction time (p=0.0042), suggests that the response follows a curvilinear trend, implying the existence of an optimal point for maximizing phenolic extraction.

The final regression equation reflects these findings, with positive linear contributions from all three main factors and negative quadratic terms, indicating a decline in response beyond certain levels.

Overall, the model is statistically valid and supports the accurate identification of optimal extraction conditions to maximize the recovery of phenolic compounds from *Globularia alypum L*.

Table 6 : Regression coefficients and their statistical significance

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	**194,43**	**9**	**21,60**	**13,62**	**0,0051**	**significant**
A-Extraction time	**5,71**	**1**	**5,71**	**3,60**	**0,1162**	
B-Liquid-to-solid ratio	**17,29**	**1**	**17,29**	**10,90**	**0,0214**	**significant**
C-Extraction temperature	**113,40**	**1**	**113,40**	**71,49**	**0,0004**	**significant**
AB	**0,5625**	**1**	**0,5625**	**0,3546**	**0,5775**	
AC	**11,76**	**1**	**11,76**	**7,42**	**0,0416**	**significant**
BC	**1,69**	**1**	**1,69**	**1,07**	**0,3493**	
A^2	**39,04**	**1**	**39,04**	**24,61**	**0,0042**	**significant**
B^2	**1,87**	**1**	**1,87**	**1,18**	**0,3271**	
C^2	**6,65**	**1**	**6,65**	**4,19**	**0,0960**	
Residual	**7,93**	**5**	**1,59**			
Lack of fit	**5,23**	**3**	**1,74**	**1,29**	**0,4647**	**Not significant**
Pure Error	**2,70**	**2**	**1,35**			
Cor Total	**202,36**	**14**				

III.2.5. **Effect liquid to solid ratio and extraction Time**

The 3D response surface plot illustrates the combined effect of extraction time and liquid-to-solid ratio on the TPC **(Figure 18)**. The surface displays a clear dome-shaped curvature, indicating that both factors have a significant and non-linear

influence on the extraction efficiency. TPC increases as both extraction time and solvent volume increase, reaching a maximum at approximately 20 minutes and a liquid-to-solid ratio of around 35 mL/g. Beyond these optimal values, further increases in either factor result in a gradual decline in CPT, likely due to the degradation of heat- or time-sensitive phenolic compounds or the dilution effect caused by excess solvent. The contour lines at the base of the plot reinforce the existence of this optimum region, highlighting the importance of selecting appropriate process parameters. Overall, the plot confirms that moderate extraction time and solvent volume are key to maximizing phenolic yield, while excessive values may negatively impact the extraction efficiency.

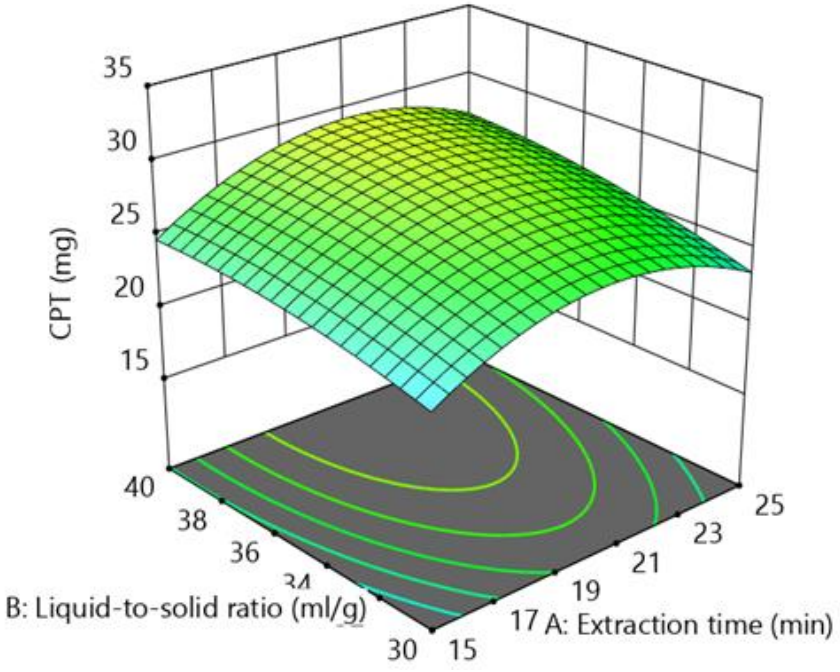


Figure 18. Response surface plot showing the effects of extraction time (X_1) and liquid-to-solid ratio (X_2).

III.2.6. **Effect liquid to solid ratio and extraction Temperature :**

The 3D response surface plot illustrates the combined effect of extraction temperature and liquid-to-solid ratio on the TPC **(Figure 19)**. The surface shows a pronounced curvature, indicating that both variables significantly and non-linearly influence the extraction efficiency. As the temperature and solvent volume increase, CPT also increases, reaching a maximum at around 47 °C and a liquid-to-solid ratio of

approximately 36 to 37 mL/g. Beyond these optimal values, a gradual decline in TPC is observed, which may be attributed to the thermal degradation of phenolic compounds at higher temperatures or the dilution effect caused by excessive solvent volumes. The contour plot at the base reinforces the presence of this optimum zone, highlighting the importance of carefully balancing both parameters. Overall, the plot demonstrates that moderately high temperatures and appropriate solvent volumes are essential to maximize the extraction of phenolic compounds while avoiding conditions that could reduce yield.

