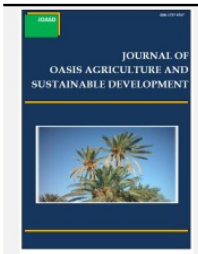

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# Effect of Different Extraction Methods on Phenolic Content, Flavonoid Levels, and Antioxidant Activities of Four Local Populations of Pea (*Pisum sativum* L.) from Southern Tunisia

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## Abstract

*Pisum sativum* L. seeds, recognized as a valuable agro-industrial by-product, are notably rich in polyphenolic compounds. However, their phytochemical composition is influenced by factors such as extraction methods, temperature and duration. This study assessed the effects of three extraction methods (Soxhlet, maceration, and ultrasound-assisted extraction) on the phytochemical profile and antioxidant activity of pea seed extracts from four oases in southern Tunisia. The Soxhlet method yielded the highest phenolic acid content and DPPH radical scavenging activity, while maceration resulted in the greatest levels of flavonoids and tannins. Ultrasound-assisted extraction demonstrated superior antioxidant activity overall, particularly in reducing power and ABTS scavenging, with the P2 population displaying the most promising results. These findings reinforce the potential of pea seeds for applications in both medicinal and food industries.

## 1. INTRODUCTION

Phytochemicals, particularly polyphenols, have garnered increasing attention due to their health benefits and powerful antioxidant properties, positioning them as potential nutraceuticals capable of preventing diseases (BenYakoub et al., 2018). Polyphenols, which are secondary plant metabolites, are characterized by the presence of aromatic rings and hydroxyl groups, giving them notable antioxidant capacity, particularly through their ability to neutralize free radicals by donating hydrogen atoms or electrons (Shahidi and Naczk, 2003; Zagoskina et al., 2023). This antioxidant capacity is crucial given the role oxidative stress plays in the development of diseases such as cancer, diabetes, atherosclerosis, and neurological disorders (Vona et al., 2021). However, the extraction of these compounds is complex and influenced by several factors, such as the type of solvent, extraction method, and storage conditions (Antolovich et al., 2002). Common solvents used for polyphenol extraction include water, ethanol, methanol, and acetone. The structural diversity of polyphenols and their interactions with other food components make precise extraction and quantification challenging, hindering the development of a universal method suited to all phenolic compounds.

Each extraction technique for bioactive compounds from *Pisum sativum* has its own strengths and drawbacks. Soxhlet extraction, for example, is highly effective in isolating a large quantity and variety of compounds within a relatively short period, while also allowing solvent reuse and eliminating the need for filtration or centrifugation. However, its use of high temperatures can result in the degradation of sensitive phytochemicals like polyphenols (Mansouri et al., 2022). To address this, alternative methods such as maceration, which is widely used due to its simplicity (Obhanin et al., 2024), and ultrasound-assisted extraction,

which breaks down plant cells and enhances solvent interaction without excessive heat, have been explored (Martinez et al., 2024; Bin Mokaizh et al., 2024). Extracting polyphenolic compounds poses a particular challenge, as they are vulnerable to degradation from high temperatures, light, and oxygen. Therefore, selecting the right extraction method and optimizing the process is crucial to maximizing yields while preserving the biological activity of these compounds (Dai & Mumper, 2010; Gil-Martin et al., 2022).

The genus *Pisum sativum*, commonly known as the pea, is an important agricultural crop with substantial nutritional value. Widely grown in temperate regions, peas are cultivated for both human consumption and animal feed. Among the various species within the genus, *Pisum sativum L*. is the most commercially significant, valued for its edible seeds and pods (Smýkal et al., 2012; Shavanov., 2021**).** Peas are rich in protein, essential amino acids, fiber, and micronutrients, making them a critical component of food security and human nutrition (McPhee, 2003; Thavarajah et al., 2023**).** Furthermore, peas contribute to sustainable agriculture by fixing atmospheric nitrogen, which enhances soil fertility and reduces the need for chemical fertilizers (Foyer et al., 2016; Kumar et al., 2021). Major production regions include the Mediterranean Basin, as well as temperate areas of Asia and Europe, where *Pisum sativum* plays a vital role in local economies and global agricultural practices (Duke, 1981).

The aim of our study is to evaluate the phenolic compounds present in four populations of pea (*Pisum sativum*) from the arid regions of southern Tunisia, utilizing three different extraction methods. Given the growing interest in bioactive compounds for their health benefits, this research focuses on comparing the efficiency and yield of phenolic extractions from these local pea populations. The extraction techniques employed include maceration, Soxhlet extraction, and ultrasound-assisted extraction. Each method was selected for its unique advantages in extracting phenolic compounds, particularly in terms of efficiency, temperature sensitivity, and compound preservation. This comparison is critical for identifying the most effective and sustainable method to maximize the recovery of phenolics while maintaining their bioactive properties, especially in crops grown in harsh, saline environments.

