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1 **Microscopic imaging as a tool to target spatial and temporal extraction of**
2 **bioactive compounds through ultrasound intensification.**

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B. Khadhraoui^{1,2}, A.S. Fabiano-Tixier², E. Petitcolas²,
P. Robinet¹, R. Imbert¹, M. El Maâtaoui³, F. Chemat^{2*}

7 (1) Laboratoires Arkopharma, laboratoire d'étude des substances naturelles, 06510 Carros,
8 France

9 (2) Avignon University, INRA, UMR408, GREEN Extraction Team, 84000 Avignon, France

10 (3) Avignon University, Qualisud UMR95, F-84000 Avignon, France

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12 **ABSTRACT**

13 Unravelling a chain of events in ultrasound-assisted extraction (UAE) of bioactive compounds
14 from plants has to start with a detailed description of deconstruction at macroscopic and
15 microscopic scale. The present work aims to study the impacts and interactions of UAE on the
16 extreme complexity and diversity of plants structures. Three plant species were selected for
17 their difference in specialized structures and their spatial distribution of secondary
18 metabolites: bitter orange leaf (*C. aurantium* L.), blackcurrant leaf (*R. nigrum* L.), and
19 artichoke leaf (*C. scolymus* L.). Different microscopic techniques (Cyto-histochemistry,
20 stereomicroscopic analysis, Scanning Electron Microscopy (SEM)) have been used to
21 understand the complexity of plant structures and to highlight ultrasound-induced impacts
22 especially on metabolites storage structures, with a neat comparison with conventional
23 “silent” extraction procedure. The main results indicate that spatial UAE impacts are strongly
24 related to plant structures’ properties (morphology, thickness, etc.) and particularly to the
25 nature and the chemical constitution of their storage specialized structures. From a temporal
26 point of view, for all studied leaves, observed mechanisms followed a special order according
27 to structures and their mechanical resistance level to ultrasound (US) treatment. Microscopic
28 mapping of metabolites and structures should be considered as a decision tool during UAE to
29 target intensification process.

30

31 **Keywords:** Ultrasound-Assisted Extraction; microscopic observations; mechanism;
32 specialized structures; bioactive compounds.

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34 **Corresponding author:** Farid CHEMAT (farid.chemat@univ-avignon.fr)

35

36 **1. Introduction**

37

38 Plant cells can be considered as highly performant ‘biofactories’ which produce a myriad
39 variety of high-value products including essentially primary metabolites (lipids, proteins,
40 carbohydrates) and secondary metabolites (terpenes, anthocyanins, polyphenols, etc.) [1,2].

41 Contrarily to primary metabolites, secondary metabolites do not have a vital role for plants;
42 whereas they have a key role in ensuring chemical communication with their environment
43 [1,2]. They also represent a valuable protection against abiotic and biotic conditions [3,4]. The
44 biosynthesis of those compounds is a highly complex process. Primary metabolites are the
45 precursors of secondary metabolites via three principal photosynthetic metabolic processes
46 namely nitrogen metabolism, fatty acid metabolism, and carbohydrate metabolism. Those
47 three basic pathways generate a large variety of secondary metabolites such as flavonoids,
48 tannins, terpenes, anthocyanins, alkaloids, etc.

49 Secondary metabolites can be stored at all aerial and underground plant parts (leaves, stems,
50 roots, etc.). Moreover, plants possess several specialized storage structures which can be
51 found in all plant parts [5]. Two major classes of these specialized structures can be
52 distinguished according to their localization [6]: Glandular Trichomes (GT) and Non-
53 Glandular Trichomes (NGT) are localized at the surface; internal glands (IG) are found in the
54 inner tissues of the mesophyll. These structures can adopt a myriad of sizes and shapes (long
55 hairs, highly branched, etc.). There are mainly two types of GT: Capitate Glandular
56 Trichomes (CGT) and Peltate Glandular Trichomes (PGT). These structures are mainly
57 distinguished by their morphology (size and stalk length) and mode of secretion [7].
58 Terpenoids seem to predominate GT structures [8, 9]. IG are frequently present in the
59 Rutaceae family, as for instance in *Citrus aurantium* leaves [10]. As for resin ducts, they are
60 mainly present in the Pinaceae family [3].

61 It is thus important to consider the great variety of natural products and the complexity of
62 their biosynthesis and storage processes within the plant kingdom but also within the plant
63 itself. This implies that extraction techniques should take into account this complexity to
64 improve extraction yield, selectivity and rate, and to ensure a targeted extraction of these
65 secondary metabolites of great interest in food, pharmaceutical, nutraceutical and cosmetic
66 industries [8, 11-13].

67 Extraction procedures range from conventional techniques (Soxhlet extraction, Clevenger
68 extraction, maceration) to new “green” technologies such as the Ultrasound-Assisted
69 Extraction (UAE). The positive contribution of power ultrasound (US) in solid-liquid
70 extraction of natural products has been widely studied and proved throughout literature [14-
71 19]. US-related performances gain consists of the amelioration of extraction yields, the
72 acceleration of extraction kinetics as well as respecting the environment and providing
73 extracts of higher quality [20-26]. Mechanisms behind UAE efficiency started to be unraveled
74 allowing a better understanding of US-generated impacts. Erosion, sonoporation, shear forces,
75 fragmentation, capillary effect and detexturation are the main reported mechanisms [27].
76 Those different mechanisms were generally observed individually, but a combination of these
77 six mechanisms has been recently reported [28]. A special order was noticed and is presumed
78 to be specific to the studied plant, and thus implying the necessity to further study the
79 ultrasonic behavior in solid-liquid extraction according to the specificities of not only US but
80 also plant matrices.

81 Within this objective, the present study aims to identify US impacts and mechanisms in the
82 case of three plant species: bitter orange (*Citrus aurantium* L.), blackcurrant (*Ribes nigrum*
83 L.) and artichoke (*Cynara scolymus* L.) leaves. UAE was compared to conventional procedure
84 (silent). The surfaces of untreated and treated leaves were investigated by stereomicroscopic
85 analysis and Scanning Electron Microscopy (SEM). Cyto-Histochemical study was carried
86 out to study the spatial distribution of natural products (caffeic acid derivatives and
87 flavonoids). US mechanisms, for each plant, were concluded on the basis of these different
88 observations to point out differences/similarities according to plant structural properties.

89

90

91 **2. Materials and methods**

92 **2.1.Plant material**

93 Dry leaves of *Citrus aurantium* L. (bitter orange), *Ribes nigrum* L. (blackcurrant) and *Cynara*
94 *scolymus* (artichoke) leaves were provided by Arkopharma (Carros, France). **Leaves were**
95 **collected in France in April 2018. They were dried at room temperature before being used**
96 **without any prior pretreatment.** Their initial moisture content ranged from 7.2 ± 0.4 % to $7.9 \pm$
97 0.6 %.

98 **2.2. Localized sonication**

99 To assess **US**-induced impacts on plant tissues, localized sonication was carried out using the
100 system presented in supplementary material (**figure S1**). Leaves were individually submitted
101 to the ultrasonic field emitted by an ultrasonic probe (1kW, UIP 1000 hdt, Hielscher
102 Ultrasonics GmbH, Germany). In each experiment, the dry leaf was fixed in a perforated disk
103 at 0.5 cm from the emitting probe. Localized sonication was performed at 20 kHz and
104 41.7 ± 0.4 W.cm⁻². Two different **US** durations (30 and 60 min) were applied to follow the
105 evolution of **US**-induced impacts over time. In all experiments, demineralized water was used
106 as a solvent. Its temperature was maintained at 25 ± 1 °C with a cooling system connected to
107 the double-jacket reactor. Conventional “silent” procedure (**CV**) was carried out using the
108 same system without **US** for 60 min. Each experiment was carried out in triplicate.

109 **2.3. Microscopic characterization procedures**

110 **2.3.1. Stereomicroscopic analysis**

111 Studied leaves were analyzed before and after each treatment (initial, localized sonication,
112 and **CV** procedure) using a Nikon AZ100 stereomicroscope (Nikon, Netherlands) equipped
113 for fluorescence (excitation filter: 465-495 nm, emission filter: 515-555 nm) to reveal the
114 presence of naturally fluorescent compounds. This stereomicroscopic investigation of leaves’
115 surfaces aimed to identify their initial characteristic structures as well as the induced structural
116 modifications/alterations. Leaves photos were taken at the same illumination conditions. All
117 microscopic observations were done in triplicate.

