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Optimization of microwave assisted extraction of bioactive flavonolignan - silybinin

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Abstract: In this paper, a novel method of microwave assisted extraction (MAE) used for the extraction of a potent hepatoprotective bioactive silybinin, as a main flavonolignan from *Silybum marianum* is presented. The extracts were quantified for silybinin by HPLC. To prove the efficiency of the proposed MAE technique, it was compared with traditional methods like Soxhlet, maceration and stirring extraction. If the extraction yield obtained from MAE was to be considered as 100% performance level then, 12 h of Soxhlet extraction and 24 h of maceration and stirring extraction could attain 79.6%, 26.3% and 35% performance (in terms of extraction yield of silybinin) efficiency with high degree of reproducibility. The optimum extraction conditions were 600 W microwave power, 12 min extraction time (spread over two extraction cycles of 6 min each), 80% v/v ethanol as the extraction solvent, 20 min preleaching time and 25:1 (mL/gm) as the solvent to material loading ratio. A synergistic mechanism of heat and mass transfer was proposed to account for the accelerated extraction due to microwave.

Keywords: Microwave assisted extraction; silybinin; soxhlet; HPLC

1. Introduction

Silymarin is a standardized extract from the milk thistle *Silybum marianum* (L.) Gaertn that has been used as a medical remedy for almost 200 years [1]. In particular, it is used as a therapeutic agent in many types of acute and chronic liver diseases; in United States, 10-15% of patients attending liver disease clinics reported having taken milk thistle derivatives [2]. In addition, silymarin protects experimental animals from various hepatotoxicants such as CCl₄, acetaminophen and phalloidin, and has been shown to have anticholestatic properties as well [3]. Silymarin is composed of a mixture of several flavonolignans, with the most important being silybinin, isosilybinin, dehydroisosilybinin, silidianin and silichristin. The extract contains also a few flavonoids, mainly taxifolin and quercetin

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[1]. All these compounds account for 65% - 80% of the whole extract content, with the remaining fraction being a chemically not well defined fraction, composed mostly of polymeric and oxidized polyphenolic compounds [4,5]. Silybinin ($C_{25}H_{21}O_{10}$), which forms the bulk amount of the silymarin complex, is actually a mixture of two diastereoisomers in approximately 1:1 proportion. It is the main flavonolignan of silymarin, and has been proposed to be its major active component [4,5,6]. Silymarin and silybinin prevent lipid peroxide formation in liver cells, mainly due to their free radical scavenging properties [7]. Silymarin also has antifibrinogenic properties, and is able to increase the synthesis rate of rRNA by activating RNA polymerase I; this enhances the biosynthetic apparatus, thus increasing the synthesis rate of both structural and functional proteins [8].

Phytochemicals extracted from plant materials are of great importance to the pharmaceutical and the dietary supplement industries. Even though extraction is the starting step in qualitative and quantitative analysis of medicinal plant constituents, but until date very substantial amount of work has been done to improve the efficiency of this crucial step. An incomplete extraction process producing a poorly prepared extract is sufficient to produce the most erroneous results even with the best chromatographic system [9]. Ideally, an extraction procedure should be exhaustive with respect to the constituents to be analyzed, rapid, simple, inexpensive and with high degree of automation. Usually, the traditional techniques like soxhlet, maceration, reflux and hydrodistillation, which have been used over decade's forms the first choice for extraction of phytochemicals. However, these techniques also suffer from severe drawbacks such as long extraction time and low efficiency particularly when trace amount of compounds are present. Moreover, many natural products are thermally unstable and could be degraded with increasing temperature during the extraction. The use of large volumes of organic solvent associated with conventional methods are detrimental to environment and their subsequent disposal also becomes an issue of concern. In this respect, a procedure that could obtain most of the effective constituents in a shortest processing time with low production cost and using minimum organic solvent will be an ideal technology. Modern techniques, such as microwave assisted extraction (MAE), in recent times has proved to be a promising ideal extraction tool for the extraction of phytochemicals from botanicals [9, 10]. Many reports on the beneficial effects of MAE with respect to medicinal plants have been published, with significant improvements over conventional extraction methods offering much lowered extraction time and enhanced efficiency [9,10]. Compared with the traditional methods, MAE has many advantages, such as shorter extraction time, lesser solvent consumption, higher extraction rate and better products with lower cost. Direct interaction of microwaves with the free water molecules presents in the glands and vascular systems, causes a tremendous increase in internal pressure inside the plant cell due to evaporation of the internal moisture content which result in the subsequent rupture of the plant tissue and the release of the active compounds into the organic solvent [10]. Therefore, MAE is an interesting alternative to conventional extraction methods, especially in the case of botanical extractions.

