Extracting and Measuring Lapachol in Devil’s Pomegranate Trees by Microwave-assisted Extraction and Gas Chromatography

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Abstract

Natural Lapachol is a naphthoquinone first extracted from the plant Tabebuia avellanedae. Many therapeutic effects including anti-tumor, anti-inflammatory, and anti-allergic properties have been attributed to this plant. Therefore, it is important to introduce a suitable method for the rapid and accurate extraction and quantification of this drug in various samples. The purpose of this study was to optimize extraction conditions of lapachol from the inner bark of the Devil’s Pomegranate using microwave energy and to analyze the derived materials by employing gas chromatography. In this research, the four important factors of duration of microwave irradiation, volume of the extraction solvent, extraction temperature, and chemical composition of the extraction solvent were studied. Results indicated extraction conditions significantly influenced lapachol extraction its efficiency. Extraction efficiency was affected by the parameters of extraction temperature, duration of irradiation, and volume and chemical composition of the extraction solvent. After optimizing experimental conditions, extraction efficiency of 97 percent was achieved. Furthermore, the limit of detection of lapachol in plant samples was 0.1 microgram per milliliter. Given these results, the mentioned optimization method reduced extraction duration, lowered the volume of the solvent used, increased extraction efficiency, and enhanced the quality of the derived extract and, therefore, it can be used to extract the small amounts of lapachol from drug and plant samples.

Introduction

Lapacho is a tree with flowers of various tones belonging to the Bignoniaceae family. The bark of the tree is used in herbal medicine. In 1981, the FDA gave lapacho a clean bill of health. Lapacho contains 3-7 percent lapachol, which is the most important therapeutic substance in lapacho. In Southern Iran, a plant similar to lapacho and of the same family, called the Devil’s Pomegranate grows the bark of which, according to reports published by researchers, contains 2-7 percent lapachol. The Devil’s Pomegranate grows in the Southern Provinces of Fars, Hormuzgan, Bushehr, and Sistan and Baluchestan. Early studies showed people for years have used the bark of this tree to relieve pain and treat wounds. Later research revealed that, like lapacho, the bark of this tree also contains 2-7 percent lapachol. As was mentioned before, the most important therapeutic substance in lapacho is lapachol, and since the Devil’s Pomegranate contains almost the same amount of lapachol as lapacho, we can expect it to have therapeutic effects similar to those of lapacho.

Lapachol was first isolated from Tabebuia avellanedae by Paterno in 1982. The presence of natural antibiotics in extracts of this plant enhances them and gives them therapeutic effects. Lapachol is a naphthoquinone with the molecular formula C_{18}H_{18}O_3 and molecular weight of 242.26 grams per mole and is soluble in organic solvents (Figure 1). It has anticancer, anti-inflammatory, anti-malarial, antiviral, antibacterial, and anti-fungal properties. Therefore, it is important to introduce a suitable method for the rapid and accurate extraction and quantification of this medicine in various samples such as body fluids, drug samples, and plant samples.

Fig. 1: Chemical structure of lapachol.
The first step in medicinal plants research, which is the starting point for the isolation and purification of chemical ingredients of plants, is to extract the active ingredients from their tissues. Plant extracts are widely used in food, pharmaceutical, and cosmetics and healthcare industries, and different extraction methods have been studied to obtain these valuable and natural compounds. Traditional extraction methods such as solvent extraction are time-consuming and require large volumes of solvents. In steam or water distillation, it takes a long time to reach the required temperature for the evaporation of volatile compounds, many of the unsaturated or ester compounds are decomposed and lost, and a substantial amount of energy and time is wasted. Methods in which chemical solvents are used for the final extraction pose the danger of poisoning by the remaining solvents. Therefore, the need has increased for new extraction methods with shorter extraction durations, less solvent consumption, and lower levels of pollution. In the past 20 years, many methods have been developed for extracting organic compounds from solid materials. Most of these methods involve stages of extraction by solvents followed by high performance gas or liquid chromatography analysis together with sensitive and selective detectors. Ultrasound-assisted extraction, supercritical fluid extraction, and microwave-assisted extraction are some of the new and rapid methods of extracting active ingredients from plant tissues. These technologies have been developed to reduce extraction duration, lower the volume of consumed solvents, increase extraction efficiency, and enhance the quality of the derived extracts. One of the simplest and most cost-effective of these is the microwave-assisted extraction method. In this technique, polar solvents such as water, methanol, or acetone are used for extracting the desired organic compounds from solid tissues. The microwave method has many advantages over customary methods including the lower extraction temperature, the lower volume of consumed solvents, and the higher recovery efficiency. Microwave-assisted extraction is one of the extraction techniques that are based on the heating of an organic solvent. The extraction substance in this method may be a liquid or a gas. The sample and the appropriate solvent (or mixture of solvents) are placed in a container and subjected to pressure applied by microwave irradiation to heat up. The extraction process is completed after 2-5 minutes, and the sample and solvent mixture is filtered after being cooled. In microwave-assisted extraction, microwaves absorbed by the sample heat it up, this heat evaporates the water in the sample and applies pressure on the cell walls and, finally, the cell walls are destroyed and the compounds are released from inside the cells into the solvent(s).

