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A possible general mechanism for ultrasound-assisted extraction (UAE) suggested from the results of UAE of chlorogenic acid from *Cynara scolymus* L. (artichoke) leaves

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Abstract

The use of ultrasound-assisted extraction (UAE) for the extraction of Chlorogenic Acid (CA) from *Cynara scolymus* L., (artichoke) leaves using 80% methanol at room temperature over 15 minutes gave a significant increase in yield (up to a 50%) compared with maceration at room temperature and close to that obtained by boiling over the same time period. A note of caution is introduced when comparing UAE with Soxhlet extraction because, in the latter case, the liquid entering the Soxhlet extractor is more concentrated in methanol (nearly 100%) that the solvent in the reservoir (80% methanol) due to fractionation during distillation. The mechanism of UAE is discussed in terms of the effects of cavitation on the swelling index, solvent diffusion and the removal of a stagnant layer of solvent surrounding the plant material.

Keywords: chlorogenic acid, ultrasound-assisted extraction, general extraction mechanism, *Cynara scolymus* L.

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1. Introduction

The significance of ultrasound-assisted extraction (UAE) is clearly shown from the results of a simple search on Science Direct which returned 10,725 documents on this topic. However, if the search is narrowed down to the mechanism of UAE then only 15 hits are recorded and some of these fail to identify any real mechanisms. Yet the mechanism is the key if we want to understand how to apply, improve and expand the uses of such extractions which are of enormous interest to the food and nutraceutical industries. In this paper we will use UAE to extract Chlorogenic Acid (CA) from a plant waste material (artichoke leaves) and attempt to deduce a mechanism of this type of extraction.

Artichoke, *Cynara scolymus* L., is a variety of thistle and it is a cultivated for its edible flower buds. The remainder, some 90% of the plant, comprising leaves, stem etc., is not used and is waste (see Fig. 1). However this waste is rich in polyphenols, among which CA (3-O-caffeoylquinic acid, Fig. 2) is a valuable natural compound used in medicine and also as an additive in beverages, cosmetics, tea products and foods [1]. CA has antibacterial, antiviral properties, and it is a natural antioxidant and anticancer agent [2-4].

Artichoke leafs contain, among other compounds, up to 2% phenolic acids including as a main component CA. In addition the leaves contain 1,3-di-O-caffeoylquinic acid (cynarine), caffeic acid, 0.4% bitter sesquiterpene lactones of which 47-83% is cynaropicrin and a volatile oil consisting mainly of the sesquiterpenes β-selinene and caryophyllene [5].

Since the leaves of Artichoke are waste material they are a very cheap natural source of CA which currently, for laboratory use, costs around $50 per gram.

Conventional methods of solvent extraction often involve long periods at high temperature and extended extraction times leading to the possibility of oxidation and hydrolysis of phenolic compounds [6]. To overcome these drawbacks, alternative extraction methods have been introduced such as ultrasound-assisted extraction (UAE) [7] and microwave assisted extractions (MAE) [8].

UAE is a useful technique [9] that is moving towards industrial acceptance [10] furthermore it can be used equally well on either a small or large scale in the phytopharmaceutical industry.

UAE is based on cavitation i.e. the creation, growth and sudden implosion (collapse) of bubbles with tremendous energy release from each of them, sometimes referred to as hot spots [7, 11, 12]. In the bulk solvent the bubble collapse will be symmetrical but near to a surface it is asymmetric and generates a high speed jet of liquid. In the case of cavitation in an extraction system the jet hits the surface of the plant material and provides continuous

circulation of new solvent at the surface, produces a deep penetration of solvent into the plant particles, continuous solvent mixing and sometimes particle size reduction [6, 13].

The so-called hot spots generated during bubble collapse do not cause heat damage to thermal sensitive compounds because they are very short-lived with cooling rates in the range of 10^{10} K/s [11, 12]. For this reason UAE is considered to be a non-thermal technique and so UAE can be used for the extraction of thermally sensitive compounds [14, 15]. However, it should be noted that ultrasonic irradiation for periods of time longer than about 5 minutes at high ultrasonic power does generate heat. For this reason it is advisable to maintain a constant temperature in the bulk extraction liquid, via a cooling bath around the extraction vessel [16] as a means of accurately controlling the extraction temperature. Even under temperature control prolonged sonication can lead to some degradation of the targeted compounds and so the duration of UAE is also an important consideration [10]. Ultrasonic extractions of phenolic compounds (similar in structure with the targeted compound in this work) and antioxidants from citrus, grape, strawberry, olive fruit and also oil from tea seeds have been studied by various researchers [17-21].

