

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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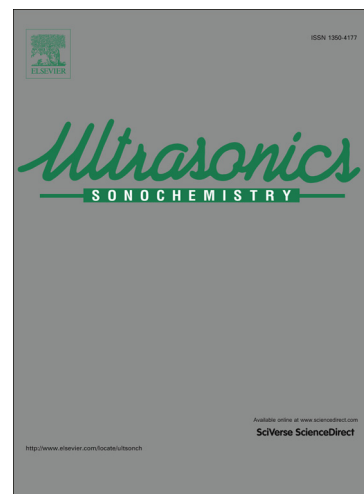
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1 **A possible general mechanism for ultrasound-assisted extraction (UAE) suggested**
2 **from the results of UAE of chlorogenic acid from *Cynara scolymus* L. (artichoke) leaves**
3

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10
11 **Abstract**

12 The use of ultrasound-assisted extraction (UAE) for the extraction of Chlorogenic Acid (CA)
13 from *Cynara scolymus* L., (artichoke) leaves using 80% methanol at room temperature over
14 15 minutes gave a significant increase in yield (up to a 50%) compared with maceration at
15 room temperature and close to that obtained by boiling over the same time period. A note of
16 caution is introduced when comparing UAE with Soxhlet extraction because, in the latter
17 case, the liquid entering the Soxhlet extractor is more concentrated in methanol (nearly 100%)
18 that the solvent in the reservoir (80% methanol) due to fractionation during distillation. The
19 mechanism of UAE is discussed in terms of the effects of cavitation on the swelling index,
20 solvent diffusion and the removal of a stagnant layer of solvent surrounding the plant
21 material.

22
23 *Keywords:* chlorogenic acid, ultrasound-assisted extraction, general extraction mechanism,
24 *Cynara scolymus* L.

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30 1. Introduction

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

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

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

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

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

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

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

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

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

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

80 **2. Materials and methods**

81 Acetonitrile and methanol (Gradient Grade HiPerSolv CHROMANORM for HPLC), water
82 (HiPerSolv CHROMANORM for HPLC), CA standard (purity $\geq 95.0\%$, CAYMAN
83 Chemical) were purchased from VWR International (East Grinstead, West Sussex, UK).
84 *Cynara scolymus* L. leaves (Romanian strain) were collected from the farm of the National
85 Research Centre-Cairo-Egypt. Collected leaves were dried at 40°C for 48 hr in a tray dryer
86 with air circulation; dried leaves were ground into fine powder and subjected to a series of
87 sieves with different range of mesh sizes. Graded material with a particle size distribution
88 $500\text{--}90\ \mu\text{m}$ was used for all experiments reported in this paper. The moisture content of this
89 material was found to be 7.5% and was determined by further drying of 1 g of powdered
90 leaves at 105°C until constant weight was obtained, The solvent used in all extraction
91 procedures was a solution of 80% methanol with 20% water, based on previous papers where
92 phenolic compounds were the target for extraction [18-20, 22-24].

93 **2.1. Extraction procedures**

94 **2.1.1. Soxhlet extraction and a note of caution**

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

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

103 **2.1.2. Heat reflux extraction**

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

106 **2.1.3. Maceration**

107 The extraction was performed at room temperature by mixing powdered *Cynara scolymus* L.
108 leaves (5g) with 100 mL 80% methanol in a sealed conical flask with samples being taken at
109 15, 30, 60 min and 24 hours. Since the majority of the extractions under UAE conditions were
110 complete in 30 minutes only the maceration results at 15 and 30 min were used for
111 comparison purposes.

112 **2.1.4. Ultrasound-assisted extraction (UAE)**

113 Powdered *Cynara scolymus* L. leaves (5g) and 100 mL 80% methanol were placed in a closed
114 250 mL Erlenmeyer flask (ca 8 cm bottom diameter) and sonicated (indirect sonication [6])
115 by immersion in an ultrasonic bath (Sonomatic 375TT 40 kHz system, power = 300W, model
116 S0375T, Langford Electronics Ltd., Coventry, UK). The extraction mixture in the conical
117 flask was kept below the water level of the bath ca 4 cm, exactly over the ultrasonic
118 transducer. The temperature of the mixture was maintained constant ($25^{\circ}\text{C} \pm 5^{\circ}\text{C}$) by using ice
119 directly added to the bath (removing the excess water to keep a constant level in the bath).
120 The temperature was monitored inside the extraction mixture using a thermocouple.