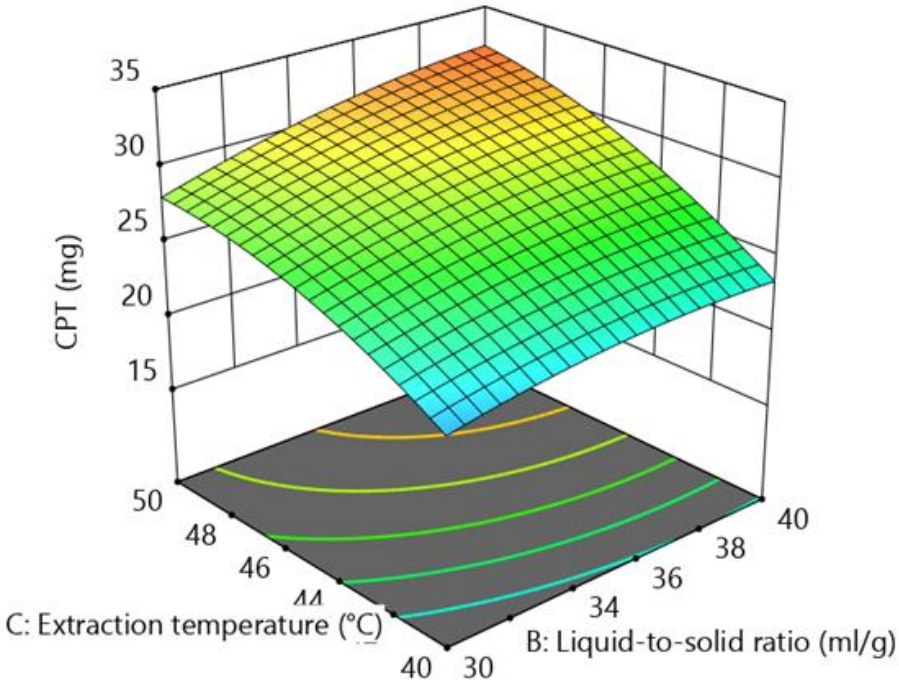


Figure 19. Response surface plot showing the effects of extraction temperature (X_3) and liquid-to-solid ratio (X_2).

III.2.7. **Effect extraction Temperature and Time**

The 3D surface plot **(Figure 20)** illustrates the interactive effect of extraction time and extraction temperature on the yield of TPC (mg). As shown, the TPC yield increases progressively with both extraction time and temperature, reaching a maximum at around 22–23 minutes and 47–48°C. This indicates that longer extraction durations and moderately high temperatures favor the release of TPC, likely due to enhanced solubility and diffusion of the compound from the plant

matrix. However, beyond these optimal conditions, particularly at higher temperatures or prolonged times, the TPC yield begins to decline. This suggests that excessive heat or extended exposure may lead to the degradation or loss of the compound. Therefore, optimizing both parameters is crucial to maximize TPC recovery while avoiding conditions that could negatively affect its stability.

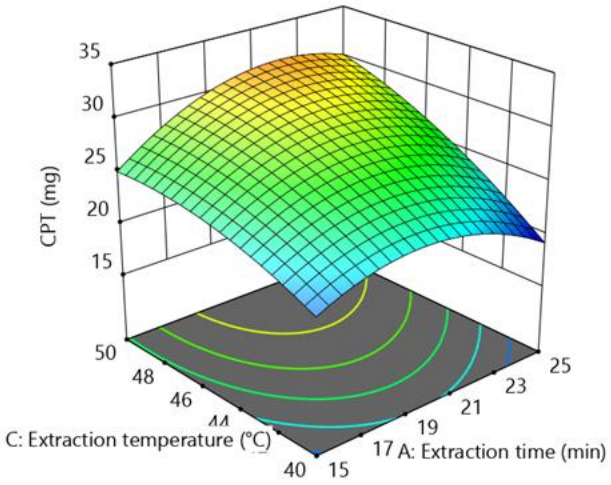


Figure 20 : Response surface plot showing the effects of extraction temperature (X_3) and time (X_1).

III.3. Free radical scavenging (RSA)

IV.3.1. **Anti-radical activity DPPH**

The part of this study is to evaluate the antiradical activity of extracts of *Globularia alypum L* using DPPH, a purple-colored free radical that reduces to a yellow compound in the presence of antioxidants. The antiradical activity of the compounds is measured by analyzing the intensity of the coloration at a wavelength of 517 nm using a spectrophotometer. This intensity of coloration is inversely proportional to the antiradical activity of the tested compounds. For this study, ascorbic acid, known as vitamin C, was used as a standard at different concentrations to calibrate the measurements. The antioxidant capacity of the studied extracts was expressed in milligrams of ascorbic acid equivalents per gram of plant material (AAEAC). This allows the antiradical activity of the different extracts to be quantified and compared. The results obtained demonstrate that all extracts exhibit significant

antiradical activities, ranging from 0,01739 to 0,02763 mg AAEAC/g of MV **(Table 4)**. These results highlight the influence of the parameters studied, such as time, volume and temperature, on the antiradical activity of the tested extracts.

The statistical analysis results provide insight into the adequacy and reliability of the regression model used to evaluate the effects of different extraction parameters on RSA. Among the tested models, the linear model is suggested based on its low p-value (0.0028), indicating a statistically significant fit. It also shows an acceptable lack of fit p-value (0.5717), suggesting that the model fits the experimental data well. The adjusted R^2 (0.6279) and predicted R^2 (0.5568) values are reasonably close, indicating good agreement between the model's predicted and observed values **(Table7)**.