In this paper we have examined in some details the mechanism of UAE of natural products using as a case study the extraction of CA from artichoke leaves.

2. Materials and methods

Acetonitrile and methanol (Gradient Grade HiPerSolv CHROMANORM for HPLC), water (HiPerSolv CHROMANORM for HPLC), CA standard (purity ≥ 95.0%, CAYMAN Chemical) were purchased from VWR International (East Grinstead, West Susses, UK). *Cynara scolymus* L. leaves (Romanian strain) were collected from the farm of the National Research Centre-Cairo-Egypt. Collected leaves were dried at 40°C for 48 hr in a tray dryer with air circulation; dried leaves were ground into fine powder and subjected to a series of sieves with different range of mesh sizes. Graded material with a particle size distribution 500–90 μm was used for all experiments reported in this paper. The moisture content of this material was found to be 7.5% and was determined by further drying of 1 g of powdered leaves at 105°C until constant weight was obtained, The solvent used in all extraction procedures was a solution of 80% methanol with 20% water, based on previous papers where phenolic compounds where the target for extraction [18-20, 22-24].

2.1. Extraction procedures

2.1.1. Soxhlet extraction and a note of caution

Ground *Cynara scolymus* L. leaves (5g) were placed in a thimble–holder and positioned in a Soxhlet apparatus. The extraction was carried out over 6 hours using 100 mL 80% methanol (water solution) in the reservoir.

However, it must be remembered that despite using 80% methanol as starting solvent, the extraction solvent in the thimble is in fact almost 100% methanol because aqueous methanol does not co-distil with water or form an azeotrope. Essentially this means that the Soxhlet method does not provide a "standard" extraction with which to compare UAE in the case of aqueous methanol solvents.

2.1.2. Heat reflux extraction

Powdered *Cynara scolymus* L. leaves (5g) was extracted using 100 mL 80% methanol under reflux for different time intervals, 15 and 30 min.

2.1.3. Maceration

The extraction was performed at room temperature by mixing powdered *Cynara scolymus* L. leaves (5g) with 100 mL 80% methanol in a sealed conical flask with samples being taken at 15, 30, 60 min and 24 hours. Since the majority of the extractions under UAE conditions were complete in 30 minutes only the maceration results at 15 and 30 min were used for comparison purposes.

2.1.4. Ultrasound-assisted extraction (UAE)

Powdered *Cynara scolymus* L. leaves (5g) and 100 mL 80% methanol were placed in a closed 250 mL Erlenmeyer flask (ca 8 cm bottom diameter) and sonicated (indirect sonication [6]) by immersion in an ultrasonic bath (Sonomatic 375TT 40 kHz system, power = 300W, model S0375T, Langford Electronics Ltd., Coventry, UK). The extraction mixture in the conical flask was kept below the water level of the bath ca 4 cm, exactly over the ultrasonic transducer. The temperature of the mixture was maintained constant (25ºC± 5ºC) by using ice directly added to 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, 600W) was employed for direct sonication extraction [6] (the horn tip position inside the extraction vessel was 1 cm below the solvent surface). The same amount of powdered *Cynara scolymus* L. leaves (5g) in 100 mL 80% methanol was placed in a round bottom flask were sonicated with the vessel immersed in a cooling mixture of ice/water. The temperature inside extraction mixture was monitored using a thermocouple.

Extractions (for both indirect and direct UAE) were carried out at room temperature (25ºC± 5ºC) for different time intervals (15, 30 and 60 min but for reasons given above only the 15 and 30 min were used for comparison). For both ultrasonic systems the effective power was calorimetrically determined [25-27] and the values are given in the Table 1.

The efficiency of extraction using the methods described above is shown in Fig. 3 in terms of the CA obtained from 5 grams of dry leaves. Note again here that the Soxhlet extraction results are not truly comparable with the others for the reasons given in 2.1.1 above.

2.1.5. General procedures for post extraction processing

After each extraction using all of the above methods, the extracts were filtered through a Fisher brand QL100, 150 mm filter paper and then the supernatant was evaporated to dryness under reduced pressure at 45°C and stored at −18°C for subsequent HPLC analysis.

