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Ultrasonic-Assisted Extraction and Conventional Extraction of Silymarin from *Silybum marianum* seeds; A Comparison.

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ABSTRACT

Ultrasonic-assisted extraction was evaluated more effective alternative to conventional maceration extraction method for the extraction of silymarin from *Silybum marianum* (L.) seeds. *Silybum marianum* (L.) seeds were extracted under indirect sonication (40 KHz water bath) and direct sonication (20 kHz probe) at different time intervals (15, 30 and 60 min). The ultrasonic-assisted extraction was compared with conventional maceration. Each extract was analysed using high performance liquid chromatography (HPLC) and the concentration of silymarin present was calculated. Results showed that increasing extraction time, increased silymarin content using all extraction methods under study. Direct sonication showed better extraction yields at all time intervals compared to indirect sonication and conventional maceration. The highest silymarin content (61.1 mg/10g of *Silybum marianum* seeds) was obtained using 20 kHz probe for 60 min extraction time.

Keywords: silymarin, *Silybum marianum*, ultrasonic extraction

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INTRODUCTION

Silybum marianum [L.] Gaertner, also known as milk thistle of the family Asteraceae, is an annual or biennial plant that is native to the Mediterranean and North Africa, but has spread to other warm and dry climates in the America, Australia, and Europe. [1, 2] *Silybum marianum* is a plant rich in phenolics. Silymarin, a standardised extract from the seeds of *Silybum marianum* contains 50–70% of flavonolignans [taxifolin, silychristin, silydianin, silybin A, silybin B, isosilybin A and isosilybin B] and other minority compounds [Fig. 1] [3].

Silymarin is advertised as a hepatoprotective, antioxidant, and free radical scavenging food supplement and has been used widely for centuries for the protection of the liver from toxic substances, treating liver damage and for the therapy of hepatitis and cirrhosis [4, 5]. Recent studies have also reported that silymarin is an effective antiviral treatment for hepatitis C virus [HCV] [6, 7]. These health-promoting properties are mainly linked to the ability of silymarin to protect the hepatocyte membrane against xenobiotic injury, which is attributed to its antioxidant potential to eliminate reactive oxygen species [ROS], it can be associated with free radical scavenging , chain-breaking activity and a reduction in ROS production [8,9]. In addition to its antioxidant properties, it has been reported to have high anti-tumor promoting activity [10] and has been linked to the prevention of skin carcinogenesis [11].

The use of conventional methods of extraction like [soxhlet, maceration, reflux and hydro distillation] which have been used over decade's, forms the first choice for extraction of phytochemicals among researches, however, these methods, lack selectivity, give lower yields, time consuming and present safety concern and environmental risk. In addition the possibility of oxidation and/or hydrolysis of target extracted compounds, for example, phenolic compounds, increase upon long exposure to high temperature and long extraction times [12].Accordingly and to overcome these drawbacks, alternative methods of extraction have been used for replacing the conventional one, for example, the use of ultrasonic assisted extraction, pressurized solvent extraction and microwave assisted extraction. Among these, Ultrasonic assisted extraction [UAE] is a technology that can be used both on a small and large scale in industry for the extraction of natural products [13]. Many reports on the beneficial effects of UAE with respect to natural products have been published, with significant improvements over conventional extraction methods offering much lowered extraction time and enhanced efficiency [14, 15].

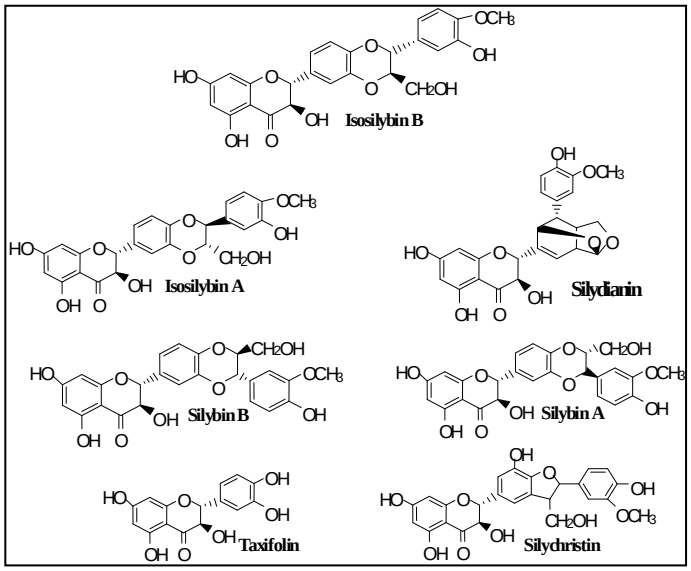

Figure 1: Chemical structures of silymarin components

Compared with conventional methods, UAE has many advantages, such as, increasing the extraction efficiency, shorter extraction time and less consumption of solvent used, these advantages is contributed to the mechanical effect of ultrasonic waves allowing greater penetration of solvent into the sample matrix,

