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# OPTIMIZATION OF MICROWAVE EXTRACTION METHOD OF TOTAL POLYPHENOLS FROM *MELISSA OFFICINALIS* L. VITROPLANTS

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***Key words:*** *polyphenols, microwave,"in vitro" culture, extract, lemon balm*

## INTRODUCTION

Plants synthesize in addition to the basic compounds necessary to their survival (carbohydrates, proteins and lipids), a wide range of bioactive compounds of economic importance.

Polyphenols are an important class of bioactive compounds that have been shown to have numerous pharmacological effects: antioxidant (Scalbert et al., 2005; Pandey and Rizvi, 2009), antiinflammatory (Boots et al., 2008; Pastor et al., 2009), antitumoral (Kandaswami et al., 2005; Lakhanpal and Rai, 2007), antimicrobial (Cushin and Lamb, 2005; Nohynek et al., 2006) and antiviral (Likhitwitayawuid et al., 2005; Chávez et al., 2006). Several epidemiological studies have shown a correlation between the consumption of foods rich in phenolic compounds and a low risk for the development of cardiovascular and neurodegenerative diseases (Spencer, 2010; Weichselbaum et al., 2010).

The special economic importance of polyphenols as well as the rapidity of the species extinction process and the narrowing of the genetic basis of plant resources in the world stimulated the reconsideration of the vital importance of genetic resources and oriented the researches from last period on finding efficient methods of bioactive compounds extracting from plants. However, until now, the optimal conditions for polyphenols extraction have not been sufficiently investigated.

There are numerous factors that influence the extraction efficiency, such as: the extraction method, solvent type and concentration, temperature and extraction time.

Generally, the extraction can be accomplished by discontinuous processes (maceration, percolation, infusion, decoction, and new performance methods: solvent accelerated extraction, microwave assisted extraction, supercritical fluid extraction), and continuous processes (continuous extraction with organic solvents, continuous percolation, Soxhlet extraction) (Paun et al., 2011).

Among these, microwave assisted extraction (MAE) offers a number of advantages over conventional extraction methods such as short extraction times, with low energy and solvent consumption, and high extraction efficiency (Huie, 2002). This is a relatively recent technique that uses microwave energy to heat the solvent and the sample in order to increase the mass transfer rate between the dissolved substances from the sample matrix and the solvent, contributing to their easier passage into solvent. In this case, a polar solvent or a mixture of miscible polar solvents is used, due to the fact that non-polar solvents do not almost absorb the microwave radiation.

Polyphenols are dipoles that can absorb the energy of microwaves due to their hydroxyl groups. Therefore, MAE is a technique that can be used to extract these compounds (Venkatesh and Raghavan, 2004; Ajila et al., 2011).

The present study proposes the use of the microwave extraction method to obtain *Melissa officinalis* extracts rich in polyphenols.

*Melissa officinalis* L. (lemon balm) is a perennial plant that belongs to the *Lamiaceae* family. Lemon balm is native to southern Europe, but is currently acclimated throughout the world, from North America to New Zealand (Jastrzebski-Stojko et al., 2013).

The option for this species was not incidentally, the lemon balm being a particularly valuable plant from a medical point of view.

*Melissa officinalis* is used internally (as extract, tea, powder, tincture, infusion, decoction, wine, oil) and externally (in the form of local baths, cataplasms, gargle) in traditional medicine for its many pharmacological effects: antioxidant (Lara et al., 2011; Spiridon et al., 2011; Dias et al., 2012; Martins et al., 2012; Luño et al., 2014; Benedec et al., 2015), antimicrobial (Mimica-Dukic et al., 2004; Hancianu et al., 2008), antitumoral (Yoo et al., 2011; Queiroz et al., 2014; Jahanban-Esfahlan et al., 2015; Weidner et al., 2015), antiviral (Nolkemper et al., 2006; Sanchez-Medina et al., 2007; Mazzanti et al., 2008; Astani et al., 2012; Astani et al., 2014), antiinflammatory (Bounihi et al., 2013).

Much of the pharmacological action that owns this plant is due to polyphenols.

Therefore, out of the multitude of bioactive principles contained by the lemon balm, this class of

compounds have been selected to be evaluated in the present study.

Although this species is known from the point of view of the components with pharmacological potential, the introduction in the research of the *in vitro* cultures as a biological model of work and MAE as a method of extracting polyphenols will lead to obtaining of important scientific data regarding to valorization on unconventional ways of the regenerative potential of the plant cell as an expression of morphogenetic and biochemical totipotency.

The biological working model used (*in vitro* culture) is an efficient and reproducible alternative for the production of plant material with superior biological and phytosanitary qualities. This method has a number of advantages as compared to classical multiplication methods: requires a reduced quantity of biological material for the initiation of *in vitro* cultures; is much faster than *in vivo*; ensures the production of free material of pathogens by the initial disinfection of the biological material and its cultivation under aseptic conditions; requires restricted space and is carried out throughout the year; does not require application of pesticides and herbicides; creates the possibility of tracking the production of bioactive compounds under controlled conditions, etc.