118 **2.3.2. Scanning Electron Microscopy (SEM)**

119 Untreated and treated leaves were observed using a scanning electron microscope (XL30, FEI
120 Philips, France). This microscopic investigation allowed the assessment of induced impacts
121 on plant micro-structures depending on the extraction process. Contrarily to the
122 stereomicroscopic analysis, SEM observations required a prior treatment of leaves’ fragments
123 which were fixed to a metal support and covered by a carbon adhesive (sputtering apparatus,

124 BD SCS 004, BALZERS) before analysis with SEM (7 Pa, 10 kV). Experiments were
125 performed in triplicate.

126 **2.3.3. Cyto-histochemistry**

127 To better understand the complexity of plant internal tissues, it is crucial to carry out cyto-
128 histochemical analysis of initial leaves. Tissues' preparation as well as tissues' sections and
129 slides' preparation are detailed in supplementary material (figure S2). Samples were taken
130 from each leaf and small fragments (5 to 7 mm) were instantly immersed into a fixative
131 mixture (4 % paraformaldehyde, 4% glutaraldehyde and 1 % caffeic acid in a phosphate
132 buffer 0.2 M) for 48h to preserve the integrity of plant tissues. Three rinsing cycles (3x1h)
133 were then conducted to eliminate all fixative solution residues. Thereafter, samples were
134 dehydrated in graded alcohol series (70 to 100%), infiltrated (24h) and embedded in resin
135 (Technovit 7100, Kultzer). Once the resin has solidified, we proceeded to sections' cutting
136 using a rotating microtome (Supercut 2065, Leica Microsystems, Germany). The obtained
137 sections were then collected on microscope slides.

138 Regarding the staining step, two procedures were performed: (1) Neu's reagent revelation for
139 all studied leaves and (2) Periodic acid-Schiff's reagent / Naphtol blue black reagent
140 (PAS/NBB) in the case of artichoke leaf. Neu's reagent revelation aimed at investigating the
141 spatial distribution of secondary metabolites such as flavonoids and caffeic acid derivatives
142 using fluorescence. As for PAS/NBB reagent, it was used to point out structural similarities
143 and /or differences between PGT of both adaxial and abaxial surfaces of artichoke leaf. Neu's
144 reagent solution was prepared as described by Andary et al. [29]. As regards PAS/NBB
145 reagent, it was prepared following procedure described by El Maâtaoui and Pichot [30].

146 All observations were performed using Leica DMR microscope equipped with dark field,
147 bright field, phase contrast and UV-illumination (Leica DMR, Leica Microsystems, Germany;
148 excitation filter: 340-380 nm, emission: 425 nm). Sections stained with Neu's reagent were
149 analyzed using an excitation filter of 340-380 nm and an emission filter at 425 nm. Under
150 these operating conditions, leaf tissues emit different fluorescence depending on their
151 phenolic content. Flavonoids are revealed in greenish-yellow. Caffeic acid derivatives are
152 light blue [29].

153 For PAS/NBB, stained sections were analyzed using phase contrast. PAS/NBB reagent has
154 the ability to reveal polysaccharides in pink color and proteins in dark blue. All experiments
155 were performed in triplicate.

156

3. Results and Discussion

3.1. Process-induced impacts depending on plant structures

Bitter orange, blackcurrant and artichoke leaves were analyzed with SEM and stereomicroscope to identify their main secondary metabolites' containing structures and to evaluate process-related structural modifications. CV and US treatments were conducted as described in section 2.2. Investigations of these three leaves, before and after each treatment, will be presented and discussed in the following sections.

3.1.1. Bitter orange leaf (*C. aurantium*)

As presented in figure 1, the presence of large internal glands (IG) is the major property of bitter orange leaf. Those specialized structures are highly present on the adaxial surface [figures 1.1 (a,b)] as previously described in many studies [31,32]. Stereomicroscopic investigation, presented in figure 1.1.c, highlighted the abundance of highly fluorescent natural compounds emanating from the whole adaxial surface. However, IG seemed to not emit any natural fluorescence. Their secondary metabolites are presumably stored deeper in their storage cavities embedded in the inner tissues of the mesophyll.

Regarding the process-induced impacts, the leaf adaxial surface appeared to be intact after 60 min of CV procedure. As shown in figure 1.2.d and 1.2.e, adaxial cuticle and IG were undamaged [compare figure 1.2 (d,e) with figure 1.1(a,b)]. In contrast, US treatment impacted significantly the adaxial surface by inducing ruptures into the cuticle which gives access to the underlying tissues (see figure 1.3.g). Moreover, large openings were noticed in the case of IG (figure 1.3.h) which is supposed to promote solvent penetration and solubilization of their secondary metabolites. Along with these observations, fluorescence intensity has dramatically decreased after US application during 60 min compared to that of both initial leaf and leaf submitted to the CV procedure (compare figure 1.3.i with figures 1.1.c and 1.2.f).

These observations showed that 60 minutes under US impacted bitter orange leaf by two different mechanisms according to plant structures: (1) sonoporation in the case of IG and (2) fragmentation in the case of the cuticle. It is worth mentioning that sonoporation started at thirty minutes of US treatment with relatively small openings into the IG envelopes (see figure 2.2). Larger openings were then generated resulting in their total explosion at sixty minutes of localized sonication (see figure 2.3). As for fragmentation mechanism, it was firstly observed after 60 min of US application.

Similar mechanical effects were previously noticed in plant solid/liquid extraction field in the case of rosemary leaf as a part of our previous work [28]. As described in this article, US-

190 generated pores were firstly identified at 20 min of **US** application. **US**-induced fragmentation
191 has also been reported in the same study at 30 min of **US** treatment.

192 Therefore, **US** could impact different plant species through the same mechanisms such as
193 sonoporation and fragmentation. However, from a temporal point of view, **US**-induced
194 structural changes could take completely different pathways.

195 **3.1.2. Blackcurrant leaf (*R. nigrum*)**

196 SEM and stereomicroscopic investigations of blackcurrant leaf' abaxial surface are presented
197 in [figure 3](#). The main characteristics of this leaf are the presence of external **PGT** and **NGT**
198 (see [figures 3.1.a](#) and [3.1.b](#)) as previously shown by Kerslake [7]. We can notice that PGT are
199 more abundant than NGT which are mainly located in the midrib. Moreover, PGT appeared to
200 be the most fluorescent structures of leaf abaxial surface (see [figure 3.1.c](#)) suggesting a high
201 presence of naturally fluorescent compounds in those external specialized structures.

202 Regarding the process-related impacts, **CV** procedure appeared to preserve those two
203 characteristic structures as well as the integrity of the cuticle (see [figures 3.2.e](#) and [3.2.f](#)). As
204 shown in [figure 3.2.e](#), after 60 min of **CV** treatment, **PGT** have the same aspect as that of the
205 initial leaf (balloon-shape, intact envelope, etc.). This is also the case for the long **NGT** which
206 seemed to be preserved ([figure 3.2.f](#)). Those observations are in agreement with the
207 fluorescence intensity which remained stable after 60 min of **CV** procedure (compare [figure](#)
208 [3.2.g](#) with [figure 3.1.c](#)).

209 However, focusing our attention on the localized sonication, many induced impacts can be
210 identified ([figure 3.3](#)). Starting with the leaf surface, it can be noticed that the cuticle seemed
211 to be shrunk and dehydrated ([figure 3.3.i](#)). In addition, **PGT** were completely exploded after
212 60 min of **US** application giving access to their storage cavities' content and their secretory
213 cells (see [figure 3.3.i](#)). Surprisingly, **NGT** were more resistant to the ultrasonic field (see
214 [figure 3.3.j](#)). Most of those structures were undamaged after 60 min; only some **NGT** were
215 eroded ([figure 3.3.j](#)) which provides access to the secondary metabolites of the underlying
216 tissues.

217 Stereomicroscopic observations provided an excellent comparison between the **CV** procedure
218 and the localized sonication (compare [figure 3.2.g](#) with [figure 3.3.k](#)). As shown in these
219 figures, within the same leaf, the left side was submitted to the **CV** procedure for 60 min
220 ([figure 3.2.g](#)) while the right side was submitted to the ultrasonic field for the same duration
221 ([figure 3.3.k](#)). It is clear that, contrarily to the **CV** procedure, **US** application generated
222 different physical impacts on abaxial leaf surface [compare [figure 3.2 \(g,h\)](#) with [figure 3.3](#)
223 [\(k,l\)](#)]. The major noticed impacts are large ruptures into the cuticle and the explosion of PGT

224 (see [figures 3.3.k](#) and [3.3.l](#)). A drastic decrease in fluorescence intensity has also been noticed
225 indicating the decrease in the concentration of naturally fluorescent compounds.

226 Regarding the evolution of the induced impacts during **US** application ([figure 4](#)), it can be
227 noticed that the generated damage in **PGT** started at 30 min of **US** treatment with small
228 openings into their smooth envelope ([figure 4.2.c](#)). Their total explosion was observed at 60
229 min of **US** application ([figure 4.3.e](#)). However, 30 min of **US** application did not induce any
230 impact on **NGT** ([figure 4.2.d](#)), further confirming their particular resistance.

231 To summarize, blackcurrant leaves' **PGT** and **NGT** are both located in the surface; whereas
232 they had not the same resistance level to the ultrasonic field. These observations pointed to
233 the strong probability that those two external structures have different structural constitutions.
234 Hence, **US** efficiency can be limited by the resistance of some specialized structures such as
235 in the case of **NGT** of blackcurrant leaf.