increasing the contact surface area between the solid and liquid phase, and as a result, the solute quickly diffuses from the solid phase to the solvent [16,17].There are several parameters that can affect ultrasonic extraction including extraction time, ultrasonic frequency and extracting solvent.

In this study, the effect of extraction time and ultrasonic frequency using UAE for bioactive compound silymarin from *Silybum marianum* seeds is presented and compared to conventional silence maceration extraction.

EXPERIMENTAL

Materials

Silybum marianum [L.] seeds were collected from Cairo- Alexandria Desert Road [Km 160] during April 2013. The seeds of the plant were ground into fine powder using blender into fine particles with particle size 500-93 µm. Silybin and silymarin standard were purchased from [Sigma-Aldrich Company Ltd.]. Methanol [Gradient Grade HiPerSolv CHROMANORM for HPLC] and water [HiPerSolv CHROMANORM for HPLC] from VWR International [East Grinstead, West Susses, UK].

Methods

Conventional maceration

The extraction was performed at room temperature by mixing 10 g of powdered *Silybum marianum* seeds with 200 mL 80% aqueous methanol in a sealed conical flask at different time internals 15, 30 and 60 min.

Ultrasonic-Assisted Extraction

Powdered *Silybum marianum* seeds [10g] and 200 mL 80% methanol were placed in a sealed 250 mL Erlenmeyer flask [≈ 8 cm bottom diameter] and sonicated [indirect sonication [13] by immersion in an ultrasonic bath [Sonomatic 375TT 40 kHz system, power = 200W, model: S0375T, Langford Electronics Ltd., Coventry, UK]. The extraction mixture in the conical flask was kept below the water level of the bath ≈ 4 cm from the bottom of the flask, exactly over the ultrasonic transducer. The temperature of the mixture was maintained constant [25ºC± 5ºC] by using ice directly into the bath [removing the excess water to keep a constant level in the bath]. The temperature was monitored inside the extraction mixture using a thermocouple.

A 20 kHz probe [Sonics & Materials Inc., Vibracell VCX600, 750W] was employed for direct sonication extraction [13] [the horn tip position inside the extraction vessel was 1 cm under the solvent level]. The same amount of powdered *silybum marianum* seeds [10g] in 200 mL 80% methanol was used. Extractions were carried out at different time intervals [15, 30 and 60 min] at room temperature [temperature monitored by a thermocouple inside extraction mixture by using an ice cooling bath around the extraction vessel to keep the temperature constant at 25ºC± 5ºC.

After each extraction using [maceration and UAE], the collected extracts were filtered throughout a Fisher brand QL100, 150 mm filter paper, then the supernatant was evaporated till dryness under reduced pressure at 45°C, weighed, and stored at −18°C for HPLC analysis.

All data presented are average values ± standard deviation of three independent experiments.

Preparation of standards

Reference solutions of silymarin flavonoids: Methanolic solution of standard silymarin was used in order to determine the retention time of silymarin content.

Reference solutions of silybin as external standard: An accurately weighed quantity of standard [silybin] was dissolved in methanol and diluted with methanol to obtain solutions with known concentrations [1000, 800, 600, 400, 20 µg/mL].

Determination of Silymarin content [HPLC analysis]

High performance liquid chromatography [Shimadzu Prominence series HPLC] was used to determine the chemical composition of each extract as well as the standards. HPLC consists of DGU-20A5 degasser, LC-20AD pump, SIL-20A injector, CTO-20AC oven, SPD-M20A detector, Hi Chrome C18 250x4mm 5 µm column and a CBM-20Alite controller. Data was analysed with Shimadzu LC solution version 1.23 software.

The mobile phase used was 0.5:35:65 phosphoric acid: methanol: water [solvent A] and 0.5:70:30 phosphoric acid: methanol: water [solvent B] at a flow rate of 1 mL/min, The oven [Shimadzu Prominence CTO-20AC] was set at 30 °C. The elution profile is given in Table 1. Detection was carried out by monitoring the absorbance signals at 288 nm.