121 A 20 kHz probe (Sonics & Materials Inc., Vibracell VCX600, 600W) was employed for
122 direct sonication extraction [6] (the horn tip position inside the extraction vessel was 1 cm
123 below the solvent surface). The same amount of powdered *Cynara scolymus* L. leaves (5g) in
124 100 mL 80% methanol was placed in a round bottom flask were sonicated with the vessel
125 immersed in a cooling mixture of ice/water. The temperature inside extraction mixture was
126 monitored using a thermocouple.

127 Extractions (for both indirect and direct UAE) were carried out at room temperature ($25^{\circ}\text{C}\pm$
128 5°C) for different time intervals (15, 30 and 60 min but for reasons given above only the 15
129 and 30 min were used for comparison). For both ultrasonic systems the effective power was
130 calorimetrically determined [25-27] and the values are given in the Table 1.

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

134 **2.1.5. General procedures for post extraction processing**

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

138 **2.1.6. Analytical procedure**

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

144 The analytical method was adapted from British Pharmacopeia [28], the mobile phase was
145 0.5% phosphoric acid in water (solvent A) and 0.5% phosphoric acid in acetonitrile (solvent
146 B) at a flow rate of 1.2 mL/min. The elution profile is given in Table 2.

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

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

153 **3. Results and discussion**

154 **3.1. Extraction mechanism**

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

278 It must be borne in mind however that ultrasonic exposure time and input power have
279 significant effects on extraction yield. These should be monitored because extraction using a
280 high energy ultrasonic probe although faster than using an ultrasonic bath can have an
281 unwelcome effect. Prolonged sonication using the probe produces some decomposition of
282 extracted compound (CA in our case) due to the higher and locally concentrated acoustic
283 power delivered to the extraction mixture. The ultrasonic power generated by the equipment
284 in the extraction mixture itself can be determined using standard calorimetric methods [25-
285 27].

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

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

291 4. Conclusion

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

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

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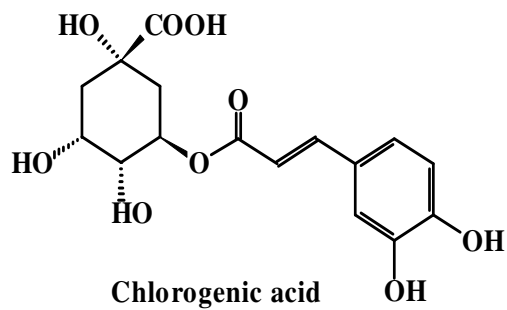
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Fig. 2. Chemical structure of chlorogenic acid

