

1 **Detection and toxicity evaluation of pyrrolizidine alkaloids in medicinal**
2 **plants *Gynura bicolor* and *G. divaricata* collected from different**
3 **Chinese locations**

4
5 by Jian Chen^{a,b}), Han Lü^{a,c}), Lianxiang Fang^d), Weilin Li^{*a}), Luc Verschaeve^{e,f}),
6 Zhengtao Wang^d), Norbert De Kimpe^b), Sven Mangelinckx^{*b})

7
8 a) Institute of Botany, Jiangsu Province and Chinese Academy of Sciences, Nanjing
9 210014, China

10 b) Department of Sustainable Organic Chemistry and Technology, Faculty of Bioscience
11 Engineering, Ghent University, Coupure links 653, B-9000 Ghent, Belgium

12 c) The Jiangsu Provincial Platform for Conservation and Utilization of Agricultural
13 Germplasm, Nanjing 210014, China

14 d) Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese
15 Medicine, Shanghai 201203, China

16 e) Department of Biomedical Sciences, University of Antwerp, Universiteitsplein 1, B-
17 2610 Wilrijk, Belgium

18 f) Toxicology Unit, Scientific Institute of Public Health, J. Wytsmanstreet 14, B-1050
19 Brussels, Belgium

Corresponding Authors

(phone: +32 9 2645951; fax: +32 9 2646221; e-mail: sven.mangelinckx@ugent.be)

(phone: +86 25 84347002; fax: +86 25 84347081; e-mail: lwlcnbng@mail.cnbg.net)

20 **Abstract**

21 Two edible plants in Southeast Asia, *Gynura bicolor* and *G. divaricata*, are not only
22 known to be nutritive but also useful as medicinal herbs. Previous phytochemical
23 investigation of *G.* species showed the presence of hepatotoxic pyrrolizidine alkaloids
24 (PAs), indicating the toxic risk of using these two plants. The present study was
25 designed to analyse the distribution of PA components and tried to evaluate the
26 preliminary toxicity of these two *G. species*. Eight samples of *G. bicolor* and *G.*
27 *divaricata* from five different Chinese locations were collected and their specific PAs
28 were qualitatively characterized by applying an UPLC-MS/MS spectrometry method.
29 Using a pre-column derivatization HPLC method, the total retronecine ester-type PAs
30 in their alkaloids extracts were quantitatively estimated as well. Finally, their
31 genotoxicity was investigated with an effective high-throughput screening method
32 referred to as Vitotox™ test and their potential cytotoxicity was tested on HepG2 cells.
33 It was found that different types of PAs were widely present in *G.* species collected
34 from south of China. Among them, no significant genotoxic effects were detected with
35 serial concentrations through the present *in vitro* assay. However, the cytotoxicity
36 assay of *Gynura* plants collected from Jiangsu displayed weak activity at the
37 concentration of 100 mg/mL. It is important to note that this research validates in part
38 the indication that the use of *G.* species requires caution.

39 **Keywords:** *Gynura bicolor*; *Gynura divaricata*; Pyrrolizidine alkaloids; Genotoxicity;
40 Cytotoxicity.

41 **Introduction.**

42 The genus *Gynura* belongs to the family Asteraceae, comprising approximately forty
43 species mainly distributed in Asia, Africa and Australia, of which ten species were
44 recorded in the South of China [1]. Many *Gynura* species, such as *G. bicolor*, *G.*
45 *divaricata* and *G. procumbens* are edible plants native to Asia, from which the aerial
46 parts are consumed in salads or as tempura in Chinese and Japanese restaurants for
47 their rich content of iron, calcium, vitamin A, etc [2-4]. From 2010, both *G. divaricata*
48 and *G. procumbens* have been approved as new source of food by the Minister of
49 Health of the People's Republic of China. Moreover, *Gynura* plants have been widely
50 used as folk medicines for the treatment of inflammation [5], hypertension [6],
51 hyperglycaemia [7] and cancer [8]. Among the species, *G. bicolor* and *G. divaricata*
52 attracted more attention for their use for the prevention and treatment of diabetes
53 mellitus in China. As demonstrated from a study on natural herbs used in the
54 traditional Chinese medical system for treatment of diabetes, a tea made from the
55 fresh leaves of *G. bicolor* and/or *G. divaricata* was found to have excellent
56 hypoglycemic effects [9].

57 In previous phytochemical reports, the presence of volatiles [10], phenolics [11],
58 anthocyanins [2], glycosides and norisoprenoids [12] was reported in *G. bicolor*.
59 Meanwhile, studies demonstrated that phenolics [13] and cerebroside [14] were
60 found in *G. divaricata*. However, a serious concern exists regarding *Gynura* plants due
61 to the presence of pyrrolizidine alkaloids (PAs) [15-16], which were reported to be

62 hepatotoxic, genotoxic, and carcinogenic [17-20]. One earlier phytochemical
63 investigation of *G. divaricata* showed the presence of integerrimine and usaramine,
64 classified as retronecine type-pyrrolizidine alkaloids (RET-PAs) [16], indicating the toxic
65 risk for using this as functional food and folk medicine. According to data, over 10,000
66 human cases worldwide were reported of poisoning by consumption of PAs-containing
67 herbal products or food [17]. The World Health Organization issued a Health and Safety
68 Guide on utilization of hepatotoxic PAs-containing herbs to prevent the risk of human
69 exposure to these alkaloids with the dose limit of 15 µg/kg body weight per day [21],
70 while obviously more stringent guidelines were established in Western developed
71 countries, for instance the internal exposure of PAs is restricted to 1 µg of PAs per day
72 in Germany [22] and Belgium proposes a limit of PAs in herbs be set at 1 µg/g of herb
73 [23].

74 Despite the various benefits from repeated consumption, limited safety information is
75 available on the use of *G. species* [24]. To fill up a part of this knowledge gap, in this
76 study, a specific investigation focusing on profiling of PAs in the aerial parts of *G. bicolor*
77 and *G. divaricata* harvested from different Chinese locations at different times (Table
78 1) was performed, through a method involving ultra-performance liquid
79 chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) analysis.
80 Furthermore, an improved pre-column derivatisation high-performance liquid
81 chromatography (HPLC) method was applied to analyse the total RET-PAs content of
82 these samples of *G. species*. To study their potential toxicity, the genotoxicity was

83 evaluated using a high-throughput bacterial Vitotox™ assay, which proved to be very
84 useful in the study of the genotoxicity of plants and extracts as well as for synthetic
85 drug leads [25-27]. In addition, the total PAs extracts of *G. bicolor* and *G. divaricata*
86 collected from Nanjing were further utilized to test their potential cytotoxicity on
87 HepG2 cells. The purpose of the present research was to characterize the PA
88 composition of *G. bicolor* and *G. divaricata*, and to study the geographical patterns of
89 PAs across the plant distributional area. Finally their potential toxicity was evaluated
90 based on the screening of genotoxicity and cytotoxicity assessment.