In this research, the possibility of extracting and purifying lapachol from the Devil’s Pomegranate with the help of microwave energy (that is widely used in extraction of compounds from plant tissues) was studied. The reasons for using this method were that the solvent rapidly and completely penetrates lapachol-containing cells, extraction and removal of lapachol is carried out with greater efficiency, and extraction duration is substantially less compared to common methods. Analytes were quantified using gas chromatography, and effective parameters were determined. Moreover, the effects of the parameters of irradiation duration, solvent volume, extraction temperature, and of the chemical structure of the extraction solvent on extraction efficiency were investigated.

**MATERIALS AND METHODS**

*Chemicals and reagents:*

Required solvents and chemicals with suitable analytical purity were bought from the Merck Company, and lapachol and antherquinone with 98 percent purity from the Aldrich Chemical Company. The purchased methanol, acetonitrile, n-hexane, and acetone were used without any further purification. Deionized distilled water prepared by a Purelab UHQ machine (manufactured by the Elga Company in England) was used in all experiments.

*Equipment used:*

A model Varian 3600 CP gas chromatograph equipped with a flame ionization detector attached to a CP-SIL-5 capillary column was used. A model OPGU-220 hydrogen generator manufactured by the Shimadza Company produced the required hydrogen gas for the detector and the needed oxygen was provided by a Zero Air capsule made by the Sina Chemical Analysis Company. An 85-milliliter model MARS X (1200 Watt, 2450 GHz) microwave system made by the CEM Company (Mathews, NC, USA) equipped with 14 extraction cells (made of thick Pyrex glass with protective liner made of TFM), a temperature control sensor, and a solvent leak detecting sensor was used. This system could tolerate maximum pressures and temperatures of 200 psi and 200 degrees Celsius. A model HR-200 digital balance made by the AND Company of Japan was used to weigh the analytes, and a model 6-15H Petrotest centrifuge machine made in Germany with the maximum speed of 1200 rpm for centrifuging the samples.

*Microwave-assisted extraction process:*

Two grams of dry powdered bark of the Devil’s Pomegranate were poured into each of extraction containers and different volumes of an extraction mixture of n-hexane and methanol (from 20 to 70 milliliters)
were added to the containers. After placing the temperature control sensor in one of the containers, they were transferred into an oven. The extraction process was carried out at 30 percent of the oven’s maximum capacity at a pre-determined temperature. In the designed temperature schedule, first the oven reached from room temperature to the selected one in two minutes. At the end of the extraction duration, the extracting process was stopped and a number of the containers were removed according to the pre-set plan. The extraction process of the remaining containers was then continued up to a specified time. Finally, the remaining containers were removed from the oven and cooled, the lids were taken off, and the contents were centrifuged, filtered, and kept for quantification.

Separation and identification of compounds with the help of a gas chromatograph:

Analytes were separated on a CP-SIL-5 column (length of 25 meters, internal diameter of 0.32 millimeter, and stationary phase film thickness of 0.52 micron). The carrier gas helium was used with a constant flow of 2 milliliters per minute. At first, oven temperature was raised to 100°C and maintained at this temperature for two minutes. It was then raised at the rate of 10°C/min until it reached 180°C, and kept constant for one minute. In the last stage, the temperature was raised at 20°C/min until it reached 220°C and kept constant for two minutes. The temperature of the flame ionization detector, and that of the injection port, was set at 250°C.

RESULTS AND DISCUSSION

Effects of the extraction solvent:

In the microwave-assisted extraction technique, selection of the solvent depends on its ability to absorb microwave and on its dissipation factor. Therefore, non-polar solvents such as aliphatic hydrocarbons, despite being good solvents for aromatic compounds, cannot be used alone and must be utilized along with a polar solvent such as dichloromethane, acetone, or methanol. In this research, a mixture of n-hexane and methanol was employed as suitable extraction solvents. On the one hand, n-hexane allows easy dissolution of lapachol, and on the other hand, methanol is able to absorb microwave energy because it has a permanent dipole moment. Therefore, the n-hexane and methanol solvent mixture was used at different volume/volume ratios. The Diagram in Figure 2 shows the effects of the quantity of n-hexane in the extraction mixture on lapachol extraction efficiency. As can be seen in this diagram, the 40:60 ratio of n-hexane to methanol (v/v) yields the highest efficiency. At higher methanol levels, the possibility of the dissolution of the desired compound is reduced, leading to lower extraction efficiency. At higher n-hexane levels, the ability to absorb microwave energy decreases and, hence, the 10-minute extraction duration is not long enough for the temperature fluctuations and penetration of solvent molecules into the solid matrix and for the removal of the desired compound (which leads to a reduction in extraction efficiency).