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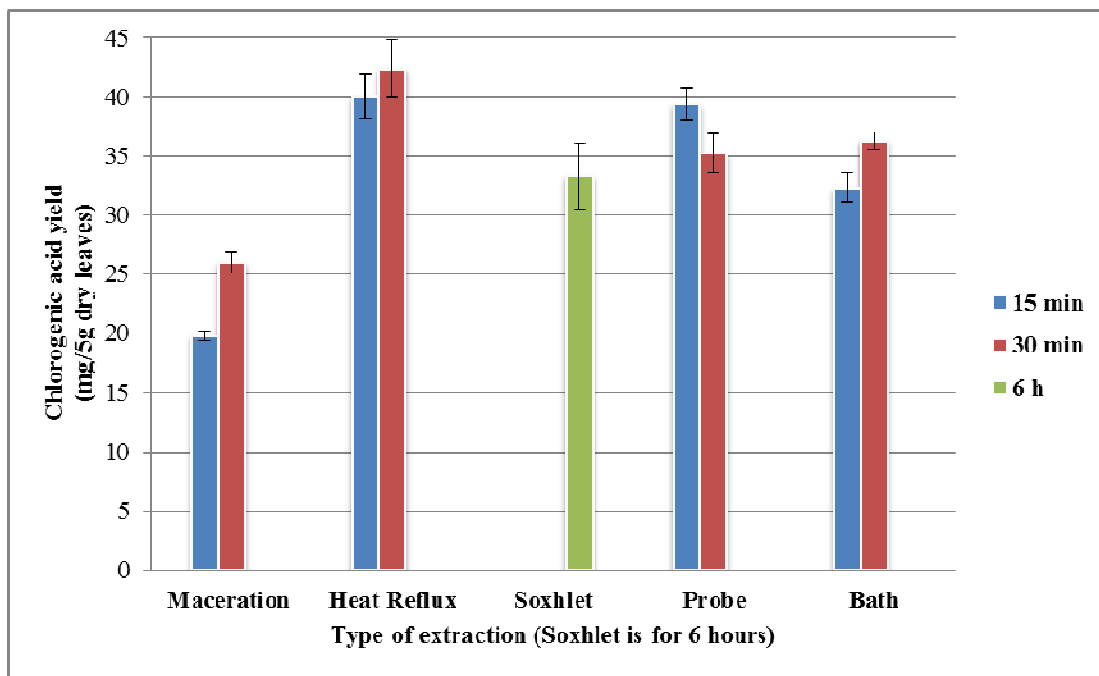
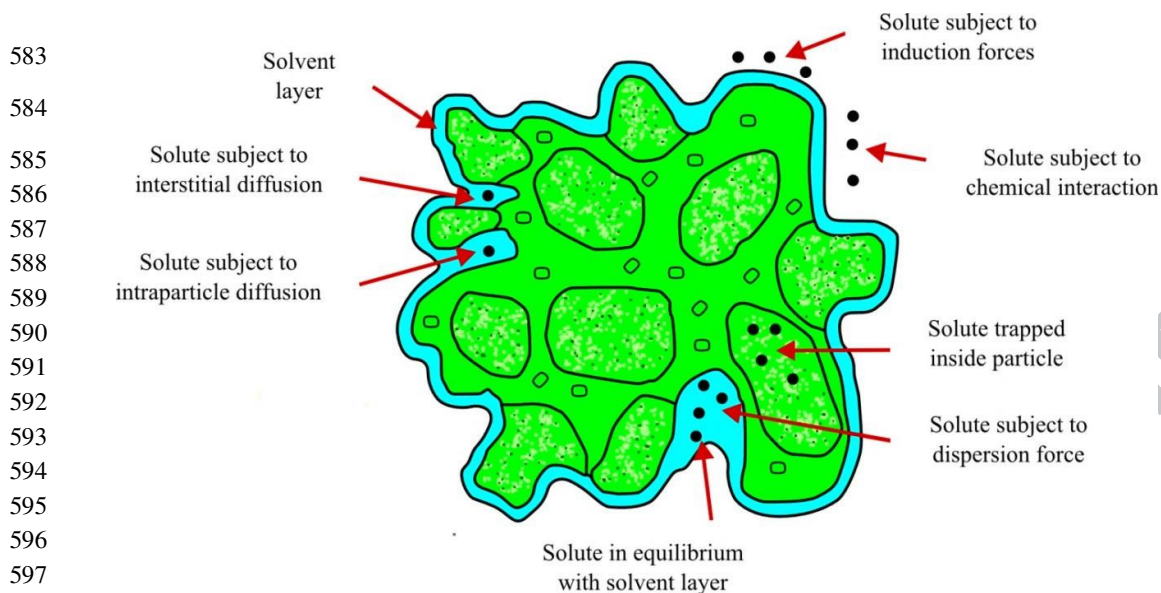


Fig. 3. Conventional compared with ultrasound-assisted extraction in terms of chlorogenic acid extraction efficiency (mg/5 grams dry leaves)