91

92 **Results and Discussion.**

93 ***Identification of PAs from two G. species.***

94 PAs are esters composed of necic acids and necine bases. According to the structure
95 of the necines, PAs can be classified into three types, namely retronecine-, otonecine-
96 and platynecine-type PAs [28]. It has been demonstrated that the use of tandem mass
97 spectrometry in multiple reaction monitoring mode, allows the screening for
98 toxicologically relevant PAs of different structure types. Retronecine-type PAs exhibit
99 the characteristic fragments at m/z 120 and 138, while otonecine-type PAs have m/z
100 150 and 168 as specific fragments. Furthermore, PA structure types with different
101 classes of esterification and oxidation can also be determined based on diagnostic
102 fragmentations [29]. The occurrence of additional fragments of m/z 136 and 118,
103 besides m/z 138 and 120, is diagnostic for *N*-oxides of retronecine-type PAs. In this

104 study, pyrrolizidine alkaloid profiles (structures shown in [Figure 1](#)) of *G. bicolor* and *G.*
105 *divaricata* from the same genus, were comprehensively analyzed by UPLC-MS and
106 UPLC-MS/MS. As shown in [Table 2](#), a total of twenty-seven PAs consisting of five
107 different types were detected, using a related diagnostic fragment ions monitoring
108 method [29]. By comparing the retention time and MS/MS fragmentation with those
109 of reference compounds, peaks 20, 22 and 24-26 were unequivocally identified as
110 retrorsine, spartioidine, seneciophylline, integerrimine and senecionine, respectively
111 (see in [Figure 2](#)). These five PAs, belonging to the RET-type, were all detected to
112 produce diagnostic fragment ions at m/z 120 and 138.

113 Peaks 11, 12, 13 and 21 (m/z 352) showed the same molecular weight as retrorsine
114 and usaramine, which was previously isolated from *G. divaricata* collected from
115 Hongkong [16]. The diagnostic fragment ions m/z 138 and 120 for RET-PAs were also
116 observed for these peaks [30]. Peak 11 eluted at 4.46 min, in correspondence with a
117 peak (Rt 4.44 min) tentatively identified as usaramine in our previous study applying a
118 similar analytical method with the same chromatographic conditions except for the
119 column temperature which was 30 °C instead of 45 °C [31]. However, mucronatine is
120 a reported RET-type isomer of usaramine with the same molecular weight [32]. Thus,
121 these peaks cannot be positively identified.

122 Peak 14 (Rt 4.67 min) and 15 (Rt 4.86 min) showed the diagnostic ions at m/z 168 and
123 150 for otonecine-type PAs (OTO-PAs). Both of them had the same molecular weight
124 of 365, suggesting a pair of isomers. Upon comparison of retention times, peak 14

125 corresponded with a peak (Rt 4.69 min) tentatively identified as neosenkirkine in our
126 previous study [31]. However, in view of the differences in column temperature during
127 the chromatographic analyses and the reported isolation of crotaverrine as an OTO-
128 type isomer of senkirkine and neosenkirkine [33], such tentative assignment of peak
129 14 is not appropriate. By comparison with the retention time of the standard
130 compound, peak 15 was unequivocally characterized as senkirkine.

131 The molecular weights of peak 3 (m/z 368, Rt 3.23 min), peak 6 (m/z 350, Rt 3.63 min)
132 and peak 10 (m/z 352, Rt 4.40 min) were 16 Da higher than those of retrorsine (m/z
133 352), seneciphylline (m/z 334) and senecionine (m/z 336), respectively. They also
134 showed diagnostic fragment ions m/z 138, 136, 120, and 118 for RET-PAs-*N*-oxides [29].
135 The retention times of peak 3 and peak 10 were nearly identical to the retention times
136 of the peaks unequivocally identified by comparison with PAs standards as retrorsine
137 *N*-oxide (Rt 3.18 min) and senecionine *N*-oxide (Rt 4.36 min) eluted under identical
138 chromatographic conditions [34]. Similarly, peak 6 eluted at 3.63 min, in
139 correspondence with a peak (Rt 3.54 min) tentatively identified as seneciphylline *N*-
140 oxide in this previous study [34]. Thus, they were tentatively identified as retrorsine *N*-
141 oxide, seneciphylline *N*-oxide and senecionine *N*-oxide, respectively, although isomers
142 of the *N*-oxides cannot be excluded.

143 ***Distribution of PAs in G. species from different Chinese locations.***

144 PAs were widely found in eight samples of *G. bicolor* and *G. divaricata* collected from
145 five different Chinese locations (Table 1), *i.e.* Nanjing, Nanping, Guangzhou, Haikou

146 and Nanchang. Both *G. bicolor* and *G. divaricata* showed a variety of PA constituents,
147 in which seneciophylline, integerrimine, senecionine for RET-PAs and senkirkinine for
148 OTO-PAs were widely detected in the samples, as can be seen in [Table 2](#). The results
149 demonstrated that the PA profiles of *G. bicolor* and *G. divaricata* from Nanjing were
150 more diverse. Both senecionine *N*-oxide and the RET-PA corresponding with peak 11
151 appeared in the two *G.* species from Nanjing. Moreover, retrorsine *N*-oxide classified
152 as RET-type *N*-oxide PA was detected only in *G. bicolor* from Nanjing, while
153 seneciophylline *N*-oxide was detected in *G. divaricata* from the same location. It was
154 noted that only five different PAs were detected in *G. divaricata* collected from
155 Nanping, which was the least in variety, suggesting the influence of regional
156 differences on PAs composition in plants.