In this study, an effective time saving extraction model using microwave energy for the improved yield of bioactive compound silybinin from *Silybum marianum* is presented. The proposed extraction model has been compared with several conventional extraction methods and the effects of various experimental conditions on the extraction yield are studied (microwave power, extraction time, ethanol concentration, preleaching time solvent volume and extraction cycle) in a systematic fashion. Until now, the extraction of silybinin from *Silybum marianum* with MAE method has not been reported. The purpose of this study is to develop a novel, ecofriendly, rapid MAE method for the efficient large scale extraction of silybinin as a potential biomarker, and to evaluate the efficiency of the proposed extraction technique against conventional extraction methods for the extraction of silybinin

2. Materials and Methods

2.1 Reagents

All solvents were from S.d Fine Chemicals (Mumbai, India) and those used for HPLC were of HPLC grade. For sample and solvent filtration, 0.45 µm membrane filters (Millipore, Germany) were used, and solvents were degassed prior to use. Silybinin was purchased from Sigma (St. Louis, MO).

2.2 Apparatus

The extraction system comprised of a modified domestic microwave oven extractor equipped with a magnetron of 2450 MHz with a nominal maximum power of 700 W, a reflux unit, 10 power levels, time controller, exhaust system, beam reflector and a stirring device.

2.3 Conventional extraction techniques

Dried seeds of Silybum marianum were provided as gift sample from Indian drugs and Botanicals (Delhi, India) and were used as received without any pretreatment. A voucher specimen (BHU/Pcog/04/2008) was deposited in the herbarium of Department of Pharmaceutics, Institute of Technology, Banaras Hindu University. Seeds were milled to homogenous 40 mesh powder (selected by sieve), immediately before the experiment.

Three conventional extraction techniques namely, Soxhlet, maceration and stirring extraction were used for comparison with MAE technique. Percentage extraction yield (w/w) for silvbinin was obtained by using the formula

Percentage extraction yield (w/w) for silvinin = $\frac{1}{Mass of seed powder taken}$ Mass of silvbinin (in extracted solution) \times 100

2.4 Soxhlet Extraction

Exhaustive Soxhlet extraction was performed using a classical Soxhlet apparatus with accurately weighed 1 g of the powdered seeds (screened through sieve 40) for 12 h. Extraction was performed with 80% v/v ethanol as the extracting solvent. The dried residue was washed twice with 10 mL petroleum ether and then finally dissolved in 10 mL methanol in a volumetric flask. The solution of the samples were transferred in the HPLC vials by filtering through 0.45 µm membrane filter.

2.5 Maceration and stirring extraction

Maceration was carried out in a closed conical flask for 24 h. Stirring extraction was carried out by continuous stirring for 24 h with the help of a magnetic stirrer in a closed conical flask. In both the cases 1 g powdered drug sample (screened through sieve 40) and 100 ml 80% v/v ethanol was used as the extracting solvent. Heat was not applied in either of the cases. The suspension after maceration/stirring was centrifuged and the supernatant evaporated under reduced pressure. The residue was then washed twice with 10 mL petroleum ether, and finally dissolved in 10 mL methanol for HPLC analysis as described earlier.

2.6 Microwave assisted extraction (MAE)

For MAE accurately weighed 1 g of the homogenous powder was mixed with 25 mL ethanol. After allowing a preleaching time of 5 min the suspension was irradiated with microwave at different experimental conditions for the optimization of the extraction parameters. The sample was treated

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under microwave irradiation in an intermittent way, i.e. irradiation:cooling:irradiation. The microwave irradiation time was 1 min and cooling time of 1 min was used to cool the sample solution between two irradiations. After extraction, the samples were centrifuged at 4000 rpm $(3520 \times g)$ and the supernatant evaporated under reduced pressure. The dried residue was washed twice with 10 mL petroleum ether and then finally dissolved in 10 mL methanol in a volumetric flask. The solution of the samples were transferred in the HPLC vials by filtering through 0.45 µm HPLC filter.