Table 1: **HPLC elution profile of *Silybum marianum* extracts**

Elution Time	Solvent A %	Solvent B %
0-5	100	0
5-30	30	70
30-30.5	0	100
30.5-35	0	100
35-37	100	0

A linear calibration curve was plotted, with silybin as external standard, based on the sum of areas under silybin A and silybin B peaks versus the concentration of silybin solution. The calibration curve shown in Fig. 2 displays high linearitywith an R^2 value of 0.9991 for the range of standards used [1000-20 µg/mL].

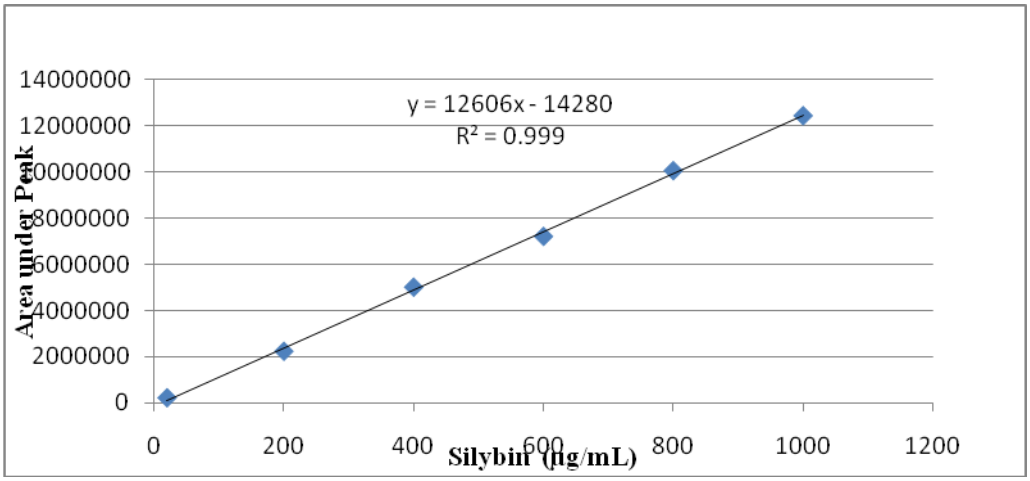

Figure 2: Silybin standard calibration curve

The percentage of each relevant component of silymarin samples were calculated as silybin equivalent against the combined areas of silybin A and silybin B as an external standard.

RESULTS AND DISCUSSION

The results of comparative total extract amount of *silybum marianum* seeds using conventional maceration and ultrasonic-assisted extraction are presented in table 2.

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Table 2: **Mass yield extract in [g/10g] of *Silybum marianum* seeds using conventional maceration and ultrasonic assisted extraction at different time intervals.**

Time [min]	Maceration	UAE	
		40 kHz water bath	20 kHz probe
15	0.34 ± 0.03	0.40 ± 0.04	0.51 ± 0.03
30	0.42 ± 0.02	0.48 ± 0.03	0.56 ± 0.06
60	0.45 ± 0.05	0.63 ± 0.02	0.69 ± 0.03

All values are ± SD of triplicates

HPLC chromatograms of silymarin standard [Fig. 3], extracts obtained using conventional maceration [Fig. 4] and UAE [Fig. 5] showed the presence of taxifolin, silychristin, silydianin, silybin A, silybin B, isosilybin A and isosilybin B at retention times of approximately [7, 14, 16, 22, 23, 26 and 27 min; respectively].