2.1.6. Analytical procedure

High performance liquid chromatography (Shimadzu Prominence series HPLC) was used to determine the chemical composition of each extract obtained. The HPLC equipment comprised of a DGU-20A5 degasser, LC-20AD pump, SIL-20A injector, CTO-20AC oven, SPD-M20A detector, HiChrome C18 250x4mm 5 µm column and a CBM-20Alite controller. The data was analysed using Shimadzu LCsolution version 1.23 software.

The analytical method was adapted from British Pharmacopeia [28], the mobile phase was 0.5% phosphoric acid in water (solvent A) and 0.5% phosphoric acid in acetonitrile (solvent B) at a flow rate of 1.2 mL/min. The elution profile is given in Table 2.

Chromatograms were recorded at 330 nm and CA quantification was carried out using a standard calibration curve of CA with the above mentioned HPLC method.

All data presented herein are average values ± standard deviation of three independent experiments and expressed as mg/5g dried leaves of *Cynara scolymus* L. leaves. The statistical significance was evaluated using independent-samples t-tests which were conducted using SPSS 17 and a P value of less than 0.05 was considered to be significant.

3. Results and discussion

3.1. Extraction mechanism

From the results obtained (Fig. 3), the best extraction of CA is obtained by heating (reflux) the leaves with solvent for 30 min. This method does present a minor problem in terms of the actual solvent composition in the extraction mixture which will be slightly more aqueous than that used initially due to an increased quantity of methanol in the vapour in the condenser.

Nevertheless ultrasound-assisted probe extraction is not significantly different from heating with solvent after only 15 min extraction time and at the lower temperature of 25°C.

Extraction using the probe system is faster than with the ultrasonic bath in the early stages because it provides almost double the ultrasonic power per mL. However, due to the higher cavitation activity the CA level decreases significantly after 15 min of ultrasound-assisted probe extraction. This is because prolonged sonication can cause degradation of some targeted compounds [29, 30]. This suggests that if there is a competition between extraction and degradation there will be an optimal extraction time for UAE [11, 31, 32].

To appreciate how UAE works it is necessary to have an understanding of the extraction mechanism. When a solvent is in contact with the dry plant material, the first phase is that it surrounds the herb particles (which are generally of very irregular shape) to create a solvent film or layer and from this stage several processes starts to occur, see Fig. 4 which is adapted from Raynie [33].

An immediate process following this first phase is the swelling of particulate matter which is due to the uptake of solvent inside the material. This will happen in almost all extraction processes because the herbs are dried before extraction to reduce the water content which otherwise would significantly change the character of the solvent used for extraction by diluting it with any water contained in the plant, see reference [34]. Drying the herb has the additional benefits of making it easier to grind and to increase storage lifetime.

The irregular shape of a vegetal particle changes while it undergoes swelling with solvent and it will expand and acquire a smoother shape. In order to simplify this discussion we will consider a spherical particle, see Fig. 5. This will allow us to focus mainly on the diffusion and rinsing/washing phases.

During the solvent swelling period of the dried material there will be a sort of dynamic interaction at the vegetal surface involving both solvent entering and materials exiting into the solvent (Diffusion 1 in Fig. 5). However at the point where the swelling reaches its maximum the dynamic interaction is reduced and solvent surrounding the vegetal particle becomes less mobile. In essence a stagnant layer of solvent is formed, surrounding the particle that will hinder the extraction efficiency by obstructing direct diffusion a process that is governed by the concentration gradient. The stagnant layer will also interfere with the rinsing and washing out processes. If the extraction is stopped at this stage there will be a rather low extraction yield. This is the reason why in the classical extraction method (i.e. maceration) very long steeping times are required (in some cases 28 days [6]).

The second step of extraction is governed by the diffusion of compounds from the solvated herb particles towards the surrounding stagnant layer (diffusion 2 outward in Fig. 5). This step is followed by the third step in which the compounds diffusion from the stagnant layer into the bulk solvent (diffusion 3 in Fig. 5). In this way the stagnant layer is effectively acting as a diffusion barrier that can hamper the extraction depending of the solvent nature and extraction method. The most commonly used extraction solvent is aqueous ethanol or methanol both of which have considerable intermolecular hydrogen bonding and the OH groups will also develop hydrogen bonds with OH and O groups in the plant cellulosic structure. This will augment the adhesion of the solvent stagnant layer around the particle. Clearly, the more adherent the stagnant layer becomes the more difficult it will be for the diffusion processes to take place. An additional factor is that the solvent stagnant layer could also cover cracks in the cells (crevices) on the outside of herbal material which will also hinder the washing out process.