157 Using the UPLC-MS/MS analytical method together with the processing application of
158 diagnostic fragment ions monitoring [[29-31, 34](#)], eighteen other unidentified PAs were
159 detected in both species as well. Despite the continuous progress in the field of
160 analysis of PAs in which HPLC hyphenated with mass spectrometry has been
161 demonstrated to be a powerful and robust tool for qualitative analysis of PAs with
162 efficient chromatographic separation and unequivocal identification of individual PAs
163 [[29](#)], these unidentified PAs remain to be characterized. This characterization is
164 challenging due to the existence of PAs stereoisomers and strongly limited availability
165 of commercial reference compounds. PA reference substances can become available
166 via classical isolation and identification of PAs from plants which was recently

167 demonstrated for *Gynura japonica* for which an efficient method for targeted analysis
168 and purification of PAs *cis/trans* isomers has been developed [15]. The present study
169 is the first report of a comprehensive analysis of PAs in *G. bicolor* and *G. divaricata*,
170 also indicating the toxic risk upon using these medicinal plants.

171 ***Quantitative analysis of RET-PAs in G. species from different Chinese locations.***

172 Among the three types of PAs, the RET- and OTO-PAs which contain a 1,2-unsaturated
173 necine base, are hepatotoxic. As described in the literature, RET-PAs are studied the
174 most because they are the most important PAs in terms of toxicity, natural abundancy
175 and reported cases of PA poisoning [28]. In the latter study, a new UHPLC-QTOF-MS
176 method useful for initial estimation of the total amount of RET-PAs in herbs using
177 retrosine as a single RET-PA standard has been developed [28]. This demonstrates that
178 methods for quantitative analysis of total amounts of RET-PAs are continuously
179 developed with focus on simplicity, specificity, reliability and analysis time. In the past,
180 we also reported on an improved, simple and specific method for quantitative analysis
181 of the total amount of RET-PAs in a plant extract based on HPLC-UV with prior
182 derivatization of the alkaloids using *o*-chloranil as reagent [35]. An advantage of this
183 method, as compared to powerful HPLC-MS methods, involves the reduced cost of the
184 equipment which broadens the potential utilization. RET-PAs were initially
185 dehydrogenized by *o*-chloranil, followed by methylation of the hydroxyl groups of the
186 necic acid moiety to yield 1-methoxy-7-methoxymethyl-2,3-dihydro-1*H*-pyrrolizine as
187 one single compound, which could be quantified by HPLC monitored by ultraviolet (UV)

188 spectroscopy at 223 nm. Neither the OTO-type nor the N-oxide PAs are prone to this
189 derivatization reaction, which made it specific for tertiary RET-type PAs. Here, this
190 method was applied for the estimation of total RET-PAs in *G.* species.

191 The applied analytical method successfully estimated the total amount of RET-PAs
192 indicating remarkable variations in eight samples of the aerial part of two *G.* species
193 (data shown in [Table 3](#)). However, like demonstrated for *Senecio madagascariensis*, it
194 is important to point out that the PAs content in samples from individual plants within
195 and among locations might vary widely [32]. At this stage, it would be speculative to
196 indicate which factors are responsible for the differences between the total amounts
197 of RET-PAs in the different samples from different locations. In contrast to *Senecio*,
198 little information on the biosynthesis and physiology of PAs of *Gynura* is available. For
199 instance, in *Senecio* the PAs are produced in the roots and preferentially accumulate
200 in the inflorescences [36]. Therefore the collecting period of the sample from the aerial
201 part of the plant, and the corresponding amount of inflorescences, might influence the
202 concentrations of PAs a lot. In the present study, when samples with a harvesting time
203 in June or July, *i. e.* before flowering, are compared, it is seen that the sample of *G.*
204 *divaricata* originating from Nanjing (GD-NJ) contained the highest concentration
205 (39.69 µg/g) of RET-PAs while *G. bicolor* from Nanping (GB-NP) showed the lowest
206 concentration (1.40 µg/g), which may relate to the differences in toxicity between
207 them. In addition, it seems that the species *G. divaricata* (GD-NJ, GD-NP, GD-GZ) always
208 contained higher amounts of RET-PAs when compared to *G. bicolor* (GB-NJ, GB-NP, GB-

209 GZ) from the same locations and harvesting time.

210 ***Genotoxicity evaluation of total alkaloids extracts derived from two G. species.***

211 One solvent sample as blank and three different dilutions of the test samples (single
212 pure standards or total alkaloids extracts) with intervals of 1:10 between dilutions
213 were tested. The genotoxic compound 4-nitroquinoline-oxide (4-NQO, 4 ppb) and
214 benzo[*a*]pyrene (BaP, 8 ppm) were used as positive controls in the absence and
215 presence of S9, respectively.

216 As an example, [Figure 3](#) shows the results of the Vitotox test for senecionine in the
217 absence of the metabolic activator S9 and the corresponding positive control (4-NQO).

218 It can be seen that S/N in the Genox strain remains between approximately 0.8 and
219 1.2 (60-240 min) and that the Cytox strain also gives values that did not considerably
220 decrease below 0.8. This means that there was no genotoxicity and no marked toxicity
221 at concentrations of 100 µg/mL and lower. The positive control 4NQO was clearly
222 genotoxic (S/N increased well above 1.5 in the Genox strain) at the non-toxic
223 concentration of 4 ppb (no decreased S/N below 0.8 in the Cytox strain). [Figure 4](#) gives
224 the results in the presence of S9. Again, there was no genotoxicity or cytotoxicity.

225 All results obtained for total alkaloids extracts from different *G. species* in the absence
226 of S9 are summarized in [Table 4](#). For all *G. bicolor* or *G. divaricata* derived total alkaloids
227 extracts, no genotoxic effects could be detected, since the S/N did not show values
228 higher than 1.5 at all concentrations of 1-100 µg/mL. However, all extracts were found
229 to be toxic or borderline toxic at 100 µg/mL in the absence of S9. An agent was

230 considered borderline toxic when S/N (Cytos) reached values slightly below 0.8. The
231 pure reference standards seneciophylline and senecionine were not genotoxic at 1-100
232 µg/mL. Here toxicity was just not reached at 100 µg/mL (S/N in Cytos strain ~ 0.8). No
233 genotoxicity or toxicity could be observed for extracts or standards at 1-100 µg/mL
234 metabolized by S9. Toxicity or 'borderline' toxicity at the concentration of 100 µg/mL
235 means that genotoxicity was tested at soluble non-cytotoxic up to a cytotoxic
236 concentration as for example required by the OECD [[e.g. OECD Guideline 471](#)].