The 2FI (two-factor interaction) and quadratic models have higher p-values (0.8822 and 0.1119, respectively), and their predicted R^2 values are significantly lower, especially for the quadratic model (0.2594), suggesting a poor predictive capability. The cubic model is marked as aliased, meaning that it cannot be properly interpreted due to insufficient data points or confounding effects.

Fit statistics further support the model's reliability. The R^2 value of 0.9106 indicates that over 91% of the variation in CPT yield is explained by the model. The adjusted R^2 (0.7496) and adequate precision (7.2548) confirm the model's significance and signal-to-noise ratio. The coefficient of variation (C.V. = 7.24%) indicates good model precision, as values below 10% are generally considered acceptable. In conclusion, the linear model provides a statistically valid and reliable fit for describing the influence of the studied parameters on CPT extraction

Table 7 : Analysis of the response surface quadratic model for DPPH

Source	Squential p-value	Lack of fit p-value	Adjusted R^2	Predicted R^2	
Linear	**0,0028**	**0,5717**	**0,6279**	**0,5568**	**Suggested**
2FI	0,8822	0,4600	0,5268	0,3291	
Quadratic	0,1119	0,7095	0,7496	0,2594	
Cubic	0,7095		0,6485		Aliased

The analysis of variance table provides valuable information on the significance of each factor and their interactions in the extraction process affecting RSA. The overall model is statistically significant ($p = 0.0354$), with an F-value of 5.66, indicating that the model as a whole effectively explains the variability in the data.

Among the individual factors, extraction temperature (C) has a highly significant effect on RSA ($p = 0.0024$), with a high F-value of 31.97, suggesting it is the most influential parameter in the model. The liquid-to-solid ratio (B) also shows a significant effect ($p = 0.0416$), whereas extraction time (A) does not significantly influence the response ($p = 0.6927$).

Interaction terms (AB, AC, and BC) exhibit no significant effects, as their p-values are all well above 0.05, indicating minimal or no interactive influence between these factors. Regarding quadratic terms, only the square of the extraction temperature (C^2) approaches significance ($p = 0.0506$), implying a possible nonlinear effect of temperature on the yield. The quadratic terms for time (A^2) and ratio (B^2) are not significant.

The lack of fit test is not significant ($p = 0.7095$), indicating that the model fits the experimental data adequately and that the unexplained variation is mostly due to pure error, not model inadequacy. Overall, the model is valid, with extraction temperature being the most critical factor influencing RSA extraction efficiency.

According to the results of the study, the values of the significant coefficients were determined and the function governing the antiradical activity of *Globularia alypum L* extracts is now well established. The mathematical model used in this study can be represented by the following relationship:

$$\mathbf{AAEAC_{DPPH}} = -0{,}28175 + 0{,}00075\,X_1 + 0{,}004525\,X_2 + 0{,}008875\,X_3 + 0{,}00001\,X_1X_2 + 0{,}00003\,X_1X_3 - 0{,}00002\,X_2X_3 - 0{,}00006\,X_1^2 - 0{,}00005\,X_2^2 - 0{,}00009\,X_3^2 \quad \textbf{(Eq.(08))}$$

Table 8 ; Regression coefficients and their statistical significance for DPPH

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0,0001	9	0,0000	5,66	0,0354	significant
A-Extraction time	5,000E-07	1	5,000E-07	0,1754	0,6927	
B-Liquid-to-solid ratio	0,0000	1	0,0000	7,41	0,0416	
C-Extraction température	0,0001	1	0,0001	31,97	0,0024	
AB	2,500E-07	1	2,500E-07	0,0877	0,7790	
AC	2,250E-06	1	**2,250E-06**	0,7895	0,4150	
BC	1,000E-06	1	1,000E-06	0,3509	0,5794	
A^2	8,308E-06	1	8,308E-06	2,91	0,1485	
B^2	5,769E-06	1	5,769E-06	2,02	0,2141	
C^2	0,0000	1	0,0000	6,56	0,0506	
Residual	0,0000	5	2,850E-06			
Lack of fit	6,250E-06	3	2,083E-06	0,5208	0,7095	Not significant
Pure Error	8,000E-06	2	4,000E-06			
Cor Total	0,0002	14				

III.3.1.1. Effect liquid to solid ratio and extraction Time

The 3D surface plot illustrates the combined effect of extraction time (A) and liquid-to-solid ratio (B) on the DPPH radical scavenging activity, expressed in terms of $AAEAC_{DPPH}$ (mg). The response surface reveals a clear curved profile, indicating a nonlinear relationship between the factors and the antioxidant activity **(Figure 21)**. As both extraction time and liquid-to-solid ratio increase, the DPPH value also increases, reaching a maximum activity at approximately 22–23 minutes and a liquid-to-solid ratio of about 36–38 ml/g. Beyond these optimal conditions, the antioxidant activity begins to decline, suggesting that overly high values for these parameters may lead to degradation of active compounds or decreased extraction efficiency. The contour lines at the base reinforce the presence of a single optimum zone. Overall, the plot indicates that moderate extraction times and higher liquid-to-solid ratios enhance antioxidant extraction, but excessive conditions may reduce activity, emphasizing the need for optimization.

