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**EXTRACTION OF POLYPHENOLS FROM BIGARADE (*CITRUS AURANTIUM* L.) LEAVES USING WATER BATH AND ULTRASOUND**

By

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A thesis submitted to the Department of Biology in partial fulfillment of the requirements for the degree of Master of Science in Biology

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# **ACKNOWLEDGEMENTS**

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# **ABSTRACT**

Thanks to their bioactive compounds, plant by-products such as bigarade leaves gained importance due to their potential capability to reduce disease risk factors caused specifically by oxidative stress and aging. The lack of convenient extraction techniques and the excessive amounts of organic solvents and energy use by conventional extraction called attention to the need of reinforcing and emphasizing the trend of “green chemistry”.

In this study, ultrasound-assisted extraction (UAE) and conventional water bath (WB) extraction of total phenolic compounds (TPC) from *Citrus aurantium* leaves were investigated along with the corresponding antiradical activity. The effects of extraction time, temperature, solid-liquid ratio and solvents (water, ethanol and methanol) were studied using both extraction techniques. The optimized extraction parameters were as follows: for the water bath, 2 hours, 60°C, solvent water at 1: 40 solid-liquid ratio. For the ultrasound: 25 minutes, 45°C, 75% ethanol and 1:50 solid-liquid ratio.

Under these optimal conditions, the yield of total phenolic compounds was improved by about 30% using UAE compared to WB, without impairing the antiradical activity. The outcomes of this study indicate that UAE is beneficial and a more cost efficient technique of extraction of bioactive compounds from bigarade leaves.

*Keywords:**Citrus aurantium,**total phenolic compounds, ultrasound-assisted extraction, water bath*

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**LIST OF ABBREVIATIONS**

|  |  |
| --- | --- |
| ATP | Adenosine Triphosphatase |
| DE | Degree of Esterification |
| DM | Dry Matter |
| DNA | Deoxyribonucleic Acid |
| DW | Dry Weight |
| EO | Essential Oil |
| FAO | Food and Agriculture Organization |
| GAE | Gallic Acid Equivalent |
| HDL | High-density Lipoprotein |
| HIV | Human Immunodeficiency Virus |
| HPLC | High-performance Liquid Chromatography |
| iNOS | Inducible Nitric Oxide Synthase |
| LDL | Low-density Lipoprotein |
| MIC | Minimum Inhibitory Concentration |

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**CHAPTER 1**

**INTRODUCTION**

1.1 Citrus Fruits

The high-demand and consumption of citrus fruits worldwide (Figure 1.1) have impacted their cultivation. They constitute one of the main agricultures, according to data from the “Food and Agriculture Organization of the United Nations” (FAO), with strong and fast development from around 30 million metric tons in the late 1960s to around more than 150 million metric tons in the early 2000s (Liu, Heying, & Tanumihardjo, 2012).



**Figure 1.1: Citrus Fruits (https://healthyeating.sfgate.com/health-benefits-citrus-fruits-7925.html)**

1.2 Citrus Fruits Bioactive Compounds

The medical benefits offered by these fruits have made them a prominent source for humans prior to the introduction of drugs and chemical treatments. There is a growing interest in the valorization of fruits and vegetables by-products because they contain various bioactive compounds namely phytochemicals that are considered as an appropriate curative approach for controlling the incidence and progression of several diseases (Mustafa, Arif, Atta, Sharif, & Jamil, 2017).

*1.2.1 Pectin*

Pectin consists of complex polysaccharides rich in residues of galactosides and is widely present in citrus fruits. It is composed of smooth and hairy chain regions that comprise hydroxyl, ester, carboxylate and amine groups (Nigoghossian et al., 2015). Pectins have been identified to display anti-mutagenic efficacy and to suppress cancer's metastasis and expansion, with no signs of inflammation or other serious side effects (Dimopoulou et al., 2019).

*1.2.2 Vitamin C*

Vitamin C (ascorbic acid) is highly known as one of the strongest water-soluble antioxidants present in citrus fruits. Reactive oxygen species (ROS) can be causative agents of an enormous number of human diseases. Antioxidant compounds offer defense against free radicals. Interestingly, vitamin C has been associated with more than 65% of antiradical and antioxidative properties in many fruits and their juices (Zulueta, Esteve, Frasquet, & Frígola, 2007).

1.4 Extraction Methods

The rising industrialization of agro-food goods, particularly those from fruits or vegetables origins, are generating by-products namely leaves, peels, seeds, bagasse or even waste water that are all viewed as agro-industrial remnants that can represent between 20% and 50% of the entire weight of the plant material (Dahmoune et al., 2013). These waste products are considered to have an added value for pharmaceutical purposes if bioactive compounds could be extracted from them or even if they could be employed as food additives or in cosmetic products (Barba, Zhu, Koubaa, Sant'Ana, & Orlien, 2016).