237 We used the Vitotox test as it is a potent screening test for genotoxicity. This assay was
238 especially designed as a rapid high-throughput genotoxicity test, which is faster and at
239 least as well performing as the Ames assay. It usually detects much lower
240 concentrations of a test compound than the Ames assay does. The fact that much
241 lower concentrations of a compound can be tested compared to other assays, as for
242 example the Ames test or micronucleus test, is one of its major advantages as this test
243 allows testing compounds in the discovery phase of a new chemical (medicinal drug
244 for example) where only very little amounts of the compound are available. It was
245 previously demonstrated on different occasions that the Vitotox test correlates very
246 well with the Ames test (much better than other tests do) and hence that it may be
247 used instead [[37-41](#)]. The Vitotox test was therefore suitable as a first rapid screening
248 of genotoxicity. It should be realized yet that no single genotoxicity test is able to cover
249 all genetic events that may lead to genotoxicity and that our conclusion on absence of
250 genotoxicity does not exclude genotoxicity by other mechanisms that are not

251 detectable in bacteria. Genotoxicity tests on medicinal plants containing pyrrolizidine
252 alkaloids for example showed that various test systems afforded different results [42],
253 and the carcinogenic as well as toxic and genotoxic effects of pyrrolizidine alkaloids
254 have been reported to vary in different species [43]. It is thus not surprising that
255 senecionine and seneciophylline elicited DNA repair synthesis according to the
256 'hepatocyte primary culture DNA repair test', suggesting that they are possibly
257 genotoxic carcinogens [43]. It was also reported that *E*-4-hydroxy-2-hexenal is a
258 hepatic metabolite of senecionine which was shown to be toxic, causing hepatic
259 necrosis *in vivo* and which can bind with deoxyguanosine to form DNA adducts [17].
260 This is not necessarily detected by a bacterial Ames or Vitotox assay that essentially
261 detects gene mutations. For example, whereas retrorsine showed positive responses
262 in both the Ames test and SOS chromotest, senecionine showed negative responses in
263 both test systems [44].

264 ***Cytotoxicity evaluation of total alkaloids extracts derived from two G. species in***
265 ***HepG2 cells assessed by cell viability assay.***

266 To rapidly evaluate the cytotoxicity of the PAs-containing plants, an appropriate cell
267 viability (MTT) assay in HepG2 cell model [24], was used in the present study for
268 screening the total alkaloids extracts of *G.* species. As shown in Table 5, the alkaloids
269 extracts of *G.* plants originating from Nanjing were selected and both of them
270 exhibited a weak cytotoxicity to HepG2 cells at the concentration of 100 mg/mL. Since
271 the sample of *G. divaricata* originating from Nanjing contained a higher concentration

272 of RET-PAs, its more significant effect on cell viability may be examined in a
273 concentration-dependent manner. On the other hand, senecionine serving as a
274 reference compound and the doxorubicin sample serving as a positive control
275 exhibited comparable cytotoxic and anti-proliferative potential at a concentration of 1
276 mM and 1 μ M, respectively. These data, in combination with e.g. hepatotoxicity data
277 from literature [17-20], suggest that caution should be considered regarding the
278 amount and duration of consumption of *G.* plants, especially for *G. divaricata* from
279 Jiangsu region for which a higher RET-PAs content was determined in the present
280 research.

281

282 **Conclusions.**

283 *G. bicolor* and *G. divaricata*, both belonging to the *Senecioneae* tribe in which possible
284 hepatotoxic pyrrolizidines could be present, have been consumed as vegetable and
285 used in folk prescription for years in China. In this paper, a comprehensive study
286 involving the qualitative analysis and estimation of PAs in *G. bicolor* and *G. divaricata*
287 was performed in combination with *in vitro* screenings for their potential genotoxicity
288 and cytotoxicity. Across the South of China, these two species were collected from
289 Jiangsu (GB-NJ and GD-NJ), Jiangxi (GB-NC), Fujian (GB-NP and GD-NP), Guangdong
290 (GB-GZ and GD-GZ) and Hainan (GB-HK) province, where they are used as
291 representative *G.* species for functional and medical usage. By comparing the
292 chromatographic behaviors and MS fragmentations with available references or

293 reported data, a distinguished difference between the PAs profiles of *G. bicolor* and *G.*
294 *divaricata* was observed according to the present method. In addition, clear
295 differences can be seen as well when comparing two samples from the same species
296 but from different locations. Specifically, both *G.* species collected from Jiangsu (GB-
297 NJ and GD-NJ) were found to contain a broader variety of PAs, while *G. divaricata*
298 originating from Fujian (GD-NP) appeared to have the least diversified PA profile.
299 Among the samples, material of *G. divaricata* collected from Jiangsu (GD-NJ) contained
300 the highest concentration of total RET-PAs through a pre-column derivatization HPLC
301 method for the estimation analysis. It is important to note that OTO-PAs, such as
302 senkirkine and its isomer, were widely distributed in both *G.* species, but they were
303 not included in the present semi-quantitative data analysis.

304 Despite the variable composition and variable concentrations of RET-PAs, all total
305 alkaloids extracts showed similar data concerning genotoxicity with some differences
306 in the results from the toxicity assay. As indicated above the Vitotox test was used here
307 as a first screening test for genotoxicity as it was previously shown to be a fast and
308 sensitive assay which correlates well with the Ames test [40-41]. It is based on SOS-
309 response and detects gene mutations, as the Ames assay does. It however does not
310 detect clastogenicity and aneuploidy or genetic effects based on mammalian
311 metabolisms that addition of S9 cannot simulate. A different response with some
312 literature data is thus not surprising. This highlights the fact that full genotoxicity
313 testing requires application of a battery of test systems covering different genotoxicity

314 events and mechanisms. Furthermore, the cytotoxicity of *Gynura* plants collected from
315 Jiangsu was tested on HepG2 cells by MTT assay and both alkaloids extracts displayed
316 weak activity at a concentration of 100 mg/mL. Therefore, it is cautiously indicated
317 that a higher content of RET-PAs and a broader variety of PAs could be correlated with
318 the toxicity of *G.* species.

319 In summary, the present work will be helpful for assessing the toxic risk of these two
320 *Gynura* species. The safety of the consumption of these two *G.* species as medicinal
321 plants warrants further investigation.

322

323 **Acknowledgement.**

324 The authors are indebted to the Special Research Fund (BOF-UGent) for a joint
325 doctorate of Jian Chen and a research grant from the National Natural Science
326 Foundation of China (No. 30900123) and a grant from Jiangsu Scientific &
327 Technological Innovations Platform (No. BM2011117).