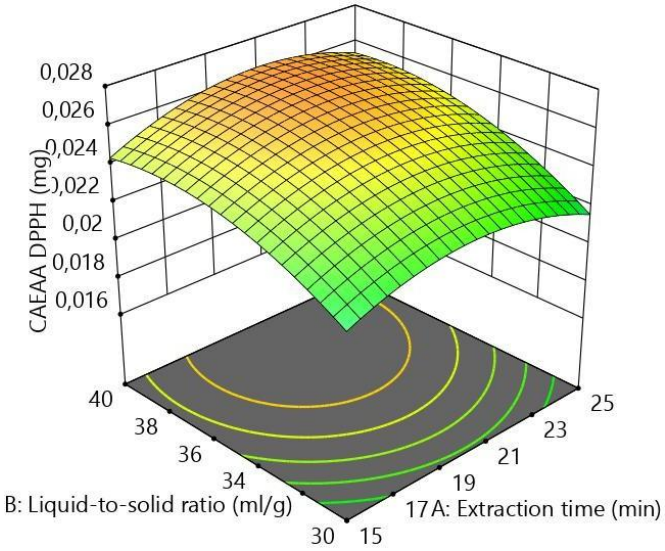


Figure 21 : Response surface plot showing the effects of extraction time (X_1) and liquid-to-solid ratio (X_2) for DPPH.

III.3.1.2. Effect liquid to solid ratio and extraction Temperature

This 3D surface plot illustrates the interactive effect of extraction temperature (C) and liquid-to-solid ratio (B) on the DPPH radical scavenging activity **(Figure 22)**. The surface reveals a clear upward trend, showing that increasing both the extraction temperature and the liquid-to-solid ratio enhances the antioxidant activity, reaching a maximum value at higher temperatures (~49–50 °C) and moderate-to-high liquid-to-solid ratios (~36–38 ml/g). The smooth curvature of the plot suggests a strong synergistic effect between these two parameters. Beyond these optimal ranges, the antioxidant activity appears to plateau, indicating that excessively high values may not further improve activity significantly. The contour map at the base confirms a single optimal zone, forming concentric curves centered around the maximum response. Overall, the results demonstrate that elevated temperatures combined with an adequate solvent volume relative to the plant material are key to maximizing the extraction of antioxidant compounds measured by DPPH activity.

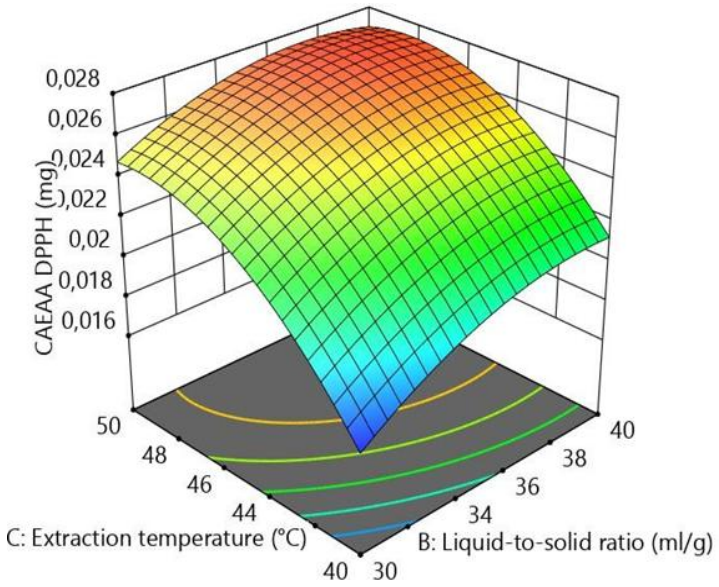


Figure 22 : Response surface plot showing the effects of extraction temperature (X_3) and liquid-to-solid ratio (X_2).

III.3.1.3. Effect extraction Temperature and Time

The 3D response surface plot illustrates the effect of extraction temperature (°C) and extraction time (min) on the DPPH radical scavenging activity, expressed as $AAEAC_{DPPH}$ (mg) **(Figure 23)**. The plot reveals that both variables significantly influence antioxidant activity. As both the extraction temperature and time increase, the DPPH scavenging activity also increases, reaching a maximum at higher levels of these two parameters (around 50 °C and 25 min). The surface appears to rise steeply, forming a clear peak, indicating a synergistic interaction between time and temperature. Beyond these optimal points, further increases are not shown but could potentially lead to a plateau or degradation. Overall, the model suggests that moderate-to-high extraction conditions are favorable for maximizing the antioxidant potential of the extract.

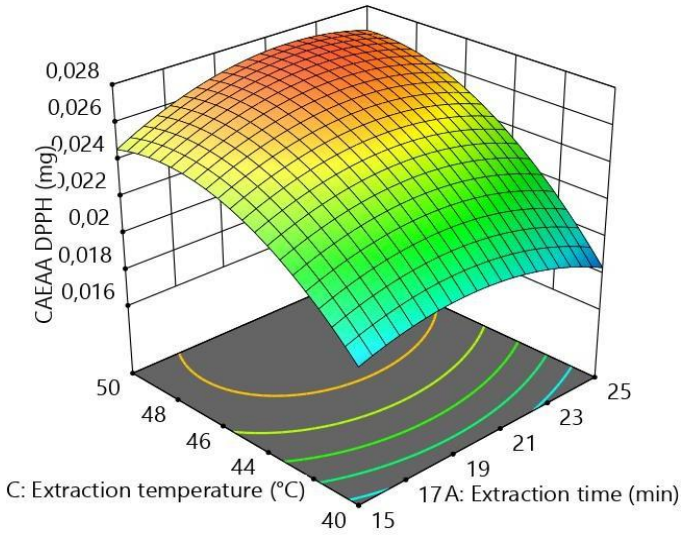


Figure 23 : Response surface plot showing the effects of extraction temperature (X_3) and time (X_1).