## 2. MATERIAL AND METHODS

### 2.1. Chemicals

The chemical reagents used in this study were primarily sourced from Sigma Chemical Company (St. Louis, MO, USA), including DPPH (1,1-diphenyl-2-picrylhydrazyl). Other reagents, such as quercetin, folin, gallic acid, sodium nitrite, aluminum chloride, chloroform, hydrochloric acid, sodium carbonate, trichloroacetic acid (TCA), ferric chloride, potassium ferricyanide, Tween 40, ferrous chloride, and potassium phosphate, were of analytical grade.

### 2.2. *Pisum sativum* samples

In June 2020, seeds of *Pisum sativum* were collected from various regions in southern Tunisia, with the specific localities detailed in Table 1. After collection, the seeds were air-dried to a constant weight, then powdered, sorted, and stored in airtight glass containers at -20°C until further analysis.

**Table 1**. Collection sites and characteristics of *P. sativum* L. seeds gathered from various arid regions in southern Tunisia.

| code | Origin | Latitude (N) | Longitude (E) | Altitude (m) |
|---|---|---|---|---|
| **P1** | Ksar Jawama, Medenine | 33°15’ | 10°11’ | 506 |
| **P2** | Ksar hallouf, Medenine | 33°15’ | 10°11’ | 506 |
| **P3** | Mareth, Gabes | 33°37’ | 10°16’ | 48 |
| **P4** | Mareth, Gabes | 33°37’ | 10°16’ | 48 |

### 2.3. Extraction methods

Three different methods were employed for the extraction of phenolic compounds: maceration, Soxhlet, and ultrasonic extraction. To avoid the use of organic solvents and align with traditional ethnomedicinal practices for pea seed consumption, water was chosen as the extraction solvent. The pea samples were air-dried under controlled conditions at a temperature of 25°C for 72 hours to preserve their integrity, followed by storage in airtight containers at 4°C to ensure the stability of their bioactive compounds and prevent any

degradation, thus ensuring reproducibility of the results.

Air-dried pea seeds (5 g) were extracted with 100 mL of water for 24 hours using the maceration method, while Soxhlet extraction was performed with seven siphons (Aguilar et al., 2018), and ultrasonic-assisted extraction involved 30 minutes in a sonication bath at 30°C (Li et al., 2019). After filtration, the extracts were lyophilized at −80°C for 48 hours and stored at +4°C until further analysis.

### 2.4. Total polyphenols and flavonoid contents

The extraction of total polyphenols and flavonoids from *Pisum* sativum seeds was carried out using the Folin-Ciocalteu method and aluminum chloride colorimetric assay, respectively Javanmardi et al. (2003). To assess the total polyphenol content, 0.5 mL of the extract was combined with 2.5 mL of Folin-Ciocalteu reagent (diluted 1:10) and left to incubate for 5 minutes. After this, 2 mL of a 7.5% sodium carbonate solution was added, and the mixture was kept in the dark at room temperature for 30 minutes. Absorbance readings were taken at 765 nm, and the polyphenol content was reported in milligrams of gallic acid equivalents (GAE) per gram of dry extract.

For flavonoid quantification, 0.5 mL of the extract was mixed with 2 mL of distilled water and 0.15 mL of a 5% sodium nitrite solution. Following 5-minute incubation, 0.15 mL of 10% aluminum chloride was added. After an additional 6 minutes, 1 mL of 1M sodium hydroxide was incorporated, and the volume was brought up with 2 mL of distilled water. Absorbance was measured at 510 nm, and the flavonoid content was expressed in milligrams of quercetin equivalents (QE) per gram of dry extract. Each analysis was performed in triplicate for precision.

### 2.5. Condensed tannin content

To assay condensed tannins, combine 1 mL of pea extract with 5 mL of freshly prepared vanillin reagent (4% vanillin in methanol and concentrated hydrochloric acid, 1:1). Incubate the mixture at room temperature for 15 minutes. Then, measure the absorbance at 500 nm using a UV-Vis spectrophotometer. Calculate the tannin concentration by referencing a standard curve of catechin and express the results as catechin equivalents per gram of extract (Tlili et al., 2014). Each analysis was performed in triplicate for precision.