328

329 **Experimental Part**

330 ***Plant material.*** Eight samples of the aerial part of *G. bicolor* and *G. divaricata* from
331 five different Chinese locations were collected ([Table 1](#)). The plants were authenticated
332 by Professor Guo Rong-lin at the Institute of Botany, Jiangsu Province and Chinese
333 Academy of Sciences, Nanjing (China). The voucher specimens (510918-1~8) were
334 deposited in the herbarium, Institute of Botany, Jiangsu Province and Chinese

335 Academy of Sciences.

336 **Sample preparation.** The samples were dried in an oven at 50 °C for 6 h until
337 constant weight and pulverized to a powder. The powder (25 g) of each sample was
338 macerated in 200 mL of 80% (v/v) aqueous ethanol for 30 min, and then extracted
339 under reflux in a water bath for 2 h. The solution was filtered and evaporated under
340 vacuum to afford a crude extract. The extract was suspended in 100 mL of 2%
341 hydrochloric acid solution and filtered. After extraction with two times 100 mL of
342 diethyl ether, the acid aqueous layer was adjusted to pH 10-11 with ammonia and
343 partitioned twice with 100 mL of dichloromethane. The dichloromethane extracts
344 were combined, evaporated under vacuum and finally dissolved in 5 mL of methanol.
345 1 mL of the above methanol solution was diluted 10 times and passed through a 0.22
346 µm membrane filter to provide the total alkaloids (TA) solution for qualitative analysis,
347 Vitotox™ assay and cytotoxic evaluation.

348 Monocrotaline, senkirkine, retrorsine, spartioidine, senecionine, seneciphylline,
349 clivorine, isoline, integerrimine, jacobine, jaconine and jacoline (purity higher than 95%
350 by HPLC), used as reference compounds, were obtained from Shanghai R&D Center for
351 Standardization of Traditional Chinese Medicines (Shanghai, China).

352 **Qualitative analysis by UPLC-MS/MS.** The analysis was carried out using a Waters
353 Acquity UPLC™ system (Milford, USA) including a binary solvent manager, automatic
354 sampling manager, column compartment and a Micromass ZQ 2000 mass
355 spectrometer (Waters Corp., Milford, USA), equipped with an electrospray interface

356 (ESI). The TQD™ system (Waters Corp., Milford, USA) was applied when executing the
357 MS/MS analysis.

358 The chromatographic separation was performed on a Waters Acquity UPLC BEH C₁₈
359 column (100 × 2.1 mm, 1.7 μm), connected with a guard column (Vanguard 5 × 2.1 mm,
360 Waters Corp., Milford, USA). A linear gradient elution of acetonitrile (A) and 10 mM
361 ammonium formate modified by the addition of 0.1% (v/v) 25% ammonia solution (B)
362 was used. The gradient programmer was applied according to the following profile: 5-
363 20% A at 0-5 min, 20-40% A at 5-7 min, 40-90% A at 7-10 min, 90% A at 10-13 min, 5%
364 A at 13-15 min. The flow rate was 0.5 mL/min and the column was maintained at 45
365 °C. Each injection volume was 2 μL.

366 The ESI-MS spectrometer was operated in positive ionization mode with scan range
367 from m/z 150 to 650. The optimized MS conditions are listed as follows: source
368 temperature, 150 °C; desolvation temperature, 450 °C; capillary voltage, 3.5 kV; cone
369 voltage, 45 V. Nitrogen was used as the source of desolvation gas (900 L/h) and cone
370 gas (50 L/h); low mass resolution, 15; high mass resolution, 15. When executing
371 MS/MS fragmentation using the TQD (triple quadrupole mass analyzer) spectrometer
372 with the mass range from m/z 100 to 650, the collision energy was set at 30 eV, the
373 collision gas (helium) flow was set at 0.1 L/h while the low and high mass resolution
374 for the MS/MS function were set at 13. The software Waters Masslynx V4.1 station
375 was applied for the data processing and statistical analysis.

376 ***Estimation of total RET-PAs by HPLC.*** 4 mL of the above methanol solution (See

377 Sample preparation) was added to a test tube containing 1 mL of 1.6 mM of *o*-chloranil,
378 and mixed for 5 min, then left standing for 4 h at 45 °C in a water bath. The weight loss
379 caused by heating was replenished with methanol. The reaction mixture was filtrated
380 through a 0.45 µm membrane filter and 10 µL of the solution was injected for
381 quantitative analysis [35].

382 The HPLC system was a Dionex Ultimate 3000 (California, USA) consisting of a
383 quaternary pump, on-line degasser, auto-sampler, and RS column compartment
384 coupled to a diode array detector. An Ultimate (Welch Materials, Maryland, USA) C₁₈
385 column (250 × 4.9 mm, 5 µm) at a column temperature of 25 °C was used for all
386 analyses. The injection volume was 20 µL. Chromatographic separation was carried out
387 using an isocratic elution consisting of methanol and aqueous 0.01% triethylamine
388 (adjusted to a pH value of 4 with formic acid) at a flow rate of 1 mL/min (40/60, v/v).
389 The wavelength of the detector was set at 223 nm. Chromeleon[®] 6.80 software was
390 used for the data analysis. A methanol stock solution of integerrimine was prepared
391 and diluted to appropriate concentrations for construction of calibration curves. Five
392 concentrations of the standard solution were derivatised as described above and
393 injected. The calibration curve was constructed by plotting the peak area versus the
394 concentration of the standard [35].

395 **Vitotox assay.** Possible genotoxicity of seneciophylline, senecionine and all total
396 alkaloid extract samples were analysed using the Vitotox test. This test is performed
397 with *Salmonella typhimurium* TA104 cells that contain a luciferase gene under