The use of ultrasonic energy offers a unique opportunity to enhance solvent extraction because of the way in which it can affect surface processes and the stagnant layer. As pointed out above in a homogenous liquid the bubbles are symmetric and their collapse in the bulk liquid is also symmetric leading to localized hot spots (Fig. 6a). However the interaction of cavitation bubble collapse in the vicinity of a solid surface is somewhat different and was observed and investigated many years ago [35]. When a bubble collapses near a surface, it deforms taking on a doughnut-shape (Fig. 6b) leading to asymmetric collapse and creating high speed solvent jets directed towards the solid surface. These jets impact onto the surface and causes damage due to a solvent–hammer type impact. This is one of the major reasons why ultrasound is so effective for surface cleaning [36, 37,38].

During UAE the jets of solvent from asymmetric bubble collapses hit the herb material with extreme high speed, disturbing the stagnant layer surrounding the material and so allowing fresh (or less loaded) solvent from the bulk medium to replace it. This is a dynamic process which continues throughout sonication. This is comparable to electrochemical processes in the presence of ultrasound where cavitation bubble collapse near to the electrode surface has been shown to reduce the diffusion layer thickness [39]. Since the collapsing bubbles are generated in large numbers the removal of the stagnant layer and its replacement with fresh (or less loaded) solvent is extremely fast and repetitive. The immediate outcome from this is that the mass transfer from bulk solvent to plant material is improved significantly and the extraction yield is elevated (all three diffusional as well as rinse out processes, being

enhanced). The most effective influence of ultrasound during UAE extraction from herbs derives from the mechanical effects of jets hitting the vegetal particles at very high speed (>400 km/h in water [40]). This is schematically represented in Fig. 7 [41].

Two other benefits arise directly from the release of cavitation energy. First, the plants cells near the surface can be broken releasing their content and secondly the jet formed during asymmetric bubbles collapse gives a better penetration of solvent into the plant particles. The jets could also contribute to cell pore enlargement acting like a micro-pump forcing the solvent into the cell where it dissolves the required compounds and transports them back into the bulk solvent [42]. The latter is the reason why ultrasound is able to produce a higher swelling index than the more traditional shaking as described in British Pharmacopoeia [28].

The swelling index of *Cynara scolymus* L. using 80% methanol as solvent was determined experimentally using both the British Pharmacopoeia (BP) procedure [28] and the corresponding ultrasonic values (in which the shaking steps, described in BP, were replaced with sonication) are shown in the Table 4. It can be seen that using ultrasound the swelling index (bath and probe) is greater than that obtained by the conventional BP procedure, confirming the important role of sonication in the UAE process.

To confirm the role of the stagnant layer we performed an experiment in which we intentionally added 100 µL of two different neutral detergents (Tween and Decon-N) to the extraction solvent (80/20 methanol/water) aiming to make the stagnant layer less adherent to the plant material and so producing differences in the extraction yield. Indeed, the resulting changes brought about by the addition detergent to the solvent which are shown in Table 3; indicate that the extraction yield is slightly increased when the stagnant layer is made less adhesive to the herbs particles.

If the mechanism that we have described is correct then there will be several parameters that have important roles in UAE:

- the extraction solvent, usually selected based on:
  - affinity to targeted compound to be extracted,
  - penetration – interaction with plant structure,
  - toxicity,
  - the degree of bonding of solvent to herbal particles the higher the bonding the more difficult it is to remove the stagnant layer;

- plant to solvent ratio (R) – usually 1 to 10 or 1 to 20 and in some cases higher but only if the targeted compound is very valuable. The higher the solvent/plant ratio the easier the diffusion processes because the stagnant layer is more easily refreshed;
- ultrasonic power, the real acoustic power (P) entering into the extraction system. Here a balance between the enhancement of extraction yield and the degradation of plant constituents by sonochemistry should be considered;
- extraction temperature (T). The higher the temperature the higher the vapour pressure of the system and so the easier cavitational bubbles will be generated. However their collapse energy is cushioned by the greater amount of solvent vapour intake and therefore the efficiency of the process may diminish;
- extraction time (t). Prolonged sonication could end up with too much cavitation induced milling of plant particles as well as degradation of targeted compounds.