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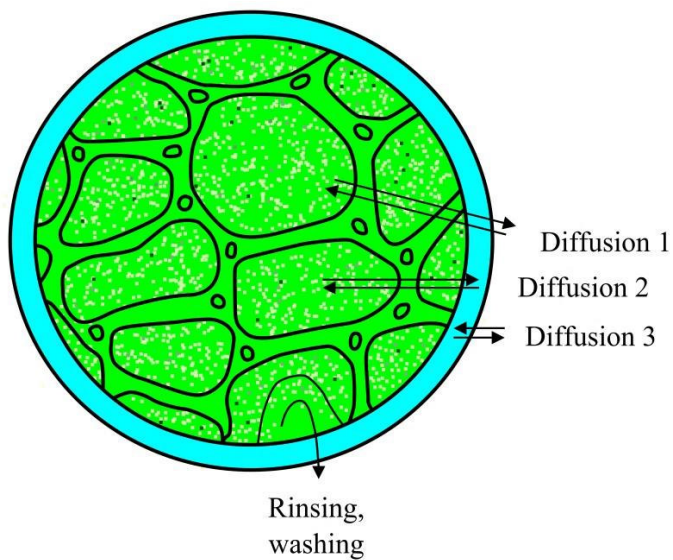


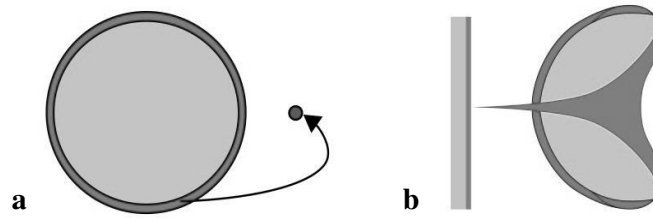
Fig. 5. Representation of a swollen vegetal particle surrounded by solvent layer

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

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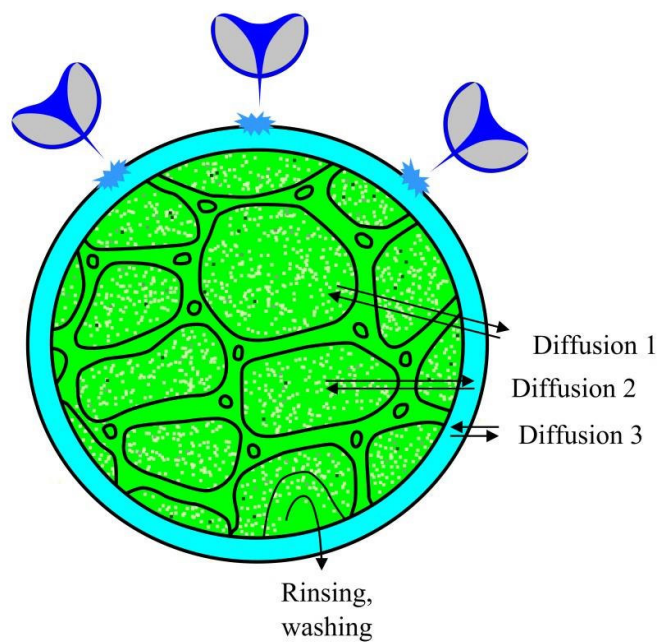


Fig. 7. Ultrasonic extraction possible mechanism

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

| Equipment | Power (W) | Power Density (W/cm³) |
|------------------|------------------|---|
| 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

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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 |

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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 μ L | 37.4 \pm 0.9 | 36.3 \pm 1.6 | 35.7 \pm 2.1 |

741 All values are \pm SD of triplicates

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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

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746 **Highlights**

- 747 • Ultrasonic-assisted extraction of chlorogenic acid from *Cynara scolymus* L.,
748 (artichoke) leaves by different extraction methods.
- 749 • A general mechanism for ultrasonic-assisted extraction is proposed.
- 750 • Ultrasonic-assisted extraction has proven to be a more effective technique than
751 conventional maceration, boiling and Soxhlet for the studied case using as solvent of
752 80/20 methanol/water.
- 753
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