398 transcriptional control of a mutated recN promoter (TA 104-recN strain or Genox
399 strain). When bacteria are exposed to a DNA damaging compound, light is emitted that
400 can easily be measured with a luminometer. An agent is considered genotoxic when
401 there is a dose-response relationship with a signal-to-noise ratio exceeding a value of
402 1.5 ($S/N > 1.5$). A second *S. typhimurium* TA 104 strain (TA104 pr1 or Cyttox strain)
403 constitutively expressing luciferase is used as a control and for measuring toxicity. In
404 this strain, increased light production indicates a non-genotoxic mechanism whereas
405 a decreased light production (S/N considerably lower than 0.8) indicates toxicity [26,
406 40]. The test was conducted according to the protocol described in the test kit which
407 is distributed by Gentaur Molecular Products (Kampenhout, Belgium). In brief, RecN2-
408 4 (Genox) and pr1 (Cyttox) *S. typhimurium* bacteria were cultivated by overnight
409 shaking at 36 °C in a rich Lysogeny broth (LB) medium (20 g LB, 1 g glucose, 0.345 g
410 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ + antibiotics/liter). The bacterial culture was then diluted 125 times with
411 poor LB medium (2 g LB, 1 g glucose, 0.375 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 4.5 g NaCl/liter) and
412 incubated by shaking for 1 h at 36 °C. Afterwards, these Genox and Cyttox cultures were
413 ready for genotoxicity testing. Therefore, cultures were diluted 10 fold with poor LB
414 medium and test compounds were added in the desired concentrations. A S9 liver
415 fraction from aroclor treated rats was added to the designated +S9 cultures to test the
416 genotoxic/cytotoxic effects of the metabolites of the extracts/compounds. The
417 bacterial suspensions were then incubated at 30 °C and the luminescent signal was
418 measured every 5 min over a period of 4 h. All calculations occur automatically and

419 are based on measurements between 60 and 240 min of incubation [26-27, 40]. Before
420 60 min of incubation the system is not yet stabilized and SOS-response, on which the
421 test is based, did not yet occurred.

422 **Cytotoxic evaluation on HepG2 cells by cell viability assay.** The cytotoxicity of PAs
423 extracts on HepG2 cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-
424 diphenyltetrazoliumbromide (MTT) staining. Briefly, 5×10^3 cells/well were plated in
425 96-well plates with 100 μ L of culture medium for 24 h, and then exposed to extracts.
426 After 72 h incubation, the culture medium was replaced with 100 μ L of fresh medium
427 including 0.5 mg/mL MTT. Following 4 h of incubation at 37 °C, this medium was
428 removed, and 100 μ L of dimethyl sulphoxide was added to each well to dissolve the
429 purple formazan crystals. The color reaction was quantified using an automatic plate
430 reader (Bio-Tek Instrument Inc, USA) at 570 nm. Each experiment was repeated three
431 times (n=3) [24].

432

433

REFERENCES

- 434 [1] Y. L. Chen, in 'Flora of China', Science Press, Beijing, **1999**, p. 309.
- 435 [2] Y. Shimizu, T. Imada, H. L. Zhang, R. Tanaka, T. Ohno, K. Shimomura, *Food Sci. Technol. Res.* **2010**,
436 *16*, 479.
- 437 [3] Y. X. Deng, Y. S. Chen, W. R. Zhang, B. Chen, X. M. Qiu, L. H. He, L. L. Mu, C. H. Yang, R. Chen, *Brit.*
438 *J. Nutr.* **2011**, *106*, 1323.
- 439 [4] C. S. Hew, B. Y. Khoo, L. H. Gam, *PLOS One* **2013**, *8*, e68524.
- 440 [5] S. L. Hsieh, C. C. Wu, C. H. Liu, J. L. Lian, *Fish Shellfish Immunol.* **2013**, *35*, 18.
- 441 [6] O. S. Abrika, M. F. Yam, M. Z. Asmawi, A. Sadikun, H. Dieng, E. A. Hussain, *J. Acupunct. Meridian*

442 *Stud.* **2013**, *6*, 199.

443 [7] K. Algariri, K. Y. Meng, I. J. Atangwho, M. Z. Asmawi, A. Sadikun, V. Murugaiyah, N. Ismail, M. U.

444 Eteng, *Asian Pac. J. Trop. Biomed.* **2013**, *3*, 358.

445 [8] A. N. Shwter, N. A. Abdullah, M. A. Alshawsh, A. Alsalahi, M. Hajrezaei, A. A. Almaqrami, S. D.

446 Salem, M. A. Abdulla, *J. Ethnopharmacol.* **2014**, *151*, 1194.

447 [9] W. L. Li, B. R. Ren, M. Zhuo, Y. Hu, C. G. Lu, J. L. Wu, J. Chen, S. Sun, *Am. J. Chin. Med.* **2009**, *37*,

448 961.

449 [10] J. Chen, A. Adams, S. Mangelinckx, B. R. Ren, W. L. Li, Z. T. Wang, N. De Kimpe, *Nat. Prod.*

450 *Commun.* **2012**, *7*, 655.

451 [11] C. Y. Chao, W. H. Liu, J. J. Wu, M. C. Yin, *J. Sci. Food Agr.* **2015**, *95*, 1088.

452 [12] J. Chen, S. Mangelinckx, A. Adams, W. L. Li, Z. T. Wang, N. De Kimpe, *Nat. Prod. Commun.* **2012**,

453 *7*, 1563.

454 [13] J. Chen, S. Mangelinckx, L. Ma, Z. T. Wang, W. L. Li, N. De Kimpe, *Fitoterapia* **2014**, *99*, 1.

455 [14] L. Chen, H.Q. Li, H. T. Song, G. G. Zhang, *Fitoterapia* **2009**, *80*, 517.

456 [15] L. X. Fang, A. Z. Xiong, X. J. Yang, Cheng W, L. Yang, Z. T. Wang, *J. Sep. Sci.* **2014**, *37*, 2032.

457 [16] E. Röder, A. Eckert, H. Wiedenfeld, *Planta Med.* **1996**, *62*, 386.

458 [17] P. P. Fu, Q. S. Xia, G. Lin, M. W. Chou, *Drug Metab. Rev.* **2004**, *36*, 1.

459 [18] P. P. Fu, M. W. Chou, M. Churchwell, Y. P. Wang, Y. W. Zhao, Q. S. Xia, G. G. da Costa, M. M.

460 Marques, F. A. Beland, D. R. Doerge, *Chem. Res. Toxicol.* **2010**, *23*, 637.

461 [19] Q. S. Xia, Y. W. Zhao, L. S. Von Tungeln, D. R. Doerge, G. Lin, L. N. Cai, P. P. Fu, *Chem. Res. Toxicol.*

462 **2013**, *26*, 1384.

463 [20] J. A. Edgar, R. J. Molyneux, S. M. Colegate, *Chem. Res. Toxicol.* **2015**, *28*, 4.

464 [21] Health and Safety Guide No. 26, In 'International programme on chemical safety pyrrolizidine

465 alkaloids', World Health Organisation, Geneva, **1989**.

466 [22] German Federal Health Bureau, in 'Bundesanzeiger, 4805', Deutsche Apotheker Zeitung, **1992**,

467 p. 1406.