IV.3.2. **Anti-radical activity ABTS**

The statistical analysis evaluates the suitability of different regression models in describing the antioxidant activity measured by the ABTS assay. The linear model is statistically significant, with a sequential p-value of 0.0235 and a non-significant lack-of-fit p-value of 0.5305, indicating that it provides a good fit without overfitting. It shows a moderate adjusted R^2 of 0.4444 and a predicted R^2 of 0.2523, making it the most reliable and suggested model among those tested. The 2FI model does not significantly improve the fit (p = 0.3938), and its predictive power remains limited. The quadratic model shows a better adjusted R^2 (0.6595), indicating an improved fit to the experimental data, but its predicted R^2 is negative (-0.0173), suggesting poor performance in predicting new responses—likely due to overfitting. The cubic model is aliased, meaning it cannot be evaluated properly due to insufficient data. Additional fit statistics show a standard deviation of 0.0050 and a relatively high R^2 of 0.8784, although this must be interpreted with caution due to the weak predicted R^2. The coefficient of variation (C.V. = 10.75%) is acceptable for biological measurements, and the Adequate Precision value of 7.0801 confirms that the model has a strong signal relative to noise. Overall, the linear model is the most appropriate for

describing ABTS activity, offering a balance between significance, fit, and predictive ability.

Table 9 : Analysis of the response surface quadratic model for ABTS

Source	Squential p-value	Lack of fit p-value	Adjusted R^2	Predicted R^2	
Linear	**0,0235**	**0,5305**	**0,4444**	**0,2523**	**Suggested**
2FI	0,3938	0,5257	0,4632	0,1533	
Quadratic	0,1704	0,7034	0,6595	-0,0173	
Cubic	0,7034				Aliased

The analysis of variance (ANOVA) and regression model for **$AAEAC_{ABTS}$** (antioxidant activity via ABTS assay) provides insight into the effects of extraction parameters on the response. The overall model has a p-value of 0.0701 and an F-value of 4.01, indicating that the model is **not statistically significant** at the 95% confidence level, although it is close to the threshold. Among the individual factors, **extraction temperature (factor C)** is the only variable that shows a **significant effect** (p = 0.0070), suggesting that it has a strong influence on ABTS activity. Neither extraction time (A) nor liquid-to-solid ratio (B) is statistically significant, with p-values of 0.5941 and 0.1238, respectively.

Interaction terms (AB, AC, and BC) and quadratic terms (A^2, B^2, C^2) are all non-significant, though the interaction between extraction time and temperature (AC, p = 0.1005) and the quadratic term for extraction time (A^2, p = 0.0581) approach marginal significance, indicating potential subtle effects.

The **lack of fit is not significant (p = 0.7034)**, which suggests that the model fits the experimental data adequately. The residual variation is small, indicating consistent experimental measurements.

The final regression equation for $AAEAC_{ABTS}$ is:

$$\mathbf{AAEAC_{ABTS}} = -0.2795 + 0.004133\ X_1 + 0.005183\ X_2 + 0.006750\ X_3 - 0.000080\ X_1X_2 + 0.000200\ X_1\ X_3 + 0.000080\ X_2\ X_3 - 0.000253\ X_1^2 - 0.000093\ X_2^2 - 0.000133X_3^2 \quad \textbf{(Eq.(09))}$$

This equation shows that the linear effects of all three variables are positive, meaning increases in time, ratio, or temperature generally enhance ABTS activity. However, the negative quadratic coefficients indicate that the response may reach an optimum and then decline at higher levels of these variables, especially for extraction time.

Table 10 : Regression coefficients and their statistical significance for ABTS

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0,0009	9	0,0001	4,01	0,0701	Not significant
A-Extraction time	8,000E-06	1	8,000E-06	0,3235	0,5941	
B-Liquid-to-solid ratio	0,0001	1	0,0001	3,42	0,1238	
C-Extraction température	0,0005	1	0,0005	19,43	0,0070	
AB	0,0000	1	0,0000	0,6469	0,4577	
AC	0,0001	1	0,0001	4,04	0,1005	
BC	0,0000	1	0,0000	0,6469	0,4577	
A^2	0,0001	1	0,0001	5,99	0,0581	
B^2	0,0000	1	0,0000	0,8128	0,4086	
C^2	0,0000	1	0,0000	1,66	0,2542	
Residual	0,0001	5	0,0000			
Lack of fit	0,0001	3	0,0000	0,5340	0,7034	Not significant
Pure Error	0,0001	2	0,0000			
Cor Total	0,0010	14				

III.3.2.1.Effect liquid to solid ratio and extraction Time

The 3D response surface plot illustrates the combined effect of extraction time (min) and liquid-to-solid ratio (ml/g) on the ABTS antioxidant activity, expressed as $AAEAC_{ABTS}$ (mg) **(Figure 24)**. The plot reveals a clear upward trend in antioxidant activity as both extraction time and liquid-to-solid ratio increase. The surface reaches a peak in the upper right corner, indicating that the highest $AAEAC_{ABTS}$ activity (≈0.028 mg) is achieved at longer extraction times (around 25 min) and higher liquid-to-solid ratios (around 40 ml/g).

However, the curvature of the surface also suggests that this trend is not strictly linear. Beyond certain levels, especially for the liquid-to-solid ratio, the increase in ABTS activity may start to plateau or decline slightly, as indicated by the gentle slope at the far end of the surface. This concave shape confirms the influence of quadratic effects,

consistent with the regression model. In summary, both extraction time and liquid-to-solid ratio positively influence the ABTS antioxidant activity, with an optimal zone near the maximum values tested. This suggests that increasing these parameters up to a certain point enhances antioxidant extraction efficiency, but overly high values may offer diminishing returns.