236 Our results showed that **US** impacted blackcurrant abaxial leaf surface by three different
237 mechanisms: (1) sonoporation resulting in the explosion of **PGT**, (2) fragmentation
238 mechanism implying large fractures into the cuticular layer and (3) erosion of some **NGT**. The
239 first mechanism noticed was the sonoporation starting at 30 min. The fragmentation
240 mechanism was observed at 60 min. However, **NGT**' erosion mechanism appeared to require
241 longer **US** treatments since it began at 60 min. It is important to note that the erosion
242 mechanism has already been reported in many studies [23, 33, 34]. It was also recently
243 identified in our previous work in the case of rosemary leaf [28]. An absolutely crucial point
244 to mention is that, in the case of rosemary leaf, mechanical erosion of **NGT** was observed
245 after only 5 min of **US** application. Total abrasion of those long and branched **NGT** was
246 noticed after 20 min contrarily to our current observations of blackcurrant leaves' **NGT**.
247 Moreover, bitter orange leaves' **IG**, embedded into the internal tissues, had the same behavior
248 as that of blackcurrant leaves' external **PGT** (see [section 3.1.1](#)) which implies the crucial
249 importance of the containing' structures constitution. These different observations proved that
250 **US**-induced impacts are strongly dependent on plant families and their different characteristic
251 structures.

252 **3.1.3. Artichoke leaf (*C. scolymus*)**

253 Observations concerning artichoke initial leaf are presented in [figure 5](#). SEM analyses pointed
254 to the likelihood between the specialized structures present in both adaxial and abaxial
255 surfaces which are **PGT** and **NGT** [compare [figure 5.1\(a,b\)](#) with [figure 5.2 \(d,e\)](#)]. It is
256 noteworthy that **NGT** are more abundant on abaxial surface where they cover densely the
257 whole surface (compare [figure 5.1.b](#) with [figure 5.2.e](#)). **PGT** of both surfaces are balloon-

258 shaped; those of abaxial surface are hidden by the massive presence of NGT. These
259 observations are in accordance with those of Brutti et al [35].

260 Stereomicroscopic analysis pointed to a higher fluorescence of abaxial surface compared to
261 that of the adaxial surface (compare figure 5.1.c with figure 5.2.f). This important
262 fluorescence could be related to the massive presence of PGT which are proved to be the most
263 naturally fluorescent structures of artichoke leaf (figure 5.2.f).

264 Figure 6 shows process-related impacts on adaxial leaf surface. As seen in this figure, having
265 undergone CV procedure (figure 6.2), adaxial leaf structures were entirely preserved: intact
266 balloon-shaped PGT and long NGT. However, stereomicroscopic observations revealed a
267 slight decrease in fluorescence intensity (compare figure 6.2.h with figure 6.1.d) which could
268 be explained by the diffusion of hydrophilic natural compounds to the surrounding solvent.
269 As regards the localized sonication (figure 6.3), induced impacts were more extensive.
270 Considering SEM investigation, it can be seen that US treatment resulted in pronounced
271 damage into the adaxial cuticle (figure 6.3.i). Many large fractures and holes were noticed,
272 thus enhancing the accessibility to the inner tissues. NGT were also impacted after 60 min of
273 US application. As shown in figure 6.3.j, most of NGT were eroded promoting the liberation
274 of their content into the surrounding solvent as well as solvent penetration into the underlying
275 tissues. Interestingly, PGT were more resistant to the ultrasonic field (figure 6.3.j). Only some
276 slight deformations into their envelopes could be noticed. Hence, longer US treatment
277 durations seemed to be required to reach the total explosion of those structures. Consequently,
278 there is evidence to suggest the hypothesis that PGT envelopes have a different constitution
279 compared to that of the cuticle and NGT, thus explaining their resistance to the ultrasonic
280 treatment. These different SEM observations were confirmed by stereomicroscopic analyses
281 showing a noticeable decrease in fluorescence intensity of the adaxial cuticle. In contrast,
282 PGT have retained their natural fluorescence [compare figure 6.3 (k,l) with figure 6.1 (c,d)].

283 To summarize, US impacted artichoke leaf adaxial surface by three different mechanisms: (1)
284 fragmentation in the case of adaxial cuticular layer (ruptures and large holes), (2) shear forces
285 inducing deformations into adaxial PGT' envelopes and (3) erosion of NGT.

286 Investigation of the abaxial leaf surface investigation is presented in figure 7. It can be noted
287 that CV procedure had not impacted leaf structures [compare figure 7.2 (e,f) with figure 7.1
288 (a,b)]. Long NGT were preserved; they covered densely the entire abaxial surface. PGT were
289 also undamaged [see figure 7.2 (e,f)]. These observations are in coherence with
290 stereomicroscopic investigation revealing constant fluorescence intensity compared to that of
291 initial leaf abaxial surface [compare figure 7.2 (g,h) with figure 7.1 (c,d)].

292 Regarding the localized sonication ([figure 7.3](#)), abaxial cuticle was extremely damaged: large
293 fractures and holes were observed. NGT were also impacted; most of them were completely
294 exfoliated ([figure 7.3.i](#)). Surprisingly, abaxial PGT were highly impacted; most of them were
295 completely exploded ([figure 7.3.j](#)) giving access to their secretory cells. Along with these
296 SEM observations, general fluorescence level has significantly dropped (compare [figure 7.3](#)
297 [\(k,l\)](#) with [figure 7.1 \(c,d\)](#)).

298 In the case of abaxial surface, US acts through four different mechanisms: (1) fragmentation
299 in the case of abaxial cuticular layer (large fractures), (2) shear forces and (3) sonoporation
300 inducing respectively deformations and pores into abaxial PGT' envelopes and (4) erosion of
301 abaxial NGT. [Figure 8](#) illustrates the gradual induced impacts during US treatment of both
302 adaxial and abaxial surfaces. This figure further outlines differences between adaxial and
303 abaxial PGT behaviors during US treatment. Indeed, thirty minutes of US application induced
304 some deformations into the adaxial PGT' envelopes (compare [figure 8.2.c](#) with [figure 8.1.a](#)).
305 These induced deformations are presumably related to shear forces generated by the
306 ultrasonic wave propagation into the liquid solvent [28]. Surprisingly, adaxial PGT having
307 undergone 60 min of US application maintained the same shape and aspect as that after 30
308 min of US application (compare [figure 8.3.e](#) and [figure 8.2.c](#)). Therefore, 60 min under US-
309 generated shear forces were insufficient to break-down their apparently resistant envelopes. In
310 the case of abaxial PGT, shear forces-related deformations appeared also at 30 min ([figure](#)
311 [8.2.d](#)). Total explosion of those structures was noticed at 60 min of US application ([figure](#)
312 [8.3.f](#)).

313 These observations suggest that adaxial and abaxial PGT' envelopes have different
314 constitutions. Cyto-chemical investigation was carried out to assess this hypothesis.
315 Microscopic observations of adaxial and abaxial PGT sections after staining with PAS/NBB
316 reagents are presented in [figure 9](#). It is apparent from this figure that adaxial and abaxial PGT
317 have the same chemical constitution (compare [figure 9.a](#) with [figure 9.b](#)). Proteins appeared to
318 be highly concentrated in the secretory cells stained in dark blue. As regards polysachharides,
319 they are mainly located in the inner part of the secretory cavity envelope revealed in pink
320 color.

321 In contrast, from a structural standpoint, adaxial PGT envelope seemed to be thicker than that
322 of abaxial PGT (compare [figure 9.b](#) with [figure 9.a](#)). It is worth remembering that abaxial
323 PGT are hidden and thus protected by the massive presence of abaxial NGT contrarily to
324 adaxial PGT which are directly exposed to the biotic and abiotic conditions (temperature, UV-

325 light, external attacks, etc.). Thus, the thickening of adaxial PGT envelopes could represent a
326 means of protection against all external disturbances.
327

328 Combining these results with SEM and stereomicroscopic observations, it can be seen that
329 thickness of adaxial PGT plays a key role in their particular resistance to the ultrasonic action.

330 **3.2.Cyto-histochemistry as a decision tool for a targeted extraction**

331 The section 3.1 was devoted to the understanding of **US**-induced impacts depending on plant
332 families/species and plant structures. It proved the importance of SEM and stereomicroscopic
333 plant analyses allowing the investigation of their characteristic structures as well as their
334 resistance level to the ultrasonic field. However, those two microscopic techniques (SEM and
335 stereomicroscopic) provide specific information limited to the surface structures such as
336 external specialized structures and the cuticular layer.

337 For example, in the case of bitter orange leaf IG, SEM and stereomicroscopic investigation
338 provided important insights into the explosion of those structures (see [section 3.1.1](#)).
339 However, there is no evidence that US application allowed the extraction of their storage
340 cavity' and secretory cells' contents. Further analyses are then required to better localize plant
341 secondary metabolites and to assess US efficiency. For such a purpose, cyto-histochemical
342 investigation was performed to better understand the spatial distribution of plant secondary
343 metabolites.

344 [Figure 10](#) shows bitter orange, blackcurrant and artichoke leaves' sections after staining with
345 Neu's reagent. As seen in this figure, for the three studied species, leaf tissues emitted two
346 different fluorescence colors depending on their phenolic contents: a greenish-yellow color
347 for flavonoids and a light blue in the case of caffeic acid derivatives. Microscopic
348 observations pointed out that the three studied leaves are highly rich in flavonoids and caffeic
349 acid derivatives. However, it is important to note that secondary metabolites' spatial
350 distribution is different depending on plant species and structures ([figure 10](#)). In the case of
351 bitter orange leaf ([figure 10.a](#)), caffeic acid derivatives are present in the cuticular layer highly
352 stained in light blue. As for flavonoids, revealed in greenish-yellow, they are located deeper in
353 the inner tissues (mesophyll tissues and IG' secretory cells) (see [figure 10.a](#)).