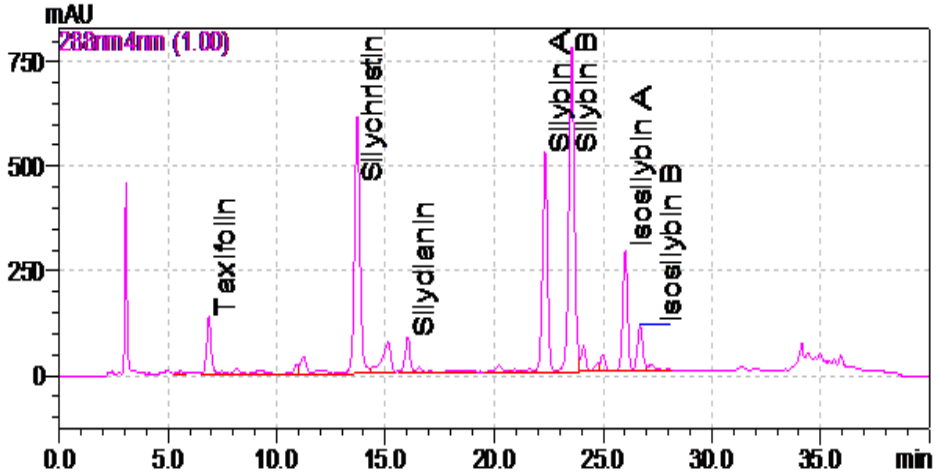

Figure 3: HPLC chromatogram of silymarin standard

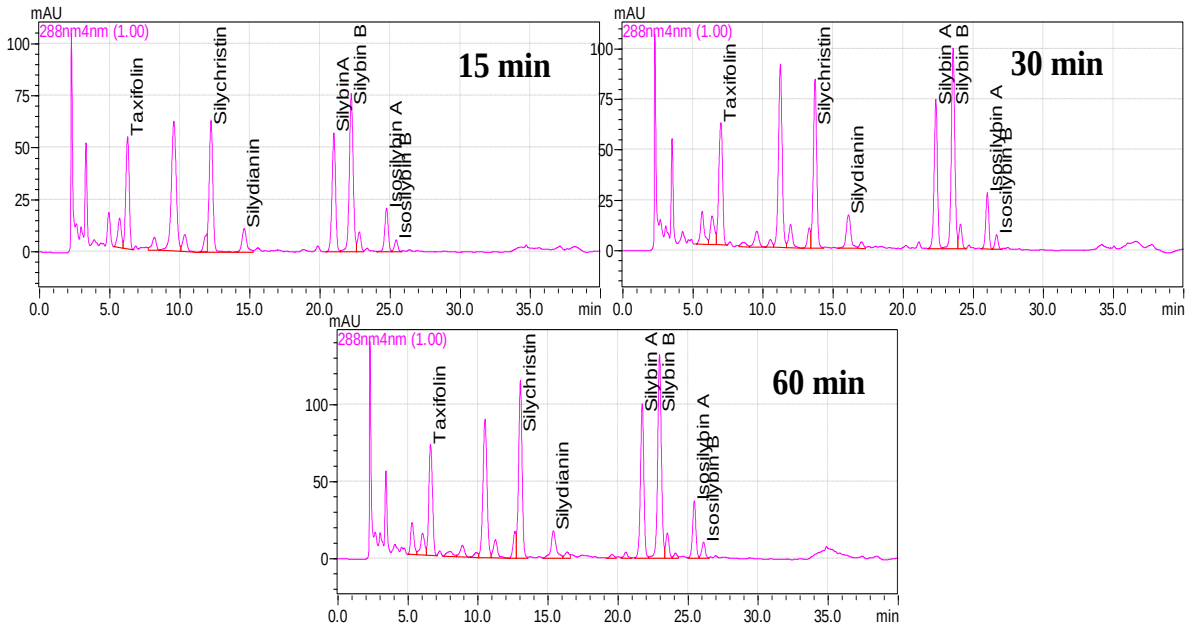

Figure 4: HPLC chromatograms of *Silybum marianum* seeds extracted using conventional maceration at different time intervals