* + 1. *Factors Affecting Phenolic Content*

Phenolic compounds diffuse on the basis of the solubility induced by their polarity in the cell. Hydrophilic molecules are primarily found in cell vacuoles, whereas other substances such as most lignins, flavonoids and water-insoluble polyphenols are retained in the cell wall by hydrophobic bonds with enzymes and polysaccharides (Galvan d’Alessandro, Kriaa, Nikov, & Dimitrov, 2012). For this purpose, it is important to choose the extraction solvent based on the solvability of the compounds targeted. Moreover, availability and affordability of polyphenols must be studied (Tomšik et al., 2016). Methanol and ethanol solvents have a significantly reduced polarity relative to water, which leads to the solvability and diffusion of phenolic contents by minimizing the solvent's dielectric constant. Previous studies, nevertheless, conclude that the use of extremely purified organic solvents could contribute to plant cell dehydration and collapse, along with denaturation of cell wall components, making it hard to extract phenolic compounds (Garcia-Castello et al., 2015).

*1.4.2 Water Bath (WB)*

Conventional extraction techniques for phenolic compounds and antioxidants such as soxhlet and maceration are considered time consuming and demand high solvent quantities (Wang & Chen, 2006).

*1.4.3 Ultrasound Assisted Extraction (UAE)*

Currently, new suggested studies aim at exploring innovative extraction systems to minimize and avoid pollution. In this framework, ultrasound assisted extraction was shown to be an innovative extraction technology that emphasizes highly advantageous consequences based on various results obtained in multiple optimized studies. Thus, it is considered currently in the field of “Green Chemistry” within the zone of “Sustainable Chemistry” because of the reduced use of energy; it is an environmental friendly system (Medina-Torres, Ayora-Talavera, Espinosa-Andrews, Sánchez-Contreras & Pacheco, 2017).

1.4.3.1 Characteristics and mode of function

UAE relies on the concept of acoustic cavitation which has the ability of breaking cell walls of the plant matrix, therefore allowing the release of bioactive compounds by forming bubbles as consequence of modifications in temperature and pressure (Tiwari, 2015). It depends on the propagating mechanical waves, made of a group of circuits determined as a mix of upper and lower pressure, recognized as contractions and rarefactions accordingly (Awad, Moharram, Shaltout, Asker, & Youssef, 2012).

1.4.3.2 Effect of parameters of UAE in performance and output

The UAE can be conducted in a continuous or pulsatile style; both of which refer to the irradiation performance (Galvan d’Alessandro, Kriaa, Nikov, & Dimitrov, 2012).

**CHAPTER 2**

**MATERIALS AND METHODS**

2.1 Sample Preparation

The leaves were harvested from Tripoli – El Mina – Port Said Street – North Lebanon during April – June, 2019. They were chosen on the basis of established criteria: the selected leaves had no signs of injury or any other damage (Figure 2.1 A). The leaves were washed in tap water to remove impurities (Figure 2.1 B) then each leaf was dried immediately using towels. Next the leaves were left to be air dried on bench papers (Figure 2.1 C) for 4 weeks. The dried leaves were then ground at room temperature using a blender (Figure 2.1 D). Thus, the obtained ground samples were stored in glass jars, sealed, coated with aluminum foil, labeled and stored away from light in the fridge (Figure 2.1 E).

**CHAPTER 3**

**RESULTS**

Effects of extraction time, temperature, solid-liquid ratio and solvent concentration on the yield of Total Phenolic Compounds TPC were investigated in this study. To our knowledge, there are no comprehensive studies on bigarade leaves that assess extraction parameters. Also, the current study encompassed the use of ultrasound for the recovery of total phenolic compounds, in order to compare results with those obtained using the conventional water bath extraction.

3.1 Dry Matter Analysis

The percentage of dry matter was calculated after drying 5 g of ground leaves in an oven at 80°C, until stabilization of the final weight. The average dry matter was 91.5% (Table 3.1).

**Table 3.1: Dry Matter Analysis**

|  |
| --- |
| DM (%) |
| Jar 1 | 91.1 |
| Jar 2 | 91.48 |
| Jar 3 | 90.43 |
| Jar 4 | 92.85 |
| **DM average** | **91.465 ± 1.02** |

3.2 Extraction Using Water Bath (WB)

*3.2.1 The Effect of Extraction Time*

The main focus of this work was to determine the time at which total phenolic compounds (TPC) of bigarade leaves reaches maximal yield. Samples were collected at different time intervals (Figure 3.1) until 4 hours.

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**APPENDIX A: Title**