In addition, because many medicinal plants are part of the spontaneous flora, harvesting from their natural habitats poses a risk of overexploitation. Thus, the introduction of medicinal plants in the culture leads to the conservation of the spontaneous flora, avoiding the destruction of some species and the disturbances that may occur in the cenologic balance of the biotope.

Another remarkable aspect observed in recent years is that climatic conditions have significantly affected the biological potential of medicinal plants, both in terms of species number and density. Also, optimal harvesting periods can not be staggered from a calendar point of view, but only phenological. The use of *in vitro* cultures for the production of medicinal plants provides the possibility of programming the production both quantitatively and as a term of obtaining according to necessities, independent of the season and the latency phenomena. In this way, harvesting can be done in the optimal period, when the content in active principles is the highest. Also, drying can be carried out immediately after harvesting, under the best conditions, or the product may be delivered for processing fresh.

Due to the different types of stress to which medicinal plants from the spontaneous flora are subjected, have appeared genetic changes with increasing frequency that lead to compromising of raw material.

Micropropagation ensures the genetic uniformity of the propagating material obtained.

Also, it has been demonstrated that by their cultivation, respecting the biological particularities of plants, the content in active principles not only maintains but in most cases is improving.

All these factors make extraction of bioactive principles from medicinal plants from spontaneous flora extremely inefficient (Atanasov et al., 2015; Ochoa-Villarreal et al., 2016) and emphasizes the need for new approaches to the production of secondary metabolites.

In this study, researchers have been carried out on the microwave assisted extraction of total polyphenols from lemon balm vitroplants, in order to optimize the extraction parameters such as type and concentration of the solvent, temperature and time of the extraction.

## MATERIAL AND METHOD

***Biological material***

In order to optimize the total polyphenols extraction from lemon balm vitroplants by MAE, it was necessary to obtain the plants by *in vitro* multiplication to ensure the useful biological material.

Thus, explants (apexes and nodal fragments) sampled from mother plants were inoculated on aseptic culture medium MS (Murashige and Skoog, 1962) supplemented with 0.5 mg/l BAP (benzylaminopurine) in order to trigger the organogenesis processes that led to the regeneration of new individuals. Cultures were grown in the growth chamber under controlled conditions (temperature $25\pm1^0$C, photoperiod 16 hours light and light intensity 3000 - 3500 lx).

***Microwave assisted extraction (MAE)***

Extractions were performed using the microwave synthesis and extraction system model MAS-II, producer Hanon Instruments - China.

The fresh plant material was triturated by adding the extraction solvent (ethanol) gradually. It was investigated efficiency of ethanol 96% and ethanol 70% on the extraction of polyphenols from *Melissa officinalis* vitroplants.

The plant material/solvent ratio was 1/10. After entire amount of solvent has been added, MAE at different temperatures (25, 40, 60°C) and extraction times (5, 10, 15 minutes) was performed. The microwave power was controlled and maintained at 250 W. Magnetic stirring at 200 rpm has been done.

The obtained extracts were analyzed in terms of total polyphenols content.

***Determination of the total polyphenols***

Dosing of the total polyphenols was performed using the Folin - Ciocalteu reagent (Singleton and Rossi, 1965). Tannic acid was used as standard, and the results were expressed in mg equivalent tannic acid (ETA)/ml of extract.

## RESULTS AND DISCUSSION

The present study aimed to establish optimal parameters for the MAE, in order to obtain plant extracts enriched in active principles. The concentration of extraction solvent, temperature and extraction time were the optimized parameters in the experiment.

***Effect of concentration of the solvent on polyphenols extraction***

The extraction solvent is an important parameter that can significantly influences the total polyphenols content in plant extracts. The capacity of extraction solvents to absorb microwaves obviously affects the efficiency of extraction. Interaction of microwaves with solvent molecules causes an increase of internal temperature and pressure within the plant, facilitating subsequent cell wall breakage and releasing of active compounds in the solvent (Zhang et al., 2008). Since most of the lemon balm compounds are polar molecules such as organic acids, alcohols, the corresponding derivatives, ethanol, methanol and water are suitable solvents for extraction.

However, water as an extraction solvent can cause extraction of water-soluble proteins and other unwanted compounds that would prevent further processing of the extract. Although in previous studies, methanol and acetone have been shown to be more efficient in extracting phenolic constituents from plant materials (Tabart et al., 2007; Tabaraki and Nateghi, 2011), the increase in human use of these compounds makes mandatory the extractions based on non-toxic solvents. Since methanol is toxic and has a similar effect to ethanol, ethanol has been selected for use in experiments to optimize the concentration of the extraction solvent.

Also, in the present study, water/ethanol mixtures were preferred as solvents because they give higher yields for polyphenols extraction compared with solvent systems with a single compound (Yilmaz and Toledo, 2006). By varying the water/ethanol ratio, the polarity of the solvents and consequently the solubility of the various phenolic compounds can be modified (d'Alessandro et al., 2012).

The results obtained showed that ten minutes extraction of total polyphenols from *Melissa officinalis* vitroplants was influenced by the ethanol concentration. It is found that ethanol 70% was more efficient for the extraction of polyphenols as compared to ethanol 96% at all extraction temperatures.