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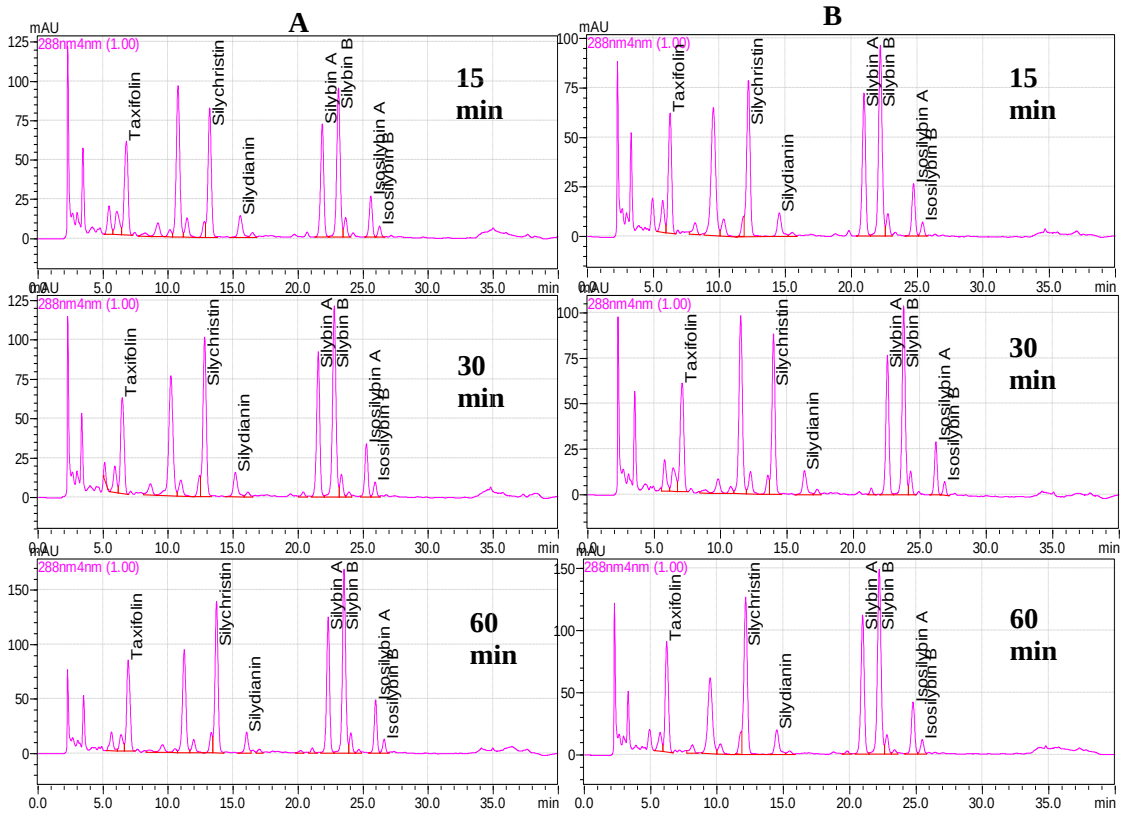

Figure 5: HPLC chromatograms of *Silybum marianum* seeds extracted using ultrasonic assisted extraction at different time intervals: [A: 20 kHz probe and B: 40 kHz bath]

Fig. 6 displays the average concentrations of silymarin, in [mg/10g] of *silybum marinaum* seeds, extracted using conventional maceration and UAE, as determined by using the calibration curve shown above.

Increasing maceration time from 15 min to 60 min, increased the yield of the extracted silymarin [19.7 and 36 mg/10 g seeds; respectively]. The maximum obtained amount was found to be after 60 min maceration.

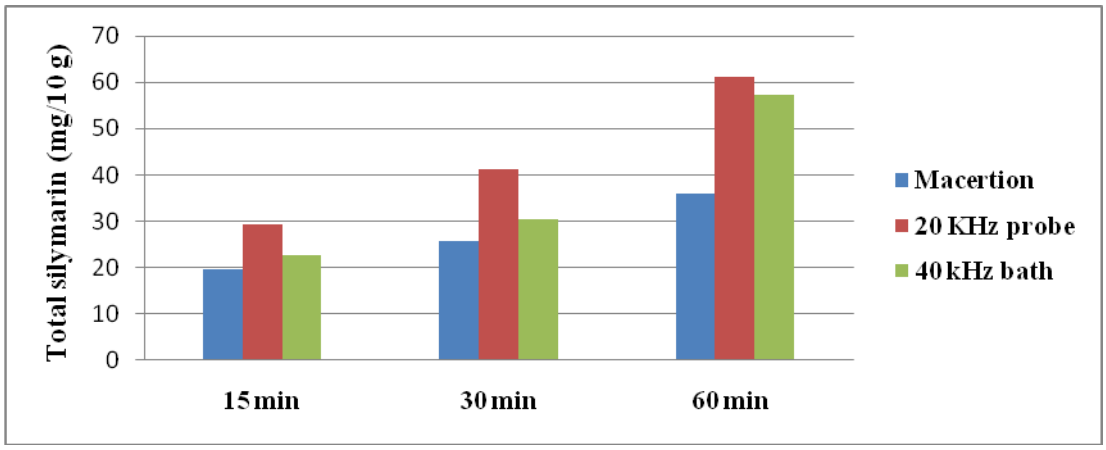

Figure 6: Total silymarin obtained using maceration and ultrasonic assisted extraction at different time intervals [all values are ± SD of triplicates]

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Similar effect was observed using UAE, as increasing the extraction time from 15 to 60 min using 20 kHz probe and 40 kHz water bath, increased the extracted silymarin content [29.4 and 61.1 mg/10 g seeds] and [22.8 and 57.8 mg/10 g seeds]; respectively.