354 Blackcurrant leaf is also rich in caffeic acid derivatives (see [figure 10.b](#)). Those compounds
355 are mainly identified in adaxial and abaxial cuticular layers. As regards flavonoids, they are
356 present in the external PGT (secretory cells and envelopes). Inner tissues (epidermal cells and
357 mesophyll tissues) were highly stained in yellowish-green indicating their high concentration
358 in flavonoids ([figure 10.b](#)). Concerning artichoke leaf ([figure 10.c](#)), flavonoids appeared to be
359 present in the entire leaf' external (NGT and PGT) and inner tissues (epidermal cells and
360 mesophyll' inner tissues). Overall, plant cyto-histochemical investigation could represent a

361 means to determine the spatial distribution of active natural compounds such as flavonoids
362 and caffeic acid derivatives and thus merits further attention.

363 **4. Conclusion and perspectives**

364 Our microscopic observations proved the complexity of plant structures. A heterogeneous
365 spatial distribution of secondary metabolites (caffeic acid derivatives and flavonoids) and a
366 diversity of their containing structures were noticed. The three studied plant species (bitter
367 orange, blackcurrant and artichoke leaves) evolve different structural properties and different
368 specialized storage structures. IG are the main characteristic of bitter orange adaxial surface.
369 NGT and PGT are present in blackcurrant abaxial leaf surface. As for artichoke leaf, NGT and
370 PGT are highly abundant in both abaxial and adaxial leaf surfaces.

371 Contrarily to the **CV** procedure which preserves plant structures, US application generated
372 several structural alterations especially on specialized structures. However, US-related
373 impacts are strongly dependent on plant species. In the case of bitter orange leaf, IG were
374 completely damaged after 60 min of US treatment. Adaxial cuticle was also highly impacted.
375 Therefore, in the case of bitter orange leaf, US acts through two different mechanisms: (1)
376 sonoporation in the case of IG and (2) fragmentation in the case of adaxial cuticle. Three
377 mechanisms were observed in the case of blackcurrant leaf: (1) sonoporation implying the
378 explosion of PGT, (2) fragmentation of the abaxial cuticular layer and (3) erosion of NGT.
379 The study of artichoke further proved the complexity of plant matrices and the considerable
380 differences observed between their two surfaces (abaxial vs adaxial). Indeed, US impacted the
381 artichoke leaf surfaces through different mechanisms. Three mechanisms were noticed in the
382 case of adaxial surface: (1) Fragmentation of the cuticular layer, (2) shear forces on the PGT
383 and (3) erosion of NGT. For the abaxial surface, four mechanisms were observed: (1)
384 fragmentation of the cuticle, (2) shear forces and (3) sonoporation on PGT, and (4) erosion of
385 NGT. This implies that adaxial and abaxial PGT have not the same resistance level which
386 confirms the complexity of plant structures behavior while being submitted to the ultrasonic
387 field.

388 Moreover, this study could be a valuable tool for assessing **UAE** performances. As an
389 example, cyto-histochemical study of bitter orange leaf before and after treatment (**CV**
390 treatment vs UAE) was carried out and presented in [figure 11](#). Considering the **CV** procedure,
391 orange bitter leaf exhibits the same structural properties compared to the initial structural
392 properties (compare [figure 11.b](#) with [figure 11.a](#)). The cuticular layer was undamaged. Plant
393 cells appeared to maintain their morphological integrity. In contrast, a noticeable decrease in

394 fluorescence intensity has been observed (compare [figure 11.b](#) with [figure 11.a](#)). Light blue
395 fluorescence of the adaxial cuticular layer had dropped dramatically compared to that of the
396 initial leaf. In the same way, the greenish-yellow fluorescence of the inner tissues has
397 decreased significantly. These two observations could be related to the diffusion of
398 hydrophilic caffeic acid derivatives and flavonoids to the extraction solvent. Regarding UAE,
399 many structural alterations can be noticed. Starting with the cuticular layer, many deep
400 fractures were identified. Moreover, significant damage has been noticed in the inner cells
401 compared to those of the initial leaf and leaf after **CV** procedure (compare [figure 11.c](#) with
402 [figure 11.a](#) and [figure 11.b](#)). This noticeable damage includes cells shrinkage, vacuole
403 regression and cell wall lysis (see [figure 11.c](#)). Together, these US-induced alterations
404 enhance solvent penetration into inner tissues as well as the release of secondary metabolites.
405 This results in a dramatic decrease in greenish-yellow and light blue fluorescence after 60 min
406 of UAE, indicating an efficient extraction of flavonoids and caffeic acid derivatives of bitter
407 orange leaf.

408 Based on our findings, spatial distribution and **temporal extraction** of secondary metabolites
409 and the comprehension of plant structural properties should be considered as a decision tool
410 for a targeted UAE which could take completely different pathways according to plant species
411 and their structural resistance level. It became important to start with microscopic analysis
412 before proceeding with US procedure on an unknown or non-studied plant. This breakthrough
413 opens the door to quick and easy methods for localizing glands and structures containing
414 bioactive compounds but also US energy and time required for detexturation. The decision
415 tool will also permit to target specific secondary or primary metabolites **providing thus a**
416 **complete and selective extraction.**

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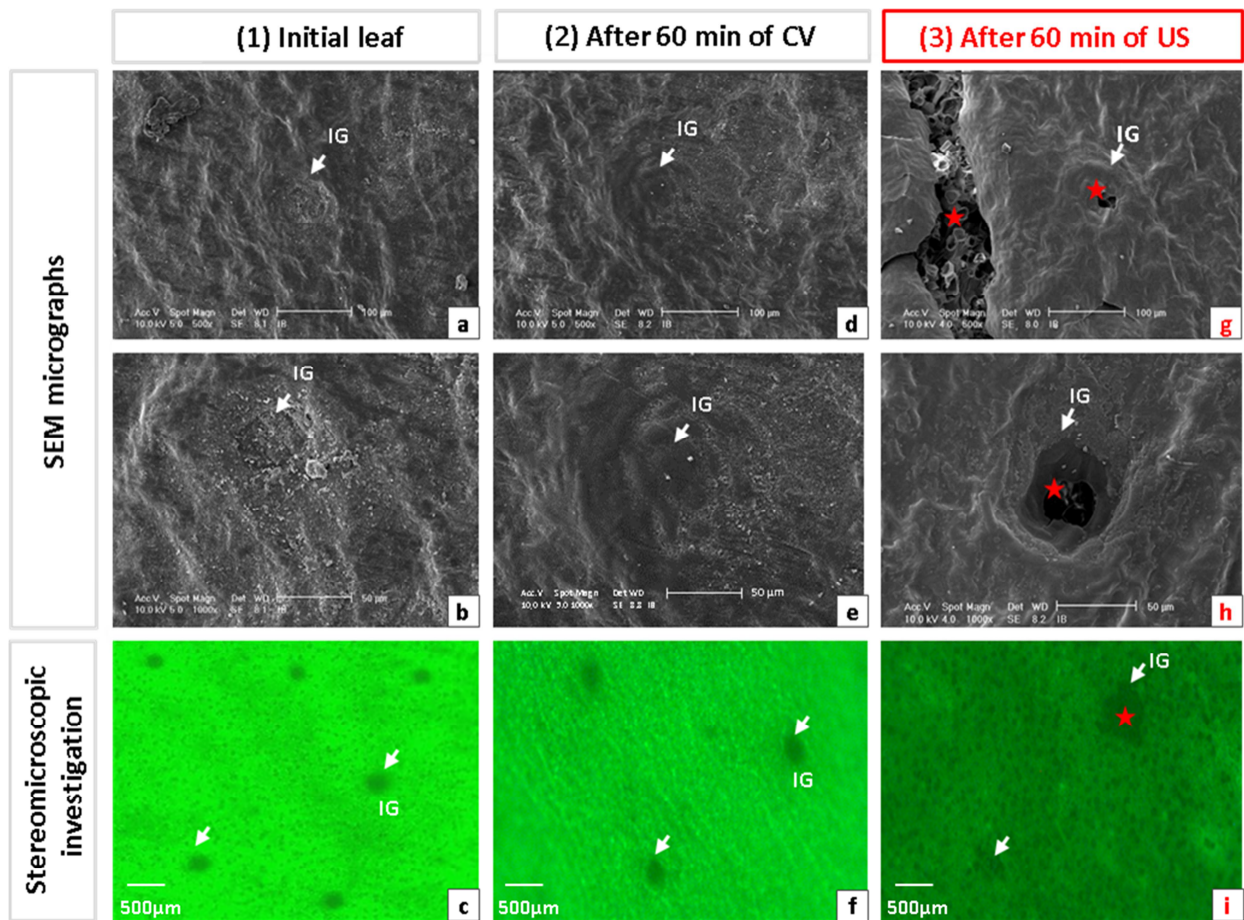
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517 204.