The total content of polyphenols varied according to the extraction temperature from 5.41 to 6.35 mg ETA/ml extract when used ethanol 70% and between 2.23 and 3.30 mg ETA/ml extract in the case of ethanol 96% (Table 1).

Table 1. Influence of the extraction solvent concentration on the total polyphenols content (values are the mean of 3 repetitions)

| Extraction time (min) | Extraction temperature (ºC) | Total polyphenols (mg ETA/ml extract) | |
|---|---|---|---|
| | | Ethanol 70% | Ethanol 96% |
| 10 | 25 | 5.41 | 2.44 |
| | 40 | 5.41 | 2.23 |
| | 60 | 6.35 | 3.30 |

From these results it can be concluded that adding a quantity of water increases the extraction efficiency. A similar result was reported in the case of MAE with solvents of the bioactive constituents of *Herba epimedii* (Chen et al., 2008). The reason for efficiency of extraction with aqueous solvents is primarily due to the water solubility of plant phenols, enhanced by the presence of a solvent which facilitates the solubilization by penetrating into the plant cell structure. In addition, the use of water in combination with alcohols led to an increase of the contact surface between the plant matrix and the solvent, which ultimately improved the yield of extraction (Chirinos et al., 2007). Therefore, ethanol 70% was chosen to be used as extraction solvent in subsequent experiments.

***Effect of time and temperature on polyphenols extraction***

The extraction of total polyphenols from the *Melissa officinalis* vitroplants was also influenced by the extraction time. Comparing the results, it can be said that, the quantity of total polyphenols increased as the extraction time increased from 5 to 10 minutes, but the extraction efficiency decreased at 15 minutes, (Table 2). Similar results have been obtained in the case of polyphenols extraction from flax seed (Beejmohun et al., 2007) and green tea leaves (Pan et al., 2003).

Longer extraction time can enhance extraction efficiency due to increased solvent diffusion in the cell (Li et al., 2003). On the other hand, longer extraction time may have additional or negative effects that lead to compounds degradation or transformation (Vongsangnak et al., 2004).

According to Fick's second law of diffusion, the final equilibrium between the concentration of the solute dissolved in the plant matrix and solvent is reached after a certain time, which means that excessive extraction time is not useful to extract more phenolic compounds, prolonging the extraction process may lead to oxidation of phenols due to exposure to light or oxygen (Chan et al., 2009). Therefore, decreasing phenolic content at certain extraction periods (in our experiments at 15 minutes) could be due to phenolic oxidation under different environmental conditions. Thus, it has been determined that the optimum extraction time was 10 minutes.

Regarding the extraction temperature, the lowest values of the total polyphenols content were obtained when the extracts have been made at a temperature of 40°C for 15 minutes (1.12 mg ETA/ml extract) and the highest quantity of total polyphenols (6.35 mg ETA/ml extract) was obtained in the case of extractions at 60°C for 10 minutes.

A higher temperature can increase the efficiency of extraction due to increased diffusion of solvent in the cell (Li et al., 2003). On the other hand, a relatively high extraction temperature led to the thermal degradation of some compounds (Vongsangnak et al., 2004). Therefore, it was concluded that the optimal temperature for obtaining high extraction yields is $40^0$C.

Table 2. Influence of temperature and time on total polyphenols extraction (values are the mean of 3 repetitions)

| Extraction time (min) | Extraction temperature ($^o$C) | Total polyphenols (mg ETA/ml extract) |
|---|---|---|
| 5 | 25 | 3.41 |
| | 40 | 2.13 |
| | 60 | 3.16 |
| 10 | 25 | 5.41 |
| | 40 | 5.41 |
| | 60 | 6.35 |
| 15 | 25 | 1.72 |
| | 40 | 1.12 |
| | 60 | 1.48 |

## CONCLUSIONS

The lemon balm vitroplants constitute a rich source of polyphenols and, also, microwave assisted extraction is an efficient method of extracting phenolic compounds from plants. These compounds can replace the synthetic antioxidants and can be used in the food, cosmetic or pharmaceutical industry.

By this study have been selected as extraction parameters of polyphenolic compounds from *in vitro* lemon balm the following: plant material/solvent ratio 1/10 (w/v), extraction solvent ethanol 70%, extraction temperature 40°C and extraction time 10 minutes.

## ABSTRACT

Polyphenols are an important class of bioactive compounds with many pharmacological effects: antioxidant, antiinflammatory, antitumoral, antimicrobial and antiviral effect.

This study aimed to optimizing the microwave assisted extraction (MAE) of polyphenols from *Melissa officinalis* vitroplants by maximizing the total polyphenols content of the extracts. The concentration of extraction solvent, temperature and time of extraction were optimized parameters in the experiment.

The following optimum extraction parameters were established: plant material/solvent ratio 1/10 (w/V), extraction solvent ethanol 70%, extraction temperature 40°C, extraction time 10 minutes, microwave power 250 W, magnetic stirring 200 rpm. By using MAE were obtained extracts rich in polyphenols which can replace synthetic antioxidants and can be used in the food, cosmetic or pharmaceutical industry.

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