### 2.6. Ferric reducing antioxidant power assay (FRAP)

The protocol, based on Yahia et al (2020), involves assessing the iron (III) reduction capacity of extracts. Extracts are prepared at various concentrations and mixed with potassium ferricyanide and phosphate buffer, then incubated at 50°C. After adding trichloroacetic acid and centrifuging, the supernatant is mixed with ferric chloride. The absorbance of the resulting solution is measured at 700 nm. Higher absorbance indicates greater reducing power, with results reported as EC50, the concentration needed to reduce absorbance by 50%. Each analysis was performed in triplicate for precision.

### 2.7. $ABTS^+$ (2,20 -azino-bis (3-ethylbenothiazoline-6-sulfonic acid))

The $ABTS^+$ radical scavenging activity was evaluated using a protocol with ascorbic acid as a reference (Yahia et al., 2020). The $ABTS^+$ radical was generated by reacting a 7 mM ABTS stock solution with 2.45 mM potassium persulfate and incubating in the dark for 12–16 hours. The resulting $ABTS^+$ solution was then diluted with methanol to reach an absorbance of about 0.70 at 734 nm. For the assay, 100 µL of each extract or ascorbic acid (used as a positive control) at different concentrations was added to 2 mL of the $ABTS^+$ solution. After incubating the mixture at room temperature for 6 minutes, absorbance was measured at 734 nm using a UV-Vis spectrophotometer. The percentage of $ABTS^+$ radical scavenging activity was determined by comparing the reduction in absorbance to the control, thereby assessing the antioxidant capacity of the samples based on their ability to neutralize $ABTS^+$ radicals.

Each analysis was performed in triplicate for precision.

### 2.8. DPPH (1,1-diphenyl-2-picrylhydrazyl)

To evaluate the free radical scavenging activity of pea extract using water as the solvent, the (1,1-diphenyl-2-picrylhydrazyl) (DPPH) assay was employed (Bersuder et al., 1998). First, a 0.1 mM DPPH solution was prepared in methanol. Pea extracts, extracted with water, were diluted to various concentrations for testing. Each extract was mixed with 3.9 mL of the DPPH solution, and the reaction was allowed to proceed in darkness at room temperature for 30

minutes. Absorbance at 517 nm was then measured using a UV-Vis spectrophotometer. The antioxidant activity was quantified by calculating the percentage reduction in DPPH radicals compared to a control without extract. The IC50 value, which represents the concentration needed to inhibit 50% of the DPPH radicals, was determined to assess the antioxidant capacity of the pea extract. Each analysis was performed in triplicate for precision.

### 2.9. Statistical analyses

All analyses for each extract were conducted in triplicate. Statistical evaluations were carried out using Xlstat for Windows (version 2016). Post-hoc multiple comparison tests were applied using Duncan's new multiple range tests, with significance established at $p < 0.05$. The results are presented as the mean ± standard deviation.

overall health. Known as the primary group of secondary metabolites in various plants, polyphenols are renowned for their potent antioxidant capabilities, effectively scavenging free radicals and helping to protect the body from oxidative damage (Halliwell and Gutteridge, 2015).

The current analysis outlines the phenolic, flavonoid, and tannin concentrations obtained through three distinct extraction methods from *P. sativum* seeds, as detailed in Table 2. The data reveal that extraction techniques significantly influenced the phenolic content. Soxhlet generally extracted the highest polyphenol levels, particularly in samples P1 and P4. Maceration was most effective for flavonoid extraction, especially in P2 and P3, and consistently produced the highest tannin content across all samples. While ultrasound was less effective for polyphenols, it showed comparable results for tannins and flavonoids, though slightly lower than maceration. Studies have demonstrated that Soxhlet extraction yields higher phenolic content in peas compared to other methods like maceration or ultrasound (Shi et al., 2022). Similar trends have been observed in other crops, such as grains and oilseeds, where Soxhlet consistently produces

**Table 2.** Total Polyphenol Content (TPC), Total Flavonoid Content (TF), and Condensed Tannin Content (CTC) in pea seeds extracted using three different methods.