2.7 HPLC analysis

The content and composition of the main flavonolignan, silybinin was analysed by using the HPLC method of Kvasnicka *et al.* [12]. Briefly, the extracts were chromatographed on a HPLC system (Waters 746, Millipore Corporation, Milford, MA,USA), equipped with a reversed phase column. A mixture of 85% phosphoric acid-methanol-water (0.5:46:64, v/v) served as mobile phase. The elution was made in an isocratic mode at a flow rate of 1ml/min, at room temperature. The eluted peaks were monitored with an UV detector (wavelength 288 nm). Pure silybinin was used as external standard for quantitative analysis. A calibration curve in the concentration range of 1 mg/mL and 0.065 mg/mL was constructed for the quantification of silybinin.

2.8 Statistical analysis

The one way ANOVA test was used to calculate the significance of the differences of the yield of silybinin [13]. The results of HPLC analysis were expressed as means of yield \pm S.D.

3. Results and Discussion

3.1 Optimization of extraction parameters

In this study, the effects of several influential extraction parameters (microwave power, irradiation time, type of solvent, solvent composition, preleaching time, loading ratio and extraction cycle) were systematically studied for set up of the optimal extraction conditions to obtain the maximum yield of silybinin. Ethanol was used as the extraction solvent.

3.2 Effect of microwave power



Figure 1. Effect of microwave power on the yield of silvbinin

*Extraction conditions: Extraction conditions: 25 mL ethanol as extraction solvent and 5 min of preleaching time

Figure 1 highlights the typical yield power plots for the extraction of silybinin. In general, the extraction efficiency was improved by raising the microwave power from 200 to 800 W. During short irradiation time, (2 and 4 min) yield of silybinin was enhanced with microwave power increasing. When the extraction solutions were heated long enough (8 min), the yields under different powers

were similar. The difference of the silybinin yield between 200 to 600 W appears more significant with short irradiation times compared to long irradiation times. Since significant increase in extraction yield was noticed at 600 W microwave power for all extraction time, hence it was considered optimum. The accelerated extraction of silybinin by increasing microwave power can be correlated to the direct effects of microwave energy on phytomolecules by ionic conduction and dipole rotation which result in power dissipated in a volumetric fashion inside the solvent and plant material and then generate molecular movement and heating. More electromagnetic energy was transferred to the extraction system quickly and improved the extraction efficiency when the microwave power increased from 200 to 800 W. Similar explanation was also given to support the effect of microwave power on the MAE of flavonoids from *Radix astragali* [13].

3.3 Effect of irradiation time



Figure 2. Effect of extraction time (irradiation time) on the yield of silybinin *Extraction conditions: microwave power: 600 W, extraction solvent: 25 ml ethanol 5 min of preleaching time.

Figure 2 shows the duration of microwave radiation of 2, 4, 6, 8, and 10 min at 600 W microwave power on the extraction yield of silybinin. Three phases were observed in the process of microwave extraction. The first phase (1) is represented by the rise in extraction between 2 min and 4 min which characterizes the first quantities extracted, located at the surface of vegetable particles. This is followed by the second phase (2) characterized by the rise in extraction yield between 4 min to 6 min representing the intern diffusion of the target analyte from the midst of the particles towards the external medium involved by the intern warming of the natural moisture located in the plant cells. The third phase (3) which begins after 6 min marks the end of the extraction process. MAE reached the highest extraction yield of 0.72% w/w when irradiation time was 6 min. However, further increase in irradiation time resulted in no improvement in the extraction performance. Similar observations were also reported for MAE of artemisnin [14] and *slavia miltiorrhiza* [11]. Since no significant difference in extraction yield was obtained between 6 min and 8 min of the extraction time, the former was considered optimum for maximum extraction.



Figure 3. Effect of ethanol concentration on the yield of silvbinin

*Extraction conditions: microwave power: 600 W, 6 min extraction time, 25 mL extraction solvent and 5 min of preleaching time.

Figure 3 shows that the yield of silybinin was greatly influenced by the aqueous ethanol concentration. Highest yield was obtained with 80% v/v ethanol concentration. Further increase in water content resulted in fall in extraction yield. Presence of some amount of water can increase the mass transfer process by increasing the relative polarity of the solvent thus improving its solubilizing capacity and through effective swelling of the plant material, thus increasing the surface area for solute solvent interaction. However, presence of excess amount of water can cause excess thermal stress due to rapid heating of the solution on account of effective absorption of microwaves by water.

3.5 Effect of preleaching time



Figure 4. Effect of preleaching time on the yield of silybinin

*Extraction conditions: microwave power: 600 W, extraction time: 6 min, ethanol concentration: 80% v/v, solvent volume: 25 mL.