468 [23] S. Dharmanada, Safety issue affecting herbs: Pyrrolizidine alkaloids. Monograph on the

469 internet. Available at <<http://www.itmonline.org/arts/pas.htm>>. Accessed Dec. 17, 2015.

470 [24] Y. H. Li, W. L. T. Kan, N. Li, G. Lin, *J. Ethnopharmacol.* **2013**, *150*, 560.

471 [25] E. E. Elgorashi, J. L. Taylor, A. Maes, J. van Staden, N. De Kimpe, L. Verschaeve, *Toxicol. Lett.*
472 **2003**, *143*, 195.

473 [26] L. Verschaeve, V. Kerstens, J. L. Taylor, E. E. Elgorashi, A. Maes, L. Van Puyvelde, N. De Kimpe,
474 J. van Staden, *Toxicol. in Vitro* **2004**, *18*, 29.

475 [27] D. Cappoen, P. Claes, J. Jacobs, R. Anthonissen, V. Mathys, L. Verschaeve, K. Huygen, N. De
476 Kimpe, *J. Med. Chem.* **2014**, *57*, 2895.

477 [28] L. Zhu, J. Q. Ruan, N. Li, P. P. Fu, Y. Ye, G. Lin, *Food Chem.* **2016**, *194*, 1320.

478 [29] A. These, D. Bodi, S. Ronczka, M. Lahrssen-Wiederholt, A. Preiss-Weigert, *Anal. Bioanal. Chem.*
479 **2013**, *405*, 9375.

480 [30] A. Z. Xiong, L. Yang, Y. Q. He, C. H. Wang, S. W. A. Bligh, Z. T. Wang, *Rapid Commun. Mass*
481 *Spectrom.* **2009**, *23*, 3907.

482 [31] X. J. Yang, L. Yang, A. Z. Xiong, D. X. Li, Z. T. Wang, *J. Pharm. Biomed. Anal.* **2011**, *56*, 165.

483 [32] D. R. Gardner, M. S. Thorne, R. J. Molyneux, J. A. Pfister, A. A. Seawright, *Biochem. Syst. Ecol.*
484 **2006**, *34*, 736.

485 [33] O. P. Suri, R. S. Sawhney, M. S. Bhatia, C. K. Atal, *Phytochemistry* **1976**, *15*, 1061.

486 [34] A. Z. Xiong, L. Yang, L. L. Ji, Z. Y. Wang, X. J. Yang, Y. Chen, X. L. Wang, C. H. Wang, Z. T. Wang,
487 *Metabolomics* **2012**, *8*, 614.

488 [35] A. Z. Xiong, L. Yang, F. Zhang, X. J. Yang, C. H. Wang, Z. T. Wang, *Biomed. Chromatogr.* **2009**, *23*,
489 665.

490 [36] C. Frölich, D. Ober, T. Hartmann, *Phytochemistry* **2007**, *68*, 1026.

491 [37] S. Muto, H. Baba, Y. Uno, *Environ. Mutagen. Res.* **2003**, *25*, 69.

492 [38] S. Muto, K. Miyata, C. Tsutsumibata, H. Daigo, T. Ooro, K. Sugiura, H. Baba, Y. Uno, In 'Topical
493 Issues in Applied Microbiology and Biotechnology'. Research Signpost, Trivandrum, **2006**, p. 1.

494 [39] W. G. E. J. Schoonen, W. M. A. Westerink, G. J. Horbach, In 'Molecular, Clinical and
495 Environmental Toxicology', Birkhäuser Verlag, Switzerland, **2009**, p. 401.

496 [40] L. Verschaeve, In 'High Throughput Screening Methods in Toxicity Testing', Wiley Publication,
497 2013, p. 213.

498 [41] W. M. A. Westerink, J. C. R. Stevenson, A. Lauwers, G. Griffioen, G. J. Horbach, W. G. E. J.
499 Schoonen, *Muta. Res.* **2009**, *676*, 113.

500 [42] E. Röder, *Pharmazie* **1995**, *50*, 83.

501 [43] H. Mori, S. Sugie, N. Yoshimi, Y. Asada, T. Furuya, G. M. Williams, *Cancer Res.* **1985**, *45*, 3125.

502 [44] S. Kevekordes, V. Mersch-Sundermann, C. M. Burghaus, J. Spielberger, H. H. Schmeiser, V. M.
503 Artl, H. Dunkelberg, *Mutat. Res.* **1999**, *445*, 81.