| | Extraction methods | | |
|---|---|---|---|
| | **Soxhlet** | **Maceration** | **Ultrasound** |
| **Total polyphenols content (mg gallic acid equivalent/g DW)** | | | |
| **P1** | 12,17±1,32$^{ab}$ | 11,22±0,25$^{b}$ | 11,98±0,33$^{a}$ |
| **P2** | 10,77±0,26$^{ab}$ | 12,47±0,44$^{ab}$ | 9,82±0,52$^{c}$ |
| **P3** | 9,52±0,05$^{b}$ | 8,47±0,13$^{c}$ | 8,12±0,1$^{d}$ |
| **P4** | 12,84±2,91$^{a}$ | 13,11±1,49$^{a}$ | 10,78±0,73$^{b}$ |
| **Total flavonoids content (mg quercitin equivalent/g DW)** | | | |
| **P1** | 2,34±0,28$^{a}$ | 1,95±0,71$^{a}$ | 2,17±0,44$^{a}$ |
| **P2** | 1,85±0,16$^{a}$ | 2,07±0,38$^{a}$ | 1,9±0,37$^{a}$ |
| **P3** | 1,11±0,29$^{b}$ | 1,54±0,17$^{a}$ | 1,41±0,28$^{a}$ |
| **P4** | 1,92±0,49$^{a}$ | 1,74±0,28$^{a}$ | 1,84±0,62$^{a}$ |
| **Condensed tannins content (mg catechin equivalent/g DW)** | | | |
| **P1** | 1,22±0,64$^{a}$ | 2,91±1,05$^{a}$ | 2,33±0,22$^{a}$ |
| **P2** | 1,09±0,04$^{a}$ | 2,34±0,97$^{a}$ | 2,41±0,37$^{a}$ |
| **P3** | 1,17±0,53$^{a}$ | 2,61±0,65$^{a}$ | 2,3±0,77$^{a}$ |
| **P4** | 1,03±0,36$^{a}$ | 2,74±0,44$^{a}$ | 2,61±0,47$^{a}$ |

*Different letters in different extracts indicate significant differences ($p < 0.05$).*

## 3. RESULTS AND DISCUSSION

### 3.1. Total phenolic, flavonoid, and condensed tannin contents

Polyphenols are gaining recognition as essential bioactive substances in plants, playing a key role in supporting cellular function and promoting

greater concentrations of polyphenols, flavonoids, and tannins due to its extended extraction process (Alara et al., 2018; Aspé and Fernández., 2011).

Maceration is a straightforward and economical extraction method that involves soaking plant materials in solvents at ambient temperature, which allows for the gradual release of bioactive compounds such as polyphenols and flavonoids. This technique is particularly advantageous for extracting heat-sensitive compounds, making it an ideal choice for certain crops (Zhang et al., 2018). In contrast, ultrasound-assisted extraction (UAE) employs high-frequency sound waves to break down plant cell walls, significantly boosting the extraction efficiency of phenolic compounds in a shorter period. Research has shown that UAE delivers higher phenolic yields compared to traditional methods, due to its ability to better penetrate plant tissues (Chukwumah et al., 2009; Bermúdez-Aguirre et al., 2011 ; González-Centeno et al. (2014); Almusallam et al., 2021).

These variations between extraction methods may be attributed to several factors, including the extraction method employed, the choice of solvents, and the geographical origin of the samples, as noted in earlier studies (Sfahlan et al., 2009; El-Chaghaby et al., 2014).

### 3.2. Antioxidant activity and correlation anlysis

The antioxidant potential of pea extracts was assessed using the FRAP, DPPH, and ABTS assays. DPPH is a stable free radical frequently used to assess the free radical scavenging ability of various samples and extraction methods. The reducing power was determined, and the EC50 values represent the concentration required to achieve 50% of maximum absorbance.

The Table 3 assesses the antioxidant activity of four pea samples (P1, P2, P3, P4) using three extraction methods: Soxhlet, maceration, and ultrasound. Ultrasound extraction consistently demonstrated the highest antioxidant activity, particularly in terms of reducing power and

**Table 3**. Antioxidant Capacity and Reducing Power of local pea seeds Extracted by Three Different Methods.

| | Extraction methods | | |
|---|---|---|---|
| | **Soxhlet** | **Maceration** | **Ultrasound** |
| **Reducing power (EC50; mg/mL)** | | | |
| **P1** | 51,23±3,32$^{b}$ | 45,12±3,59$^{b}$ | 28,91±4,11$^{b}$ |
| **P2** | 67,11±8,92$^{a}$ | 50,78±1,89$^{a}$ | 37,51±0,52$^{a}$ |
| **P3** | 47,66±1,88$^{b}$ | 39,82±3,16$^{c}$ | 27,18±1,75$^{b}$ |
| **P4** | 42,55±3,17$^{b}$ | 37,48±2,19$^{c}$ | 31,29±2,74$^{b}$ |
| **ABTS (mg of ascorbic acid equivalent/g DW)** | | | |
| **P1** | 72,24±5,12$^{b}$ | 91,74±11,01$^{b}$ | 160,23±14,07$^{b}$ |
| **P2** | 87,15±10,03$^{a}$ | 110,23±9,47$^{a}$ | 197,44±12,55$^{a}$ |
| **P3** | 69,22±3,1$^{b}$ | 80,47±8,6$^{b}$ | 150,78±6,16$^{b}$ |
| **P4** | 65,33±7,18$^{b}$ | 79,4±5,67$^{b}$ | 140,78±8,11$^{b}$ |
| **DPPH (IC50; mg/mL)** | | | |
| **P1** | 28,47±1,12$^{bc}$ | 37,15±3,21$^{ab}$ | 35,12±2$^{bc}$ |
| **P2** | 30,6±0,91$^{b}$ | 40,81±1,65$^{a}$ | 38,17±0,85$^{a}$ |
| **P3** | 33,85±2$^{a}$ | 39,5±0,54$^{a}$ | 37,4±1,2$^{ab}$ |
| **P4** | 27,45±0,74$^{c}$ | 35,11±1,32$^{b}$ | 34,78±0,34$^{c}$ |