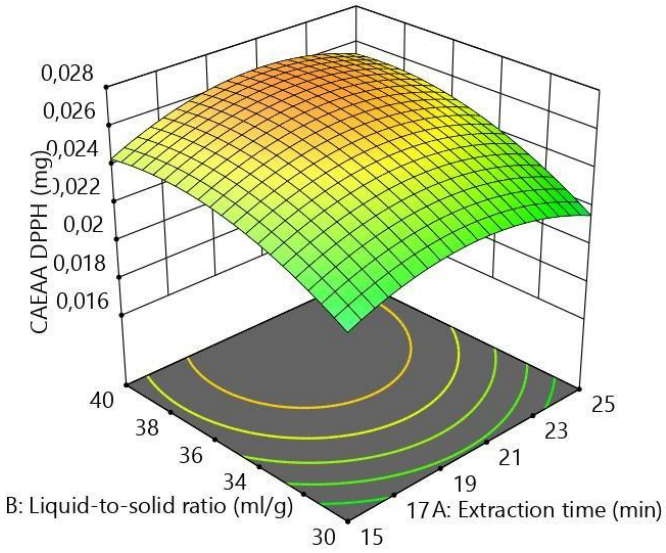


Figure 24 : Response surface plot showing the effects of extraction time (X_1) and liquid-to-solid ratio (X_2) for ABTS

III.3.2.2. Effect extraction Temperature and Time

This 3D response surface plot shows the combined effect of extraction time (min) and extraction temperature (°C) on $AAEAC_{ABTS}$ (mg), which represents the ABTS radical scavenging activity (a measure of antioxidant capacity) **(Figure 25)**.

From the plot, we observe a curved, rising surface, indicating that both extraction time and temperature significantly affect the antioxidant yield. The maximum ABTS activity (~0.028 mg) occurs at higher extraction temperatures (close to 50 °C) and longer extraction times (around 25 minutes). This suggests that elevating both parameters enhances antioxidant extraction, likely due to improved solubility and diffusion of active compounds.

The curvature also reveals a parabolic behavior, meaning that there is an optimal range beyond which further increases in temperature or time may not lead to

substantial improvement, and might even reduce the activity due to possible thermal degradation of sensitive compounds.

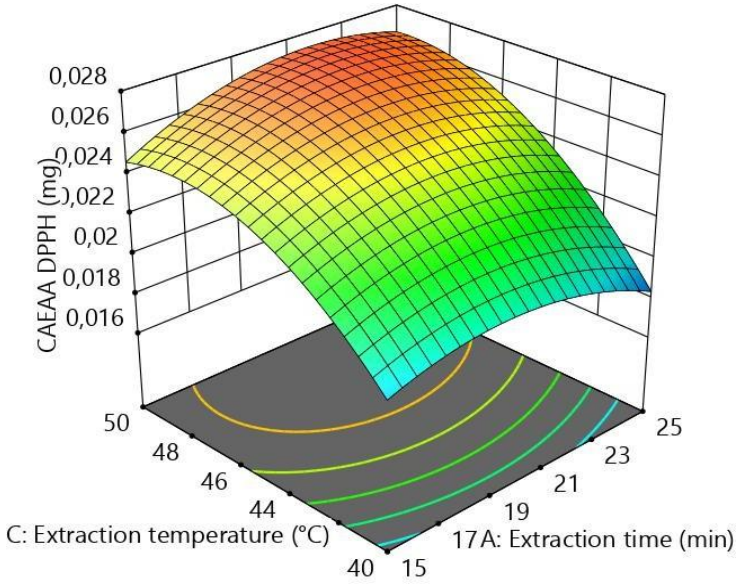


Figure 25 : Response surface plot showing the effects of extraction temperature (X_3) and time (X_1) for ABTS.

III.4. Optimal Conditions for TPC, DPPH, and ABTS

The optimization of extraction conditions using response surface methodology (RSM) demonstrated that moderately high temperature (~47–50 °C), extraction time (~22–25 min), and a liquid-to-solid ratio of 35–40 mL/g significantly enhance the recovery of total phenolic content (TPC) and antioxidant activity measured by DPPH and ABTS assays. Extraction temperature was identified as the most influential parameter across all responses, while the liquid-to-solid ratio also had a notable effect, especially in combination with temperature. The optimal TPC yield was observed at 45 °C, 20 min, and 35 mL/g, while maximum antioxidant activities for both DPPH and ABTS occurred near 50 °C and 25 min. A strong correlation was observed between TPC and DPPH activities, confirming the contribution of phenolic compounds to radical scavenging potential. ABTS showed a similar but slightly weaker correlation.

Overall, these results highlight the importance of optimizing extraction parameters to maximize the recovery of bioactive phenolics and their associated antioxidant activities.

Table 11 : Optimal Conditions and Correlations

Response	**Optimal Conditions**	**Model Fit (Adj. R^2)**	**Most Significant Factor**	**Correlation**
TPC	**45 °C, 20 min, 35 mL/g**	**0.8903**	**Extraction temperature**	**Strong with DPPH**
DPPH	**50 °C, 25 min, 38–40 mL/g**	**0.7496**	**Extraction temperature**	**Strong with TPC & ABTS**
ABTS	**50 °C, 25 min, 38–40 mL/g**	**0.4444**	**Extraction temperature**	**Moderate with TPC & DPPH**

Conclusion

General Conclusion

In recent years, many researchers have increasingly turned their attention to the biologically active compounds found in plant extracts, which are considered valuable natural sources of potentially beneficial substances. Among these medicinal plants, *Globularia alypum L*. stands out for its richness in phenolic compounds and its traditional therapeutic use.