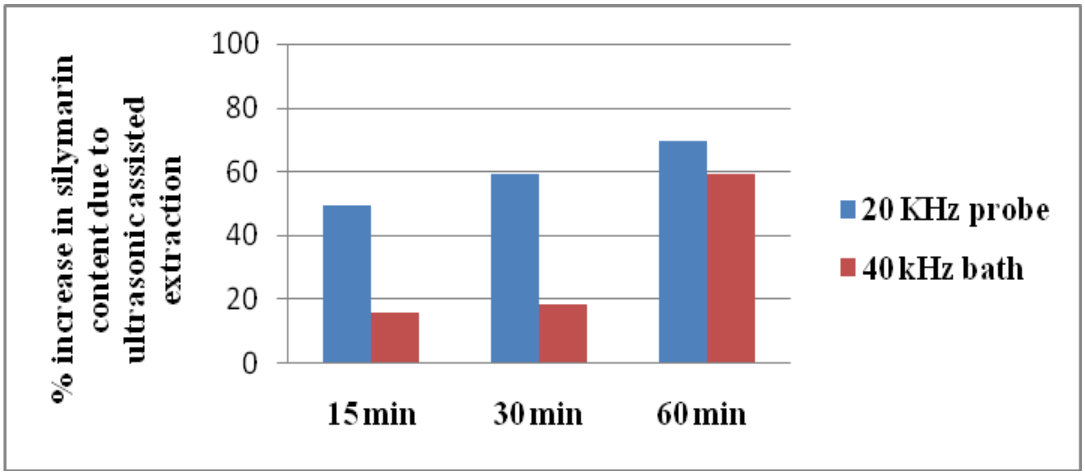

Figure 7: Percentage increase in extracted with the application of ultrasonic assisted extraction, percentage increase calculation: [difference/concentration in conventional extract] ×100 [n = 3].

Fig. 7 demonstrates the % increase of silymarin contents obtained using UAE. This increase could be attributed to the UAE mechanism of extraction, when a liquid is ultrasonically irradiated acoustic cavitation bubbles occur. Bubbles form, grow and suddenly collapse producing tremendous amounts of localised energy. In a system containing solid particles the bubbles collapse releasing high temperatures and pressures [~5000 °K and ~2000 atm] but also create a high speed jet which is directed towards the solid surface, in our case the herb particles [18, 19]. The generated ultrasonic jets hit the herb particles, with an extreme high speed allowing better penetration of solvent into the plant particles. The jets could also contribute to cells pores enlargement acting like a micro-pump forcing the solvent into the cell, where it can dissolve the compounds and transport them into the bulk solvent [20].

Also from the obtained results it was observed that 20 kHz probe showed better extraction yield over 40 kHz water bath. A standard calorimetric method has been conducted to measure the power entering a reactor by monitoring the increase of temperature introduced into the system when the ultrasound is first switched on [21].

Table 5: Power and power density of ultrasonic devices used.

Equipment	Power [W]	Power Density [Wcm-3]
20 kHz probe	20.82± 0.3	0.104± 0
40 kHz water bath	8.99± 0.1	0.045± 0

All values are ± SD of triplicates

From our results [Table 5] it is clear that the probe system provides more power per ml [> 2 times] which explain the effectiveness of 20 kHz probe in extraction over 40 kHz water bath, however further studies should be conducted to monitor the extraction efficiency of the probe system after 60 min of silymarin extraction, as prolonged sonication using the probe system may produce some decomposition of extracted compound due to the higher and locally concentrated acoustic power delivered to the extraction mixture.

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CONCLUSION

Increasing extraction times using both conventional maceration and UAE resulted in increasing silymarin contents. UAE has proven to be a more effective technique to replace conventional maceration, comparison with conventional extraction methods revealed that UAE could save a lot of time and being more efficient. 20 kHz probe system showed better extraction efficiency over 40 kHz water bath. Future work is required to monitor the prolonged extraction efficiency of silymarin from *silybum marianum* seeds using ultrasound applications.

ACKNOWLEDGMENT

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