*Different letters in different extracts indicate significant differences ($p < 0.05$).*

Both techniques have distinct benefits, with maceration suited for preserving delicate compounds, while ultrasound extraction offers a faster and more efficient process.

ABTS scavenging, with P2 showing the best results. Soxhlet extraction was most effective for DPPH scavenging, especially in P3 (IC50: 33.85 mg/mL), indicating strong antioxidant potential for this method. Maceration generally resulted in

lower antioxidant activity compared to the other techniques. Overall, ultrasound emerged as the most efficient extraction method, with Soxhlet also performing well for DPPH assays. The extraction method significantly influences the antioxidant activities of pea extracts worldwide. Research indicates that ultrasound-assisted extraction (UAE) consistently yields higher levels of phenolic compounds and enhanced antioxidant capacity compared to traditional methods. For example, a study found that UAE resulted in a greater extraction efficiency of phenolics, leading to increased antioxidant activity in green peas (Wang et al., 2020). Conversely, Soxhlet extraction, although effective for obtaining bioactive compounds, often results in lower antioxidant activity due to the thermal degradation of sensitive components Khodapanahi et al., 2012).

The results of correlation (Table 4) indicated that total phenolic and flavonoid content were strongly correlated to the antioxidant activities using a maceration method. The total flavonoid content showed a significant correlation with ABTS ($R^2$= 0.875) and reducing power assay ($R^2$= 0.853). Other papers have reported similar results (Fuentes-Alventosa et al., 2009). Moreover, researcher emphasized that the choice of extraction method could significantly impact the antioxidant capacities of different pea varieties, underscoring the importance of method selection in optimizing bioactive compound recovery (Samart et al., 2024). Overall, these findings indicate that extraction methods play a crucial role in determining the antioxidant potential of pea extracts globally.

## 4. CONCLUSIONS

The current study demonstrates that pea seeds are a valuable source of polyphenolic compounds with significant antioxidant activity, offering great potential for applications in the pharmaceutical and food industries. Therefore, countries that cultivate peas should capitalize on this agro-industrial by-product. It was clear in this study that the highest concentrations of phenolic acids and radical scavenging activity were obtained when using the soxhlet method. The results also show that maceration extraction exhibited higher flavonoid and tannins contents and Ultrasound-assisted extraction demonstrated superior antioxidant activity. It is important to recognize that future industrial applications will require addressing various challenges, particularly improving the long-term stability of polyphenolic compounds and enhancing their bioavailability. Further research should also explore the impact of additional environmental factors, like soil salinity and climate conditions, on the phenolic content and antioxidant capacity of these pea populations.

**Table 4.** Correlation Coefficients between Total Polyphenol, Flavonoid, and Condensed Tannin Contents and DPPH, ABTS, and FRAP Assays for the Three Extraction Methods

| Methods of extraction | | FRAP | ABTS | DPPH |
|---|---|---|---|---|
| **Soxhlet** | **Total polyphenol content** | -0,330 | -0,314 | -0,990** |
| | **Total flavonoid content** | 0,135 | 0,135 | -0,867** |
| | **Total condensed tannins** | 0,037 | -0,006 | 0,347 |
| **Maceration** | **Total polyphenol content** | 0,217 | 0,355 | -0,349 |
| | **Total flavonoid content** | 0,853** | 0,875** | 0,226 |
| | **Total condensed tannins** | -0,522 | -0,619* | -0,775* |
| **Ultasound** | **Total polyphenol content** | 0,088 | -0,046 | -0,730* |
| | **Total flavonoid content** | 0,317 | 0,273 | -0,473 |
| | **Total condensed tannins** | 0,340 | -0,277 | -0,473 |

*Different letters in different extracts indicate significant differences ($p < 0.05$).*

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