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519 **List of figures**520 **Figure 1.** Investigation of *C. aurantium* adaxial leaf surface.521 **Figure 2.** SEM micrographs of *C. aurantium* adaxial leaf surface during ultrasound treatment.522 **Figure 3.** Investigation of *R. nigrum* abaxial leaf surface.523 **Figure 4.** SEM micrographs of PGT and NGT of the *R. nigrum* abaxial leaf surface during
524 **US** treatment.525 **Figure 5.** Investigation of *C. scolymus* adaxial and abaxial surfaces.526 **Figure 6.** Investigation of *C. scolymus* adaxial leaf surface.527 **Figure 7.** Investigation of *C. scolymus* abaxial leaf surface.528 **Figure 8.** SEM micrographs of *C. scolymus* adaxial and abaxial surfaces during **US** treatment.529 **Figure 9.** Cyto-histochemical investigation of *C. scolymus* PGT after staining with PAS/NBB
530 reagent (phase contrast micrographs).531 **Figure 10.** Cyto-histochemical study after revelation with Neu's reagent.532 **Figure 11.** Cyto-histochemical study of *C. aurantium* adaxial leaf surface after staining with
533 Neu's reagent.



534

535 **Figure 1.** Investigation of *C. aurantium* adaxial leaf surface.

536 (1) Investigation of the initial leaf: SEM micrographs (a-b) and stereomicroscopic

537 investigation under UV-illumination (c). (2) Leaf investigation after 60 min of CV procedure:

538 SEM micrographs (d-e) and stereomicroscopic investigation under UV-illumination (f). (3)

539 Leaf Investigation after 60 min of US treatment: SEM micrographs (g-h) and

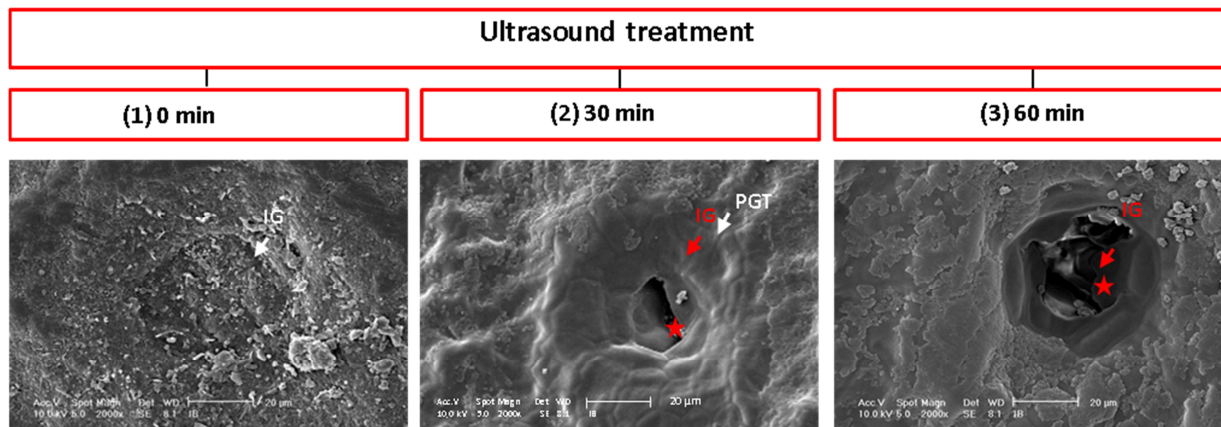
540 stereomicroscopic investigation under UV-illumination (i).

541 IG: Internal Gland; ★ : rupture/opening zone.

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546 **Figure 2.** SEM micrographs of *C. aurantium* adaxial leaf surface during **US** treatment.

547 (1) Initial leaf. (2) After 30 min of US treatment. (3) After 60 min of US treatment.

548 IG: Internal Gland; ★ : rupture/opening zone.

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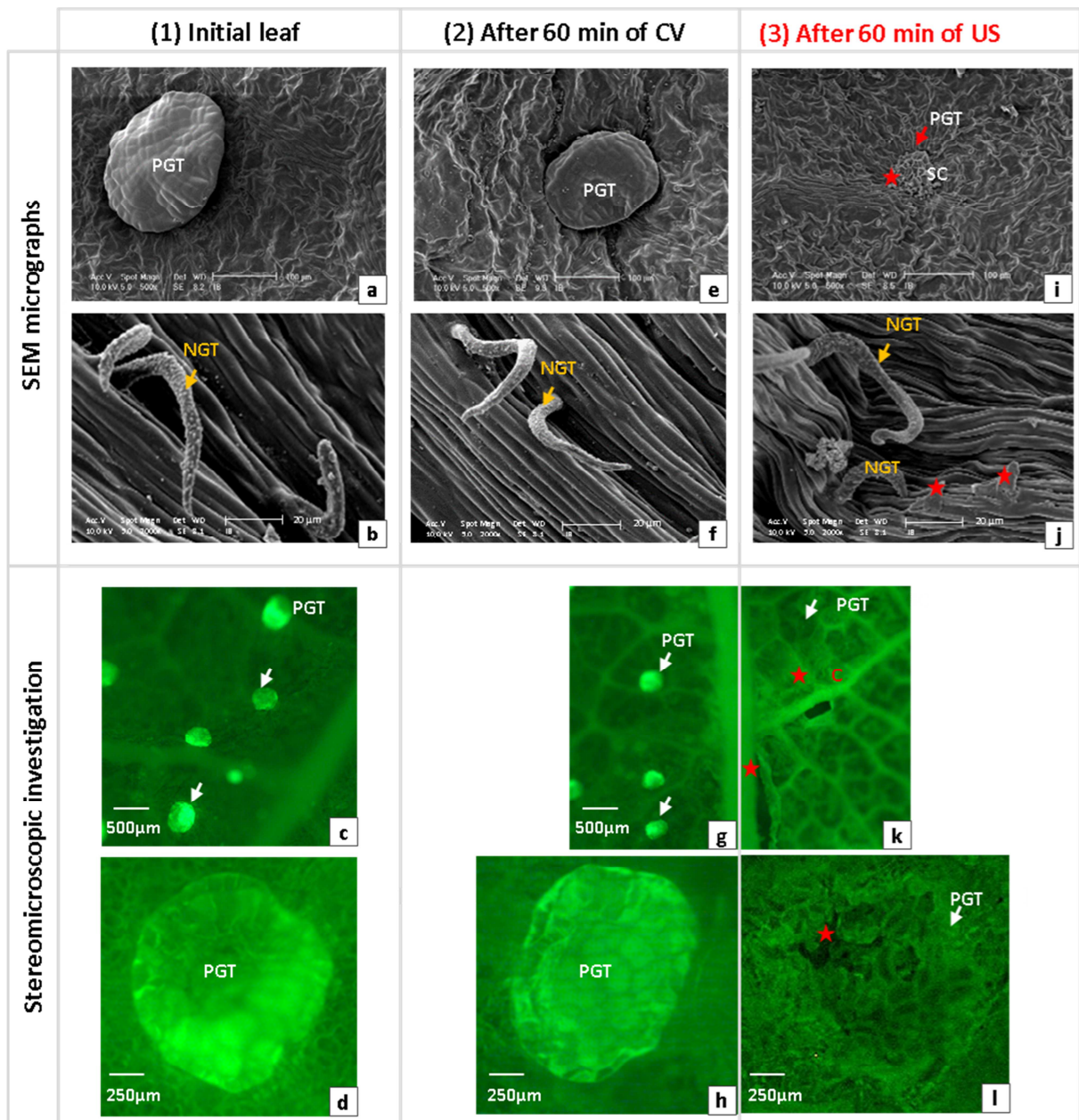
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565 **Figure 3.** Investigation of *R. nigrum* abaxial leaf surface.

566 (1) Investigation of the initial leaf: SEM micrographs (a-b) and stereomicroscopic investigation under