To optimize the extraction of these bioactive molecules, researchers applied the technique of ultrasound-assisted extraction (UAE). This method enhances extraction efficiency within a shorter time, reducing energy consumption and minimizing the degradation of thermosensitive phenolic compounds. The present study contributes to this field by aiming to optimize the extraction conditions of antioxidants from *Globularia alypum L*., focusing on both leaves and flowers as plant materials.

The phenolic content present in the extracts was quantified to assess the effectiveness of the extraction under varying experimental conditions. The experimental approach was divided into two parts. First, preliminary single-factor experiments were conducted by varying one parameter at a time while keeping the others constant. These initial tests were used to determine the appropriate range for each factor before optimization.

In the second phase, a multivariate optimization was performed using Response Surface Methodology (RSM) based on the Box–Behnken Design (BBD).

This study successfully employed RSM using Box–Behnken Design to optimize the extraction conditions of total phenolic content (TPC) and antioxidant activities (DPPH and ABTS assays) from the selected plant material. The analysis revealed that extraction temperature, liquid-to-solid ratio, and extraction time significantly influence the yield of phenolic compounds and their antioxidant potential. Among these, extraction temperature emerged as the most critical factor across all responses. Optimal extraction conditions were identified around 45–50 °C, 20–25 minutes, and a liquid-to-solid ratio of 35–40 mL/g, under which the highest TPC and antioxidant activities were achieved.

A strong correlation between TPC and DPPH activity confirms the key role of phenolic compounds in free radical scavenging. ABTS showed a similar but slightly

weaker relationship. These findings emphasize the value of process optimization in maximizing the recovery of bioactive compounds with health-promoting properties. The results provide a scientific basis for developing efficient and sustainable extraction protocols for phenolic antioxidants, supporting their application in food, pharmaceutical, and nutraceutical formulations.

As perspectives, we suggest: Applying advanced analytical techniques: To more precisely characterize the chemical composition of the extracts, the use of advanced analytical tools, such as high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) is recommended. These techniques enable the accurate separation, identification, and quantification of individual compounds within the extract, providing a clearer understanding of the phenolic profile and allowing the specific identification of active phenolic compounds.

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Annexes

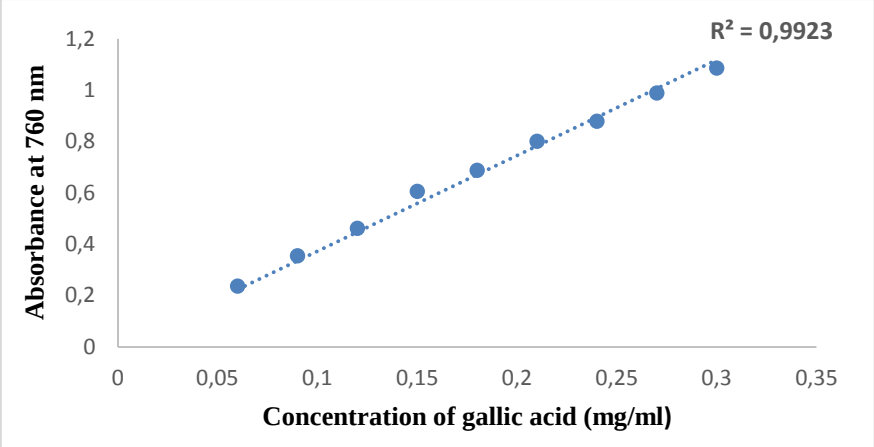
Materials and Chemicals

All chemicals and reagents used in this study were of analytical grade. The following were employed:

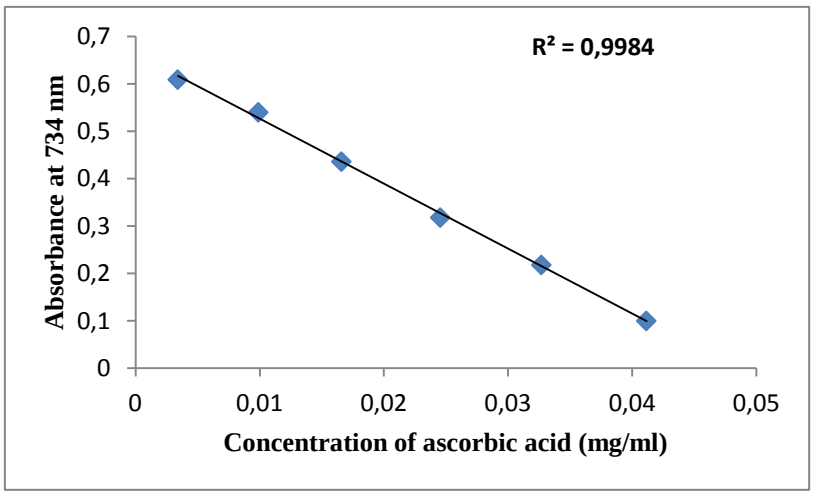
- **Methanol (CH_3OH):** extraction solvent—analytical grade (Sigma-Aldrich).
- **Folin–Ciocalteu reagent:** for total phenolic content (TPC) determination.
- **Sodium carbonate (Na_2CO_3):** used in TPC analysis.
- **Gallic acid:** standard compound for calibration curve in TPC.
- **2,2-Diphenyl-1-picrylhydrazyl (DPPH):** used for antioxidant activity assay.
- **2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS):** for antioxidant evaluation.
- **Potassium persulfate ($K_2S_2O_8$):** used in ABTS radical generation.
- **Distilled water:** for solution preparation and dilution.