Preleaching time can be defined as the contact time between sample matrix and extracting solvent before microwave irradiation. Fig 4 shows that extraction performance kept improving until preleaching time reached 20 min which was considered most favorable for enhancing the extraction yield. Further increase in preleaching time did not show any promising effect on the extraction performance. Preleaching time of 20 min allows sufficient swelling of the plant matrix. This increased hydrated status helps is bursting of the cell wall due to internal thermal stress and enlargement of the

cellular pores thus facilitating leaching of the target analyte. Similar observations were also made in the MAE of tanshinones from *Salvia miltiorrhiza* [11].

3.6 Effect of solvent to material ratio



Figure 5. Effect of preleaching time on the yield of silybinin

*Extraction conditions: microwave power: 600 W, extraction time: 6 min, ethanol concentration: 80% v/v, solvent volume: 25 mL.

The solvent volume must be sufficient to ensure that the entire sample is immersed, especially when having a matrix that will swell during the extraction process. Generally in conventional extraction techniques a higher volume of solvent will increase the extraction performance, but in MAE a higher solvent volume may give lower yield [14,15]. To investigate the influence of solvent to material ratio on the yield of silybinin, several loading ratios (25:1, 30:1, 35:1, 40:1, mL/g) were examined. Fig. 5 shows that the yield of silybinin decreased with the increasing solvent volume beyond 30:1 ml/gm. Since no significant difference in the yield of silybinin was observed between 25:1 mL/gm and 30:1 mL/gm loading ratio hence the former was selected as the optimum. This was probably due to an inadequate stirring of the solvent when the microwaves are applied at larger solvent volumes. Moreover, larger volume of solvent (80% v/v ethanol) will cause more absorption of microwave energy and thus sufficient microwave energy may not be available for facilitating the cell breakage for effective leaching out of the target analyte [16]. Similar effects were also recorded during the MAE of artemisnin [14] and tea polyphenols and tea caffeine [17].

3.7 Effect of extraction cycle

The effect of repeated and successive extractions of the residue (extraction cycle) was investigated in this experiment. The extraction conditions were set at the optimum parameters obtained so far in the study. A second successive extraction of the residue yielded further 0.26% w/w silybinin taking the final extraction yield to 1.37% w/w. The above data reflects that 81% of the extraction was over in the first extraction cycle itself. A successive third extraction did not show any presence of silybinin.

Hence the final optimum extraction conditions as obtained from the study is as follows: 600 W microwave power, 12 min extraction time (spread over two extraction cycles of 6 min each), 80% v/v ethanol as the extraction solvent, 20 min preleaching time and 25:1 (mL/gm) as the solvent to material loading ratio.

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3.8 Proposed extraction mechanism

Microwave treatment affects the structure of the cell due to the sudden temperature rise and the internal pressure increase. The higher temperature attained by the cell wall, during MAE, causes dehydration of cellulose and reduces its mechanical strength, which allows the solvent to gain an easy entry inside the cellular channels [15,18]. During the cell wall rupture process, a rapid exudation of the chemical substance within the cell into the surrounding solvents takes place. This mechanism of MAE based on exposing the analytes to the solvent through cell rupture is different from that of heat reflux extraction that depends on a series of permeation and solubilization processes to bring the analytes out of matrix.

3.9 Stability studies

Stability at the optimum conditions derived were performed by subjecting standard silybinin (at two concentration level; 0.75 mg/mL and 1 mg/mL in 80% v/v ethanol) acid to MAE for 6 min at 600 W microwave power. The recovery of silybinin was taken as the indicative marker for the stability of silybinin at the derived operating extraction conditions. Data is indicated in Table 1. Results showed that average complete recovery at the operating extraction conditions varied from 96% to 97.3% with no change in retention time of silybinin, thus abolishing any fear of thermal degradation at the selected conditions.

3.10 Repeatability

| Compound | Initial concentration (mg/mL) | Recovered concentration after MAE, mg/mL | Relative Standard Deviation (RSD%) n = 3 | Average recovery (%) |
|-----------|----------------------------------|--|--|----------------------------|
| silybinin | 0.075 | 0.073 | 0.94 | 97.33 |
| | 1.000 | 0.96 | 1.25 | 96.00 |

Table 1. Stability studies of standard silybinin under optimum MAE conditions

*Extraction conditions: 600 W microwave power, 6 min extraction time, 25 ml extraction solution volume prepared with 80% v/v ethanol.