567 UV-illumination (c-d). (2) Leaf Investigation after 60 min of CV procedure: SEM micrographs (e-f)

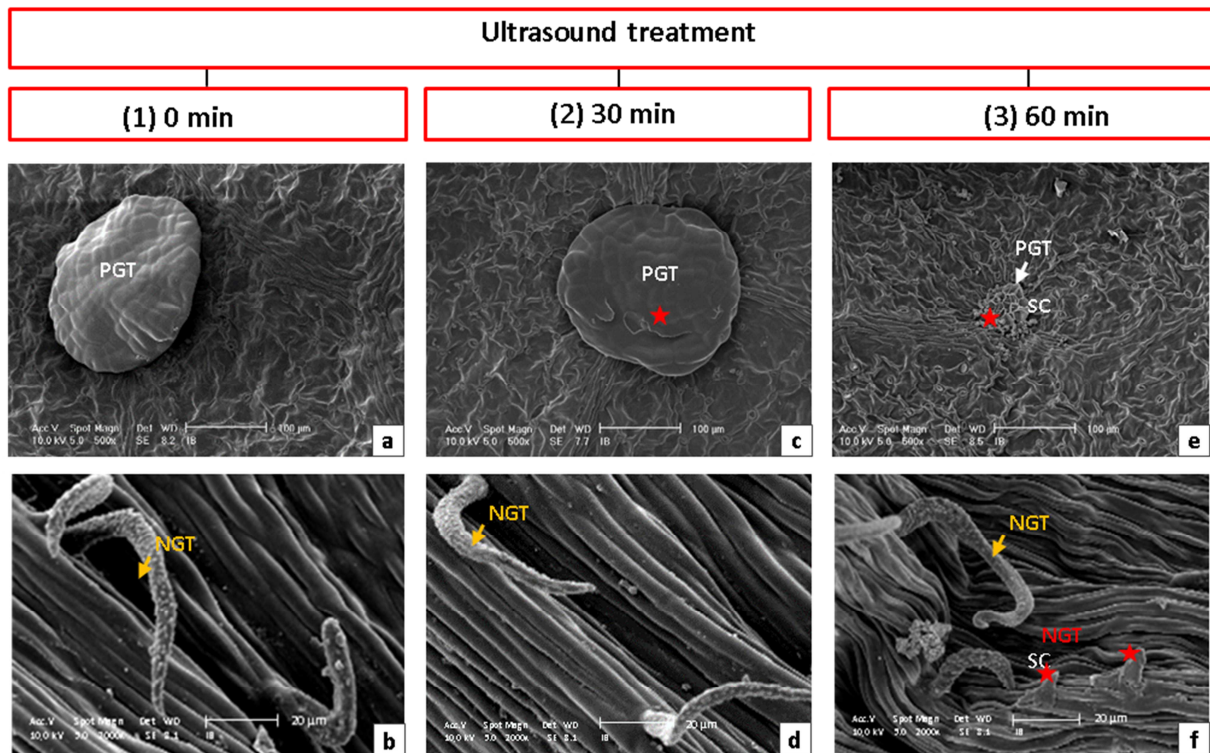
568 and stereomicroscopic investigation under UV-illumination (g-h). (3) Leaf Investigation after 60 min

569 of US treatment: SEM micrographs (i-j) and stereomicroscopic investigation under UV-illumination

570 (k-l).

571 PGT: Peltate Glandular Trichomes; SC: Secretory Cells; C: abaxial Cuticle; ★ : rupture/ opening zone.

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574 **Figure 4.** SEM micrographs of PGT and NGT of the *R. nigrum* abaxial leaf surface during
575 **US** treatment. (1) Investigation of the initial leaf: PGT (a) and NGT (b). (2) Leaf investigation
576 after 30 min of **US** treatment: PGT (c) and NGT (d). (3) Leaf investigation after 60 min of **US**
577 treatment: PGT (e) and NGT (f).

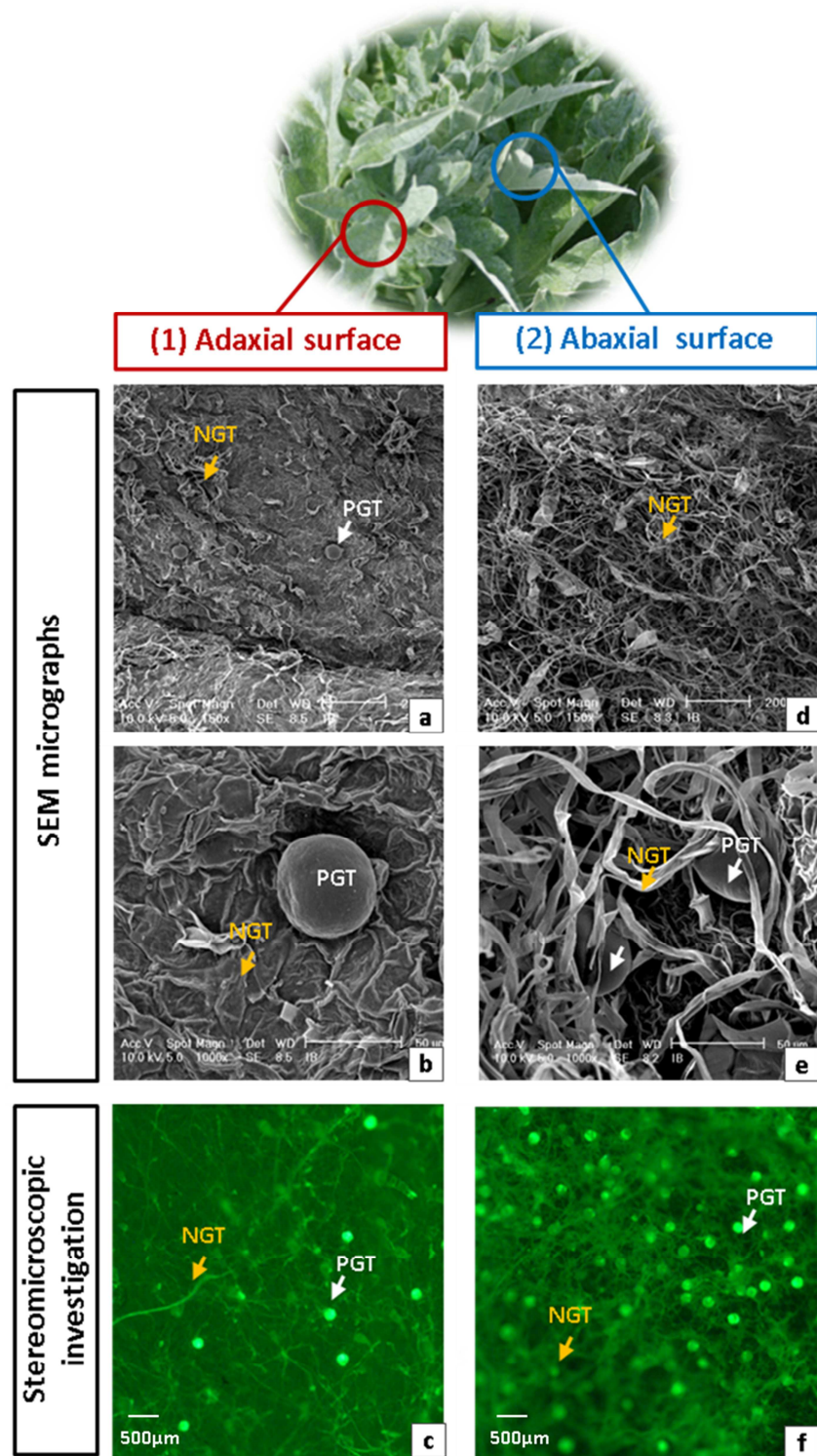
578 PGT: Peltate Glandular Trichomes; NGT: Non-Glandular Trichomes; SC: Secretory Cells;

579 ★ : rupture/opening zone.

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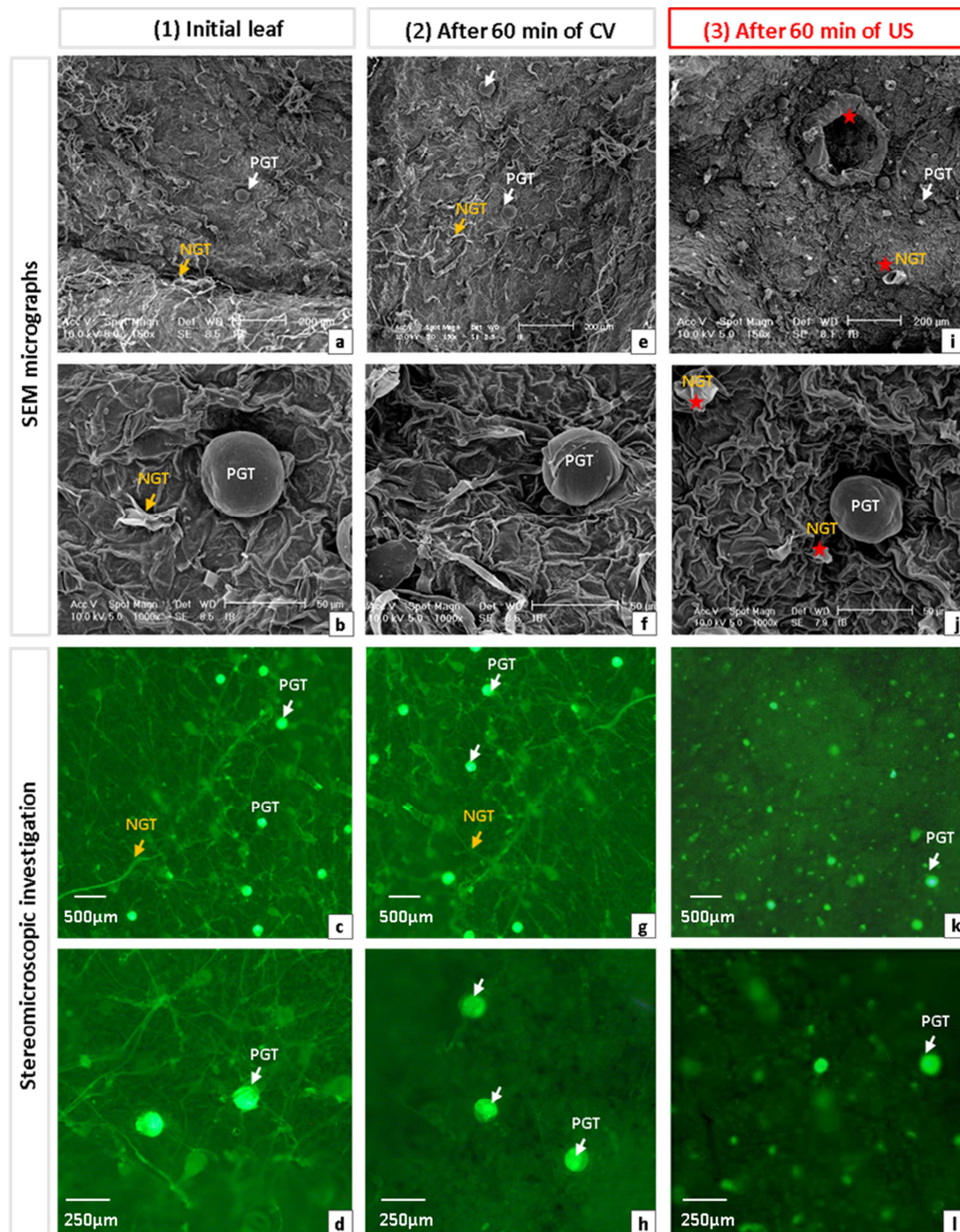
584 **Figure 5.** Investigation of *C. scolymus* adaxial and abaxial surfaces.