It is a reasonable question to ask under what circumstances UAE should be employed. One obvious consideration is that if the traditional extraction technique of maceration (in which almost no energy consumption is involved) gives an acceptable extraction yield why should we attempt UAE? There are two answers to this.

1. UAE affords shorter extraction times so that, in cases where the targeted compound is valuable and it degrades slowly in the plant during storage and extraction, – a common occurrence in practical work – then a faster extraction is desirable.
2. It is possible to use UAE to achieve a more selective extraction of the targeted component compared with maceration

If either of these applies then UAE will be the method of choice.

It must be borne in mind however that ultrasonic exposure time and input power have significant effects on extraction yield. These should be monitored because extraction using a high energy ultrasonic probe although faster than using an ultrasonic bath can have an unwelcome effect. Prolonged sonication using the probe produces some decomposition of extracted compound (CA in our case) due to the higher and locally concentrated acoustic power delivered to the extraction mixture. The ultrasonic power generated by the equipment in the extraction mixture itself can be determined using standard calorimetric methods [25-27].

Based on the proposed mechanism of UAE process and the results obtained in the present study, further studies should be conducted for the possible industrialisation or scale up of the UAE of CA from a plant waste material (artichoke leaves). Previous studies showed that the

optimized lab-scale results obtained using UAE of phenolic compounds (similar in structure to CA) was promising for extraction on an industrial scale [43, 44].

4. Conclusion

A general mechanism for an ultrasound-assisted extraction is proposed. This introduces the concept of the formation of a stagnant layer of solvent surrounding the herbal material which acts as a diffusion barrier during extraction process. This layer impairs conventional extraction but can be disrupted or removed under the influence of cavitation bubble collapse. Using UAE the best results for the extraction of chlorogenic acid CA from Artichoke leaves were obtained at room temperature using either a 20 kHz probe for 15 min or a 40 kHz water bath for 60 min. The yield of CA decreased on longer exposure to sonication with the 20 kHz probe. UAE has proven to be a more effective technique in this extraction than conventional maceration, boiling and the use of a Soxhlet for the extraction of polyphenols from *Cynara scolymus* L. leaves with a solvent of 80/20 methanol/water.

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Fig. 1. Artichoke, *Cynara scolymus* cultivated in Egypt