For the extraction process, the following laboratory equipment and materials were used:

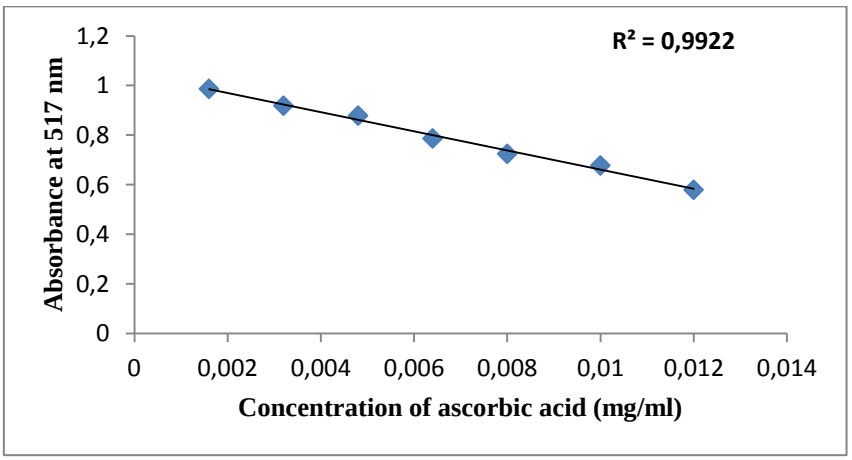
- **Ultrasonic bath** for ultrasound-assisted extraction,
- **Soxhlet extractor** for delipidation (using hexane),
- **Glassware:** beakers, volumetric flasks, measuring cylinders, filter papers, and pipettes,
- **UV-Visible spectrophotometer** for quantification of total phenolic content and antioxidant activity.



Curve stander of gallic acid (phenolic compounds)



Curve stander of ascorbic acid used in the ABTS assay



Curve stander of ascorbic acid used in the DPPH assay

الملخص

في هذه الدراسة قمنا باستخلاص البوليفينول من نبتة *Globularia alypum* L باستخدام طريقة الاستخلاص بالموجات فوق الصوتية. باستخدام طريقة متعددة المتغيرات لمنهجية استجابة السطح مع تصميم Box-Behnken سمحت لنا بتحسين ثلاثة متغيرات (درجة الحرارة ، الوقت ، حجم الميثانول) للحصول على أكبر كمية للمركبات الفينولية TPC و أحسن نشاط مضاد للأكسدة DPPH و ABTS .
لوحظت أفضل مردودية للمركبات الفينولية عند 45 درجة مئوية ، 20 دقيقة و35 مل ، بينما بلغت أقصى نشاط مضاد للأكسدة لكلٍّ من DPPH و ABTS حوالي 50 درجة مئوية و25 دقيقة .ولوحظ ارتباط قوي بين TPC وDPPH، مما يؤكد مساهمة المركبات الفينولية في إمكانية إزالة الجذور الحرة .بينما أظهر ABTS ارتباطًا مشابهًا ولكنه أضعف قليلًا .وبشكل عام، تُبرز هذه النتائج أهمية تحسين معايير الاستخلاص لزيادة مردودية الفينولات النشطة بيولوجيًا ونشاطها المضاد للأكسدة.
الكلمات المفتاحية: النشاط المضاد للأكسدة، الاستخلاص، التحسين، *Globularia alypum L*، المركبات الفينولية، بوكس بهنكن.

Abstract

In this study, we extracted polyphenols of the plant *Globularia alypum* L using ultrasonic extraction process. The use of Response surface Methodology multivariate method by Box-Behnken design allows us to optimize three variables (temperature, time and volume of methanol). In order to obtain the best yield of Total Phenolic Compounds TPC;as well as the best antioxidants activities for both DPPH and ABTS.

The optimal TPC yield was observed at 45 °C, 20 min, and 35 mL, while maximum antioxidant activities for both DPPH and ABTS occurred near 50 °C and 25 min. A strong correlation was observed between TPC and DPPH activities, confirming the contribution of phenolic compounds to radical scavenging potential. ABTS showed a similar but slightly weaker correlation. Overall, these results highlight the importance of optimizing extraction parameters to maximize the recovery of bioactive phenolics and their associated antioxidant activities.

Key words: antioxidant activity, extraction, optimization, *Globularia alypum L*, phenolic compounds, box behnken.

Résumé

Dans cette étude, nous avons extrait les polyphénols de plante *Globularia alypum* L en utilisant un procédé d'extraction par ultrasons. L'utilisation de la méthode multivariée de Méthodologie de surface de Réponse par le plan d'expérience Box-Behnken nous permet d'optimiser trois variables (température, temps et volume de méthanol). Afin d'obtenir le meilleur rendement en Teneur des Composés Phénoliques TCP; ainsi que les meilleures activités antioxydantes pour le DPPH et l'ABTS.
Le rendement optimal en TPC a été observé à 45 °C, 20 min et 35 ml, tandis que les activités antioxydantes maximales pour le DPPH et l'ABTS ont été observées vers 50 °C et 25 min. Une forte corrélation a été observée entre les activités TPC et DPPH, confirmant la contribution des composés phénoliques au potentiel de piégeage des radicaux. L'ABTS a montré une corrélation similaire, mais légèrement plus faible. Globalement, ces résultats soulignent l'importance d'optimiser les paramètres d'extraction pour maximiser le rendement des composés phénoliques bioactifs et leurs activités antioxydantes associées.

Mots clés : activité antioxydante, extraction, optimisation, *Globularia alypum* L, composés phénolique, box behnken.