585 Adaxial surface: SEM micrographs (a-b) and stereomicroscopic investigation (c). (2) Abaxial surface:

586 SEM micrographs (d-e) and stereomicroscopic investigation (f).

587 PGT: Peltate Glandular Trichomes; NGT: Non-Glandular Trichomes.

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590 **Figure 6.** Investigation of *C. scolymus* adaxial leaf surface. (1) Investigation of the initial leaf: SEM

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micrographs (a-b) and stereomicroscopic investigation under UV-illumination (c-d).

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(2) Leaf Investigation after

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60 min of CV procedure: SEM micrographs (e-f) and stereomicroscopic investigation under UV-illumination (g-

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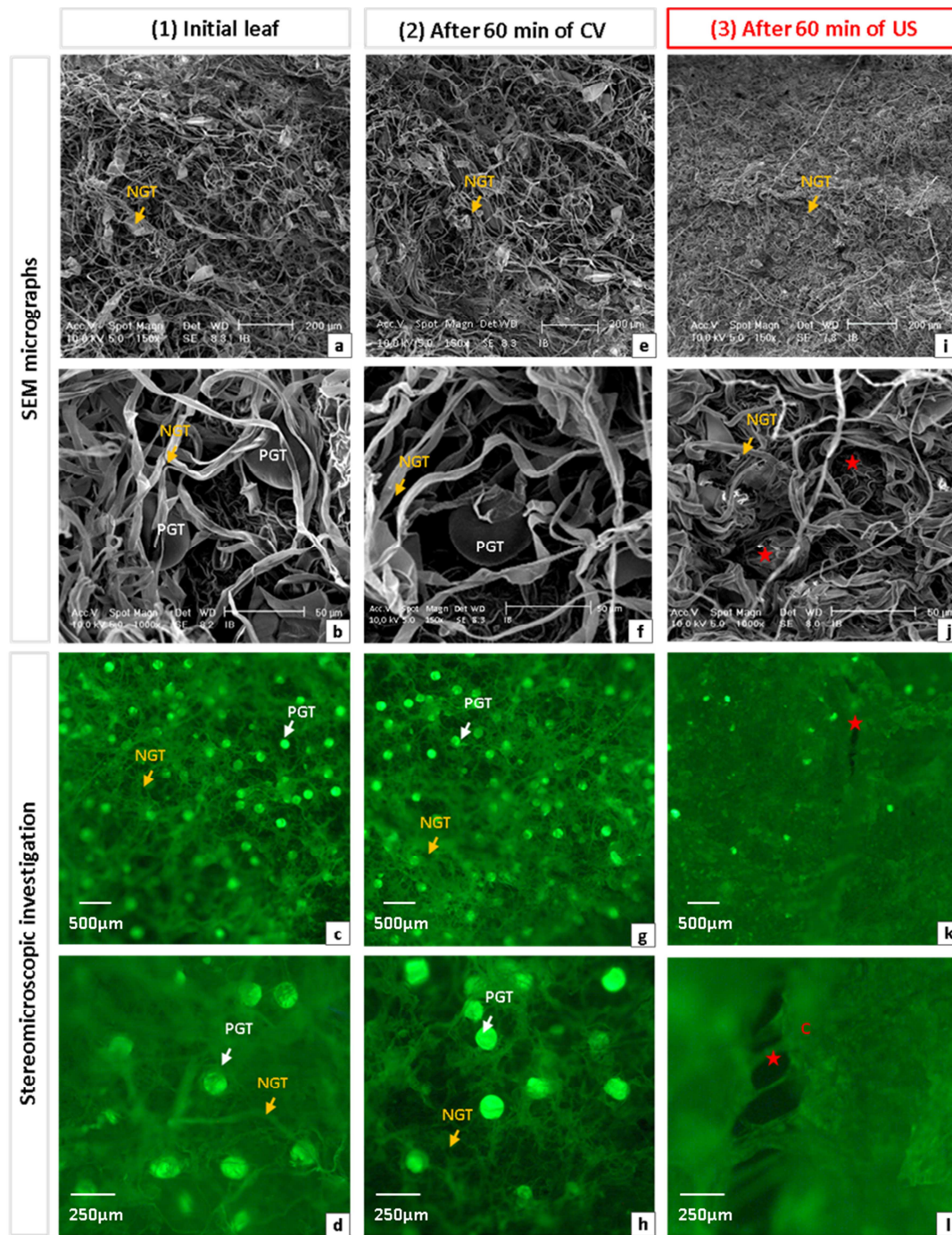
h). (3) Leaf Investigation after 60 min of US treatment: SEM micrographs (i-j) and stereomicroscopic

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investigation under UV-illumination (k-l). PGT: Peltate Glandular Trichomes; NGT: Non-Glandular Trichomes;

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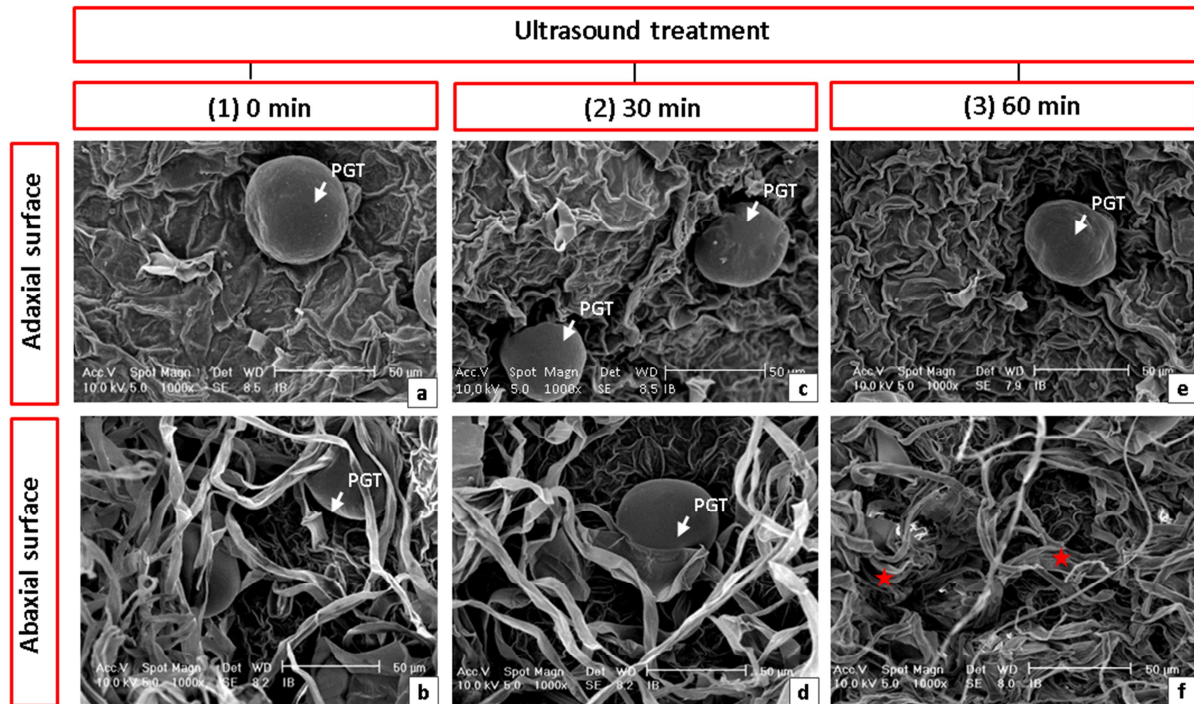
★ : rupture/opening zone.



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598 **Figure 7.** Investigation of *C. scolymus* abaxial leaf surface. (1) Investigation of the initial leaf: SEM
 599 micrographs (a-b) and stereomicroscopic investigation under UV-illumination (c-d). (2) Leaf Investigation after
 600 60 min of CV procedure: SEM micrographs (e-f) and stereomicroscopic investigation under UV-illumination (g-
 601 h). (3) Leaf Investigation after 60 min of US treatment: SEM micrographs (i-j) and stereomicroscopic
 602 investigation under UV-illumination (k-l). PGT: Peltate Glandular Trichomes; NGT: Non-Glandular Trichomes;
 603 C: Abaxial Cuticle; ★ : rupture/opening zone.