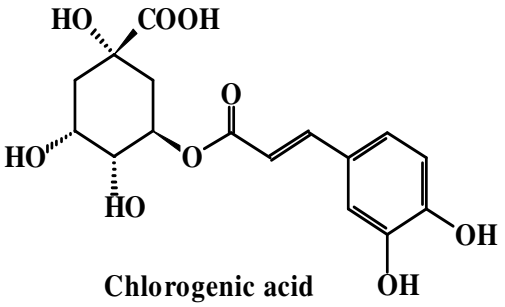

Fig. 2. Chemical structure of chlorogenic acid

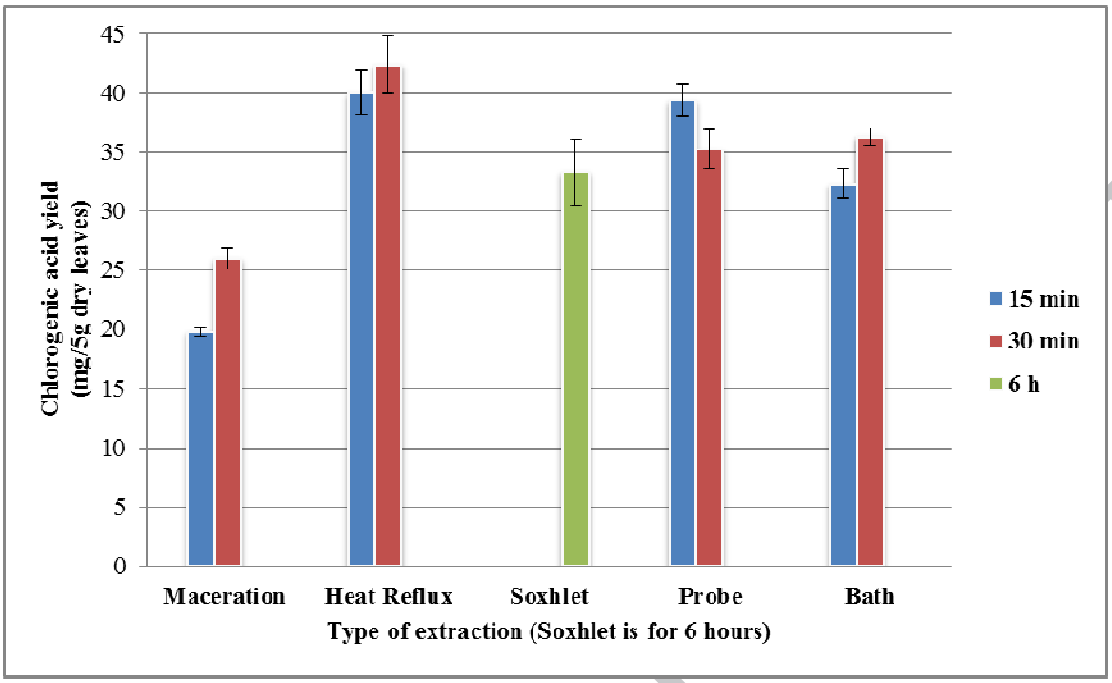

Fig. 3. Conventional compared with ultrasound-assisted extraction in terms of chlorogenic acid extraction efficiency (mg/5 grams dry leaves)

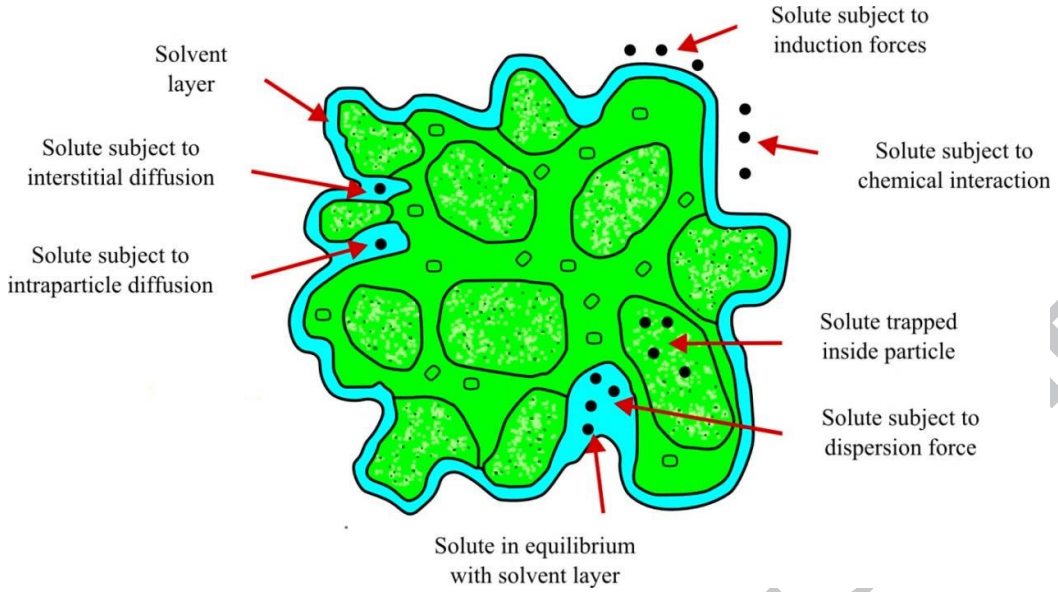

Fig. 4. The major physical/chemical processes that may occur during solvent extraction from herbal material, adapted from [33]