To determine the reproducibility of the novel extraction method five samples of 1 g each were processed under the optimum extraction conditions as obtained from the systematic study of different extraction parameters. The mean percentage extraction of silybinin obtained was found to be 1.37% w/w, which was 25.7% more efficient than 12 hrs of conventional Soxhlet extraction. The calculated R.S.D. value was 3.8%, which shows that the proposed microwave extraction method has an acceptable precision. The repeatability of the chromatographic process was also considered. An amount of 1 g sample was processed under the optimal MAE conditions. The sample was analyzed repeatedly for five times under the same chromatographic conditions. The R.S.D. of the chromatographic analysis was 0.68%.

3.11 Comparison of MAE with other conventional extraction techniques

| Extraction method | Extraction time | Solvent volume | R.S.D (%) | Response (% w/w) |
|---------------------|-----------------|----------------|------------------|---------------------|
| MAE | 6 min | 25 ml | 3.8 (n=5) | 1.37 |
| Maceration | 24 h | 100 ml | 12.4 (n=5) | 0.36 |
| Soxhlet extraction | 12 h | 100 ml | 7.6 (n=5) | 1.09 |
| Stirring extraction | 24 h | 100 ml | 11.2 (n=5) | 0.48 |

Table 2. Comparison profile of MAE with traditional extraction techniques

Response= % extraction of silybinin, w/w. Weight of the powdered leaf sample = 1 g. MAE was performed at the optimum set up as obtained in the study

The selection of an extraction method would mainly depend on the advantages and disadvantages of the processes, such as extraction yield, complexity, production cost, environmental friendliness and safety. In general, maceration and heat reflux extraction are the most frequently used extraction procedures. The drawbacks of maceration and heat reflux extraction are the large amount of solvent and long extraction time needed. Considering the expensive solvent consumption and the long extraction period, these extraction methods are not favorable from a commercial perspective. The principle of heating during MAE is based on the direct effect of microwaves on molecules by ionic conduction and dipole rotation. Ionic conduction is the electrophoretic migration of ions when an electromagnetic field is applied. The resistance of the solution to this flow of ions will result in friction and therefore heat the solution. Dipole rotation means realignment with the applied field. At 2.45 GHz, which is the frequency used in commercial systems, the dipoles align, randomize and jostle $4.9 \times$ 10⁹ times per second and this results in heating [19]. Based on that mechanism, either polar samples or polar extraction solvents are required for efficient heating. However, compared with the conventional extraction methods, MAE method showed prominent advantages with strong penetration force, high extraction efficiency, reduced extraction time, less exposure to organic solvents which can lead to better products with lower cost. In the current study, MAE was compared with the other conventional extraction techniques for the extraction of silybinin from Silybum marianum. The conditions of different techniques and their results are summarized in Table 2. It must be noted that all the extraction techniques were used under their optimized conditions. Table 2 showed that in terms of yield of target analyte, the best results were obtained by MAE, which gave significantly higher values. On extraction time, MAE was also the fastest extraction method with only 12 min of extraction time (spread over two extraction cycles of 6 min each)with preleaching time of 20 min. Stirring and heat reflux extractions are time consuming processes based on heat or mixing to increase the mass transfer rate. If the extraction yield obtained from MAE was to be considered as 100% performance level then, 12 h of Soxhlet extraction and 24 h of maceration and stirring extraction could attain 79.6%, 26.3% and 35% performance (in terms of extraction yield of silvbinin) efficiency. These features would position MAE as a valuable and cost effective technology suitable for today's highly competitive industries with growing demand for increased productivity, improved efficiency and reduced cycle time.

4. Conclusion

The effectiveness and efficient applicability of MAE technique for the extraction of bioactive compounds has been demonstrated. The technique can also be useful for the extraction of marker compounds which are present in trace amount and often get degraded when extraction is attempted through conventional techniques. Hence the proposed method can be very useful in case of chemical standardization of botanicals to meet global standards. Comparison with conventional extraction

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methods revealed that MAE could save a lot of time and electrical energy. Besides, the quantity of solvent consumed in MAE was the least which proves its environment friendly or ecofriendly feature. That means, it would save the production cost greatly. Henceforth the proposed extraction method can be called as green extraction technique with an ecofriendly edge. In addition, the green aspect of the total procedure becomes a key feature since research concerning new alternatives and new solvents in chemistry are at the moment, for earth and environment protection, a key challenge that we cannot disregard.

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