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606 **Figure 8.** SEM micrographs of *C. scolyms* adaxial and abaxial surfaces during **US** treatment.

607 (1) Initial leaf: adaxial surface (a) and abaxial surface (b). (2) After 30 min of ultrasound

608 treatment: adaxial surface (c) and abaxial surface (d). (3) After 60 min of **US** treatment:

609 adaxial surface (e) and abaxial surface (f)

610 IG: Internal Gland; ★ : rupture/opening zone.

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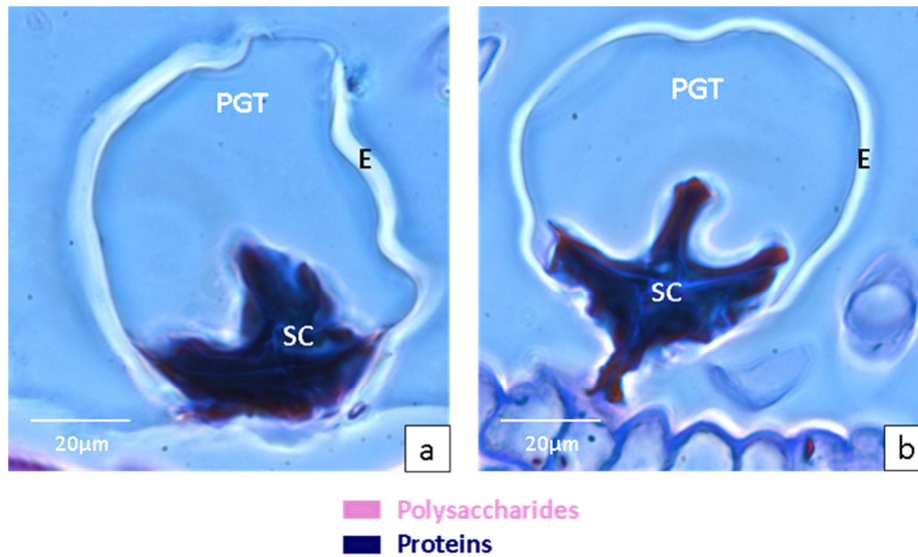
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619 **Figure 9.** Cyto-histochemical investigation of *C. scolymus* PGT after staining with PAS/NBB
 620 reagent (phase contrast micrographs).

621 (a) Adaxial PGT. (b) Abaxial PGT.

622 PGT: Peltate Glandular Trichomes; SC: Secretory Cells; E: Storage cavity' envelope

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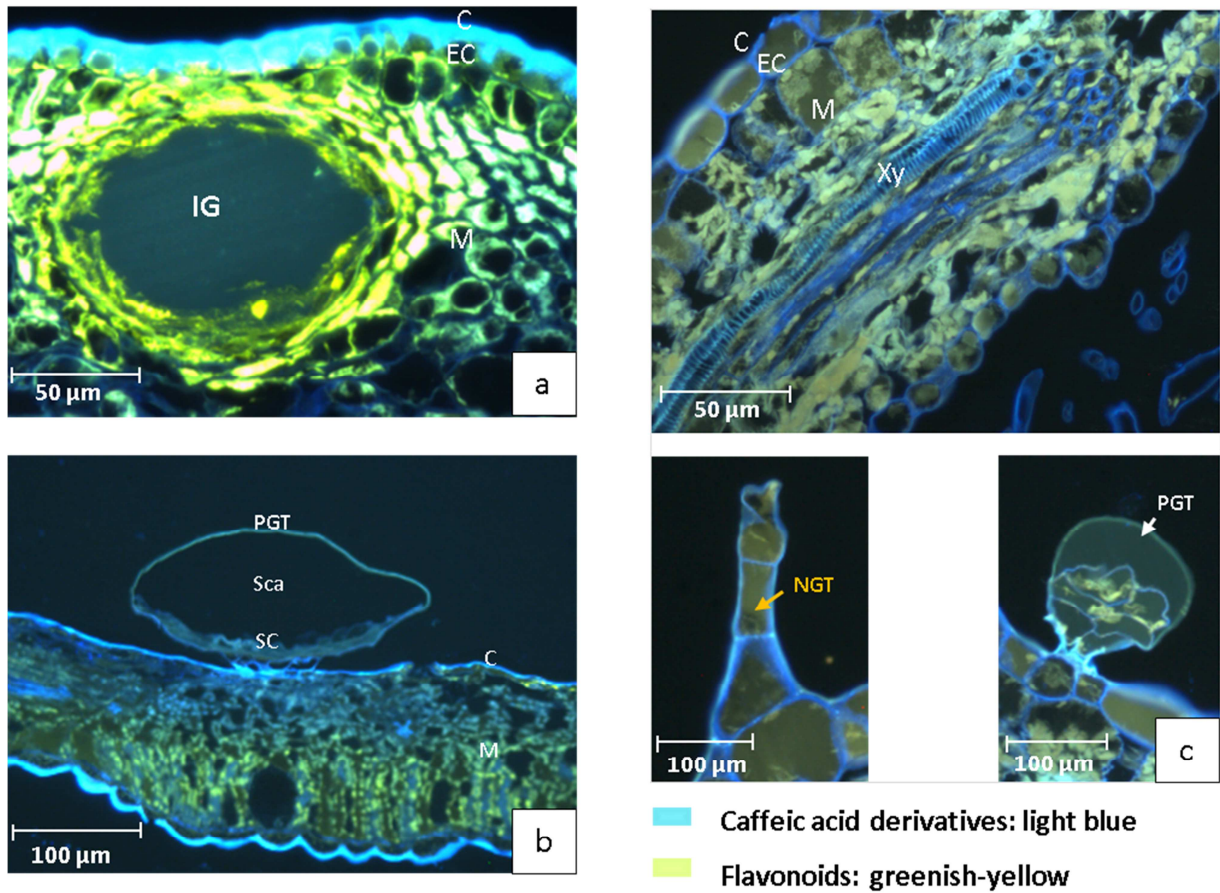
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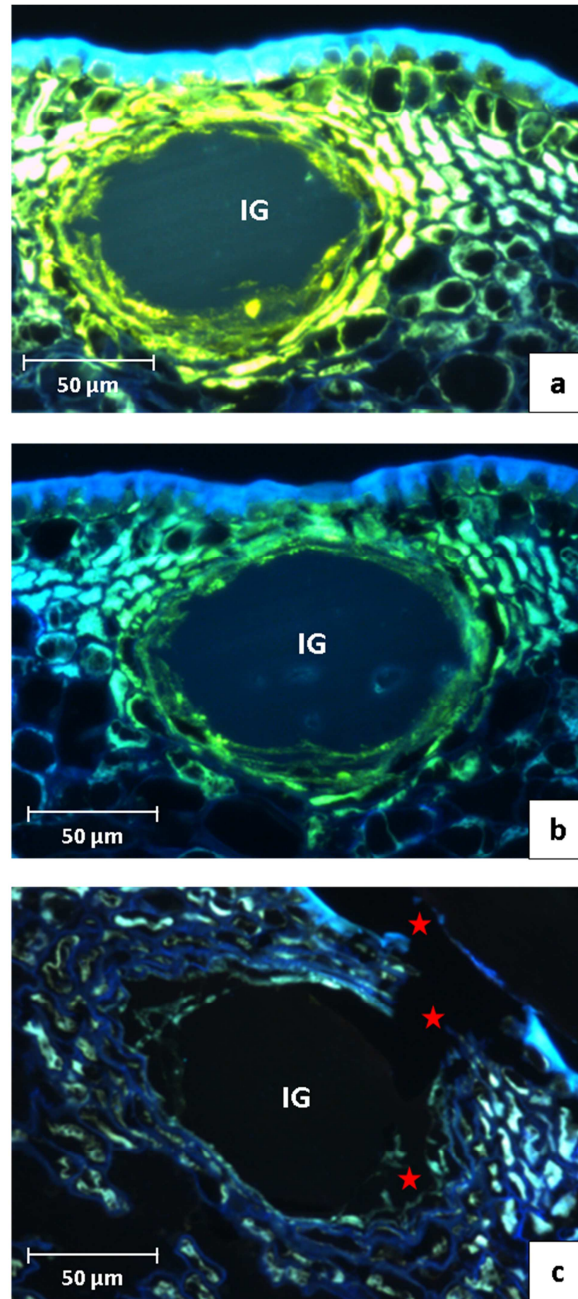
634 **Figure 10.** Cyto-histochemical study after revelation with Neu's reagent.635 (a) *C. aurantium* leaf cross section. (b) *R. nigrum* leaf cross section. (c) *C. scolyumus* leaf cross
636 section.637 PGT: Peltate Glandular Trichomes; NGT: Non-Glandular Trichomes; Sca: Storage cavity;
638 SC: Secretory Cells; C: Cuticle; EC: Epidermal Cells; M: Mesophyll; Xy: Xylem

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■ Caffeic acid derivatives: light blue

■ Flavonoids: greenish-yellow

643

644 **Figure 11.** Cyto-histochemical study of *C. aurantium* adaxial leaf surface after staining with

645 Neu's reagent. (a) Initial leaf. (b) After 60 min of CV procedure. (c) After 60 min of US

646 treatment (UAE). IG: Internal Glands; ★: rupture zone

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