504

505

506

507

508

509

510

511

512

513

514

515

516

517

518

519

520

521

522

523

524

525

526

527

528

529

530

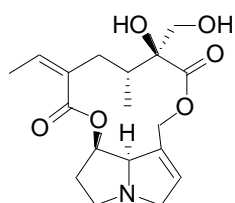
531

532

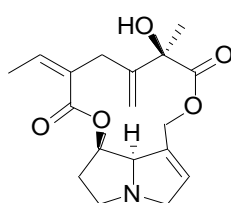
533

534

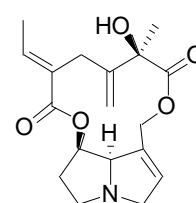
535



Retrorsine



Seneciophylline

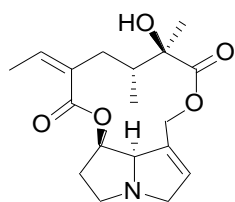


Spartioidine

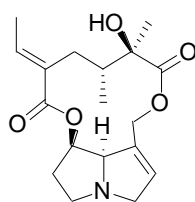
536

537

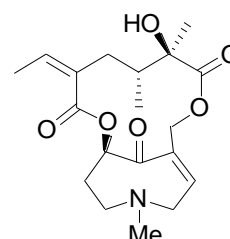
538



Senecionine



Integerrimine

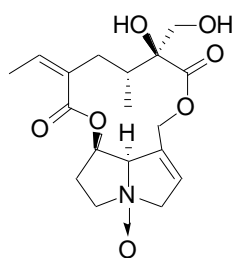


Senkirkine

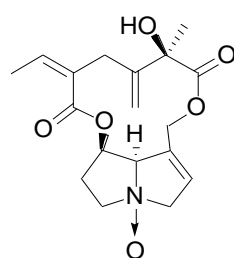
539

540

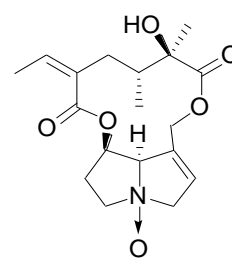
541



Retrorsine-N-oxide



Seneciophylline-N-oxide



Senecionine-N-oxide

542

543

544

545

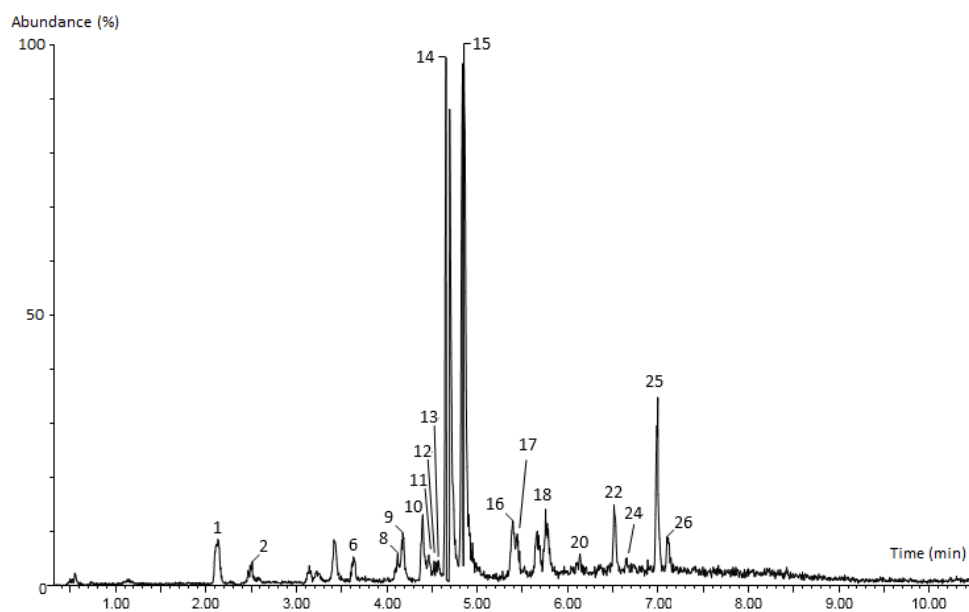
546

547

Figure 1 Structures of pyrrolizidine alkaloids determined in *G. bicolor* and *G. divaricata*

548

549



550

551 **Figure 2** Representative extracted ion chromatogram of the fingerprint of the pyrrolizidine alkaloids
552 in *Gynura divaricata* collected from Nanjing displaying the $[M+H]^+$ ions at m/z 240, 254, 268, 286,
553 334, 336, 350, 352, 364, 366, 368 and 382. Peak numbers correspond to Table 2.

554

555

556

557

558

559

560

561

562

563

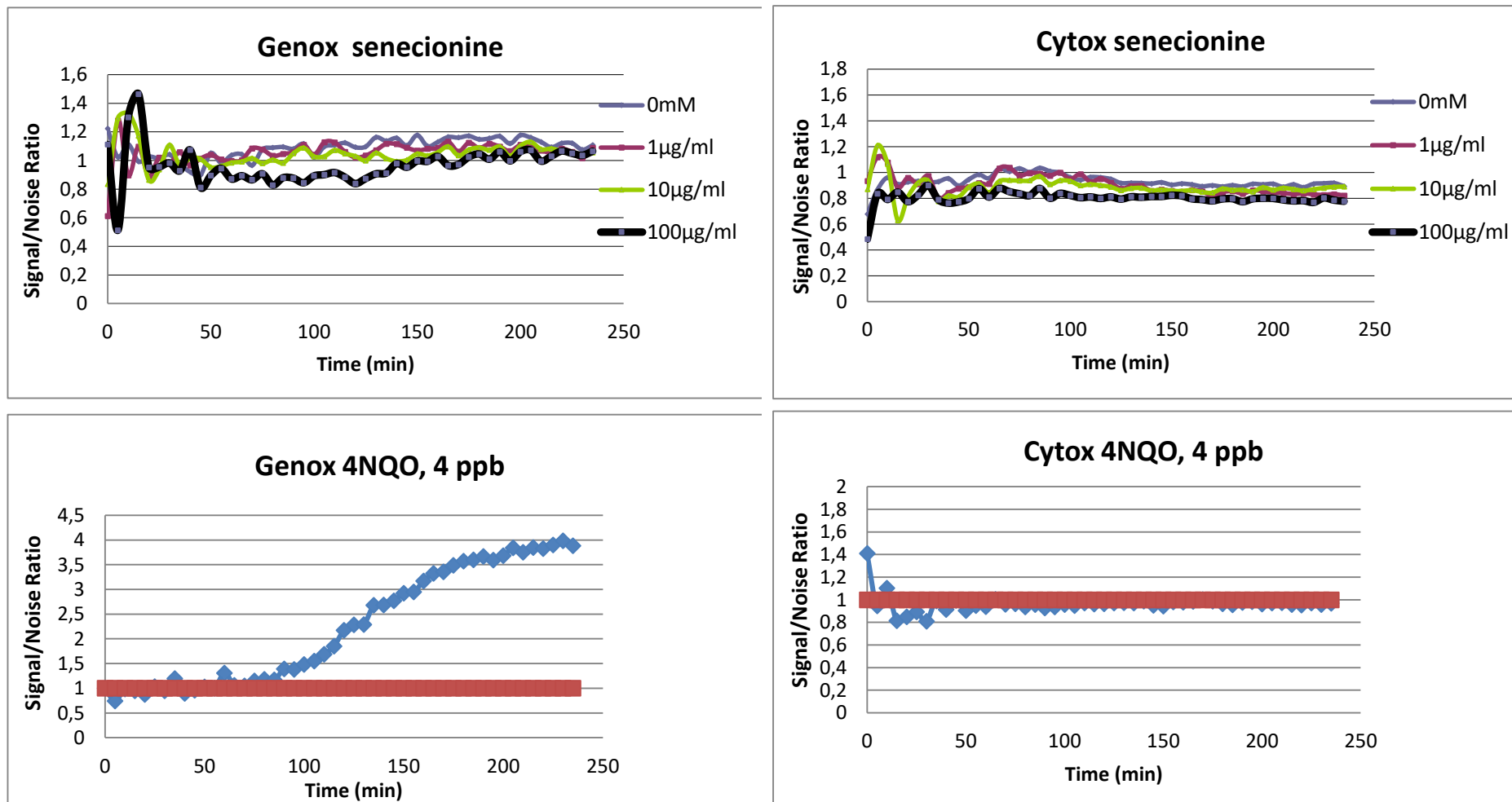
564

565

566