Fig. 2: Effects of high n-hexane levels in the extraction mixture on lapachol extraction efficiency.

Effects of extraction solvent volume:

Volumes of 10 to 70 milliliters of the n-hexane and methanol solvent mixtures were tested to determine the effects of solvent volume. Figure 3 indicates the 40-milliliter volume is suitable for achieving the highest efficiency. At lower volumes, extraction efficiency declines due to solvent saturation and, at higher volumes, the reduced efficiency can be attributed to the loss of the desired compound in the volatilization stage. Furthermore, high volumes of the extraction solvent increases analysis duration since more time will be required in the solvent evaporation stage.
Fig. 3: Effects of extraction solvent volume on lapachol extraction efficiency.

Effects of duration of microwave irradiation:
Periods of 5 to 17 minutes were tested to find the range of this factor. Figure 4 reveals that microwave irradiation for 11 minutes resulted in the highest efficiency. At lower periods, the solvent does not sufficiently penetrate into the tissue matrix and, at longer periods, efficiency declines, probably due to thermal decomposition.

Fig. 4: Effects of irradiation duration on lapachol extraction efficiency.

Effects of temperature on microwave-assisted extraction:
Extraction temperature is an important parameter in microwave-assisted extraction. In this research, temperatures from 70 to 130°C were tested. Figure 5 shows that 100°C yields better results.

Fig. 5: Effects of the applied temperature on lapachol extraction efficiency.
Effects of sample moisture:
Although some articles have reported the presence of some water in the sample increases extraction efficiency, this issue is more relevant to microwave-assisted extraction at room pressure. In this method, gas bubbles are formed inside sample tissues due to the high local heat generated by microwave irradiation, which will, in itself, expand the pores in the sample and facilitate solvent penetration into the sample and removal of the desired molecules. However, this does not apply to microwave-assisted extraction in closed containers where the pressure rises, and tests on dry and wet samples revealed efficiency declines in the presence of moisture. Therefore, dry samples were used in all experiments.

Recovery efficiency:
Recovery efficiency is a very important factor in evaluating analytic methods, and tests are conducted on samples containing known percentages of the desired compound to test the correctness and accuracy of these methods. In this section, experiments were performed on a plant samples spiked with two different concentrations of lapachol. Results are summarized in Table 1. As can be seen, recovery efficiency is at an acceptable level.

Table 1: Lapachol recovery efficiency at concentrations of 5 and 10 micrograms per milliliter.

<table>
<thead>
<tr>
<th>Spiked sample concentration (10μg.ml⁻¹)</th>
<th>Sample after extraction (5 μg.ml⁻¹)</th>
<th>Analyte</th>
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<tr>
<td>Spiked sample</td>
<td>Percentage recovery</td>
<td>Mean recovered quantity</td>
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Conclusions:
Designing and developing an extraction, separation, and quantification method for lapachol is an essential necessity given the importance of lapachol in pharmaceutical industry and its low concentration in most plant samples. Therefore, the ultimate purpose of this research was to find an efficient and simple method that uses the minimum quantity of solvents. Consideration of items such as plant matter characteristics, choice of suitable solvents, and accuracy in the extraction stages, is necessary in obtaining the best and most effective extraction method. It must also be kept in mind that high efficiency in extraction does not mean high efficiency in obtaining the desired compound. A microwave-assisted extraction method together with gas chromatography analysis was introduced for simple and rapid extraction of lapachol. Optimal conditions were studied and determined by changing one variable at a time. The highest extraction efficiency was achieved at solvent mixture volume of 40 milliliters, extraction temperature of 100°C, and n-hexane to methanol ratio of 0.4 (v/v). Reproducibility of the method was obtained at relative standard deviation of 3.1, which shows the method enjoys high correctness and accuracy. The detection limit was 0.1. Use of microwave energy can be an effective lapachol extraction method because it reduces extraction duration. In all, results indicate the optimal conditions for lapachol extraction can be employed for the extraction and quantification of lapachol at low concentrations in various samples with complex tissues such as those of the Devil’s Pomegranate.

REFERENCES