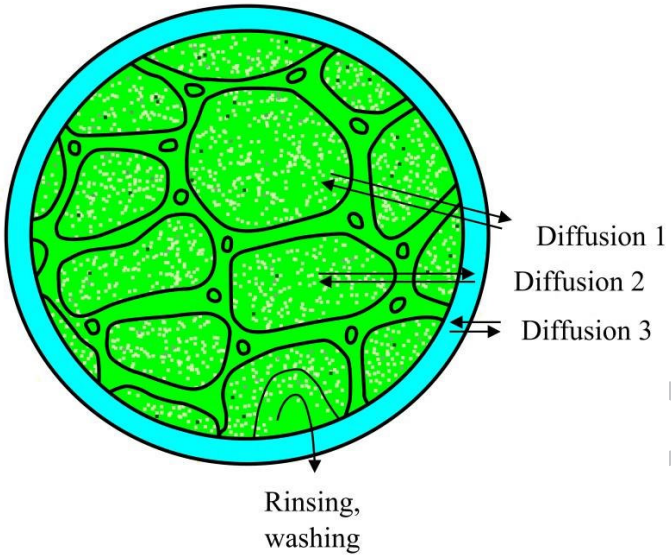

Fig. 5. Representation of a swollen vegetal particle surrounded by solvent layer

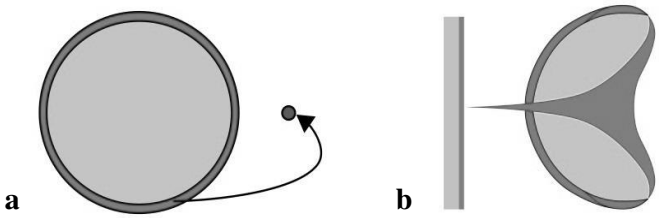

Fig. 6. Illustration of the symmetric (a) and asymmetric (b) collapse of cavitation bubbles

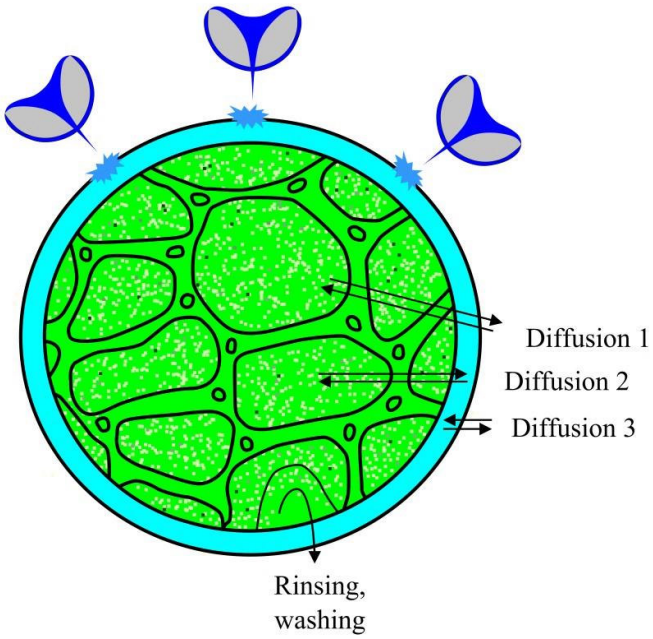

Fig. 7. Ultrasonic extraction possible mechanism

734 **Table 1.** Power and power density of ultrasonic devices used

Equipment	Power (W)	Power Density (W/cm^3)
20 kHz	20.82 ± 0.3	0.104 ± 0
40 kHz	8.99 ± 0.1	0.045 ± 0

735 All values are ± SD of triplicates

736

737 **Table 2.** HPLC elution profile

Elution Time	Solvent A %	Solvent B %
0-1	92	8
1-20	75	25
20-22	0	100
22-24	0	100
24-26	92	8
26-35	92	8

738

739 **Table 3.** Chlorogenic acid concentration mg/5g dried leaves (15 min 20 kHz probe) using
740 tween and Decon-N vs blank extraction

Volume of detergent	Tween	Decon-N	Blank Extraction (no detergent)
100 µL	37.4 ± 0.9	36.3 ± 1.6	35.7 ± 2.1

741 All values are ± SD of triplicates

742

743 **Table 4.** Swelling index (mL/g) of *Cynara scolymus* L. leaves

Procedure	80% MeOH
BP procedure	8.2 ± 0.2
Ultrasonic probe 20 kHz	10 ± 0.3
Ultrasonic cleaning bath 40 kHz	9.5 ± 0.5

744 All values are ± SD of triplicates

745

Highlights

- Ultrasonic-assisted extraction of chlorogenic acid from *Cynara scolymus* L., (artichoke) leaves by different extraction methods.
- A general mechanism for ultrasonic-assisted extraction is proposed.
- Ultrasonic-assisted extraction has proven to be a more effective technique than conventional maceration, boiling and Soxhlet for the studied case using as solvent of 80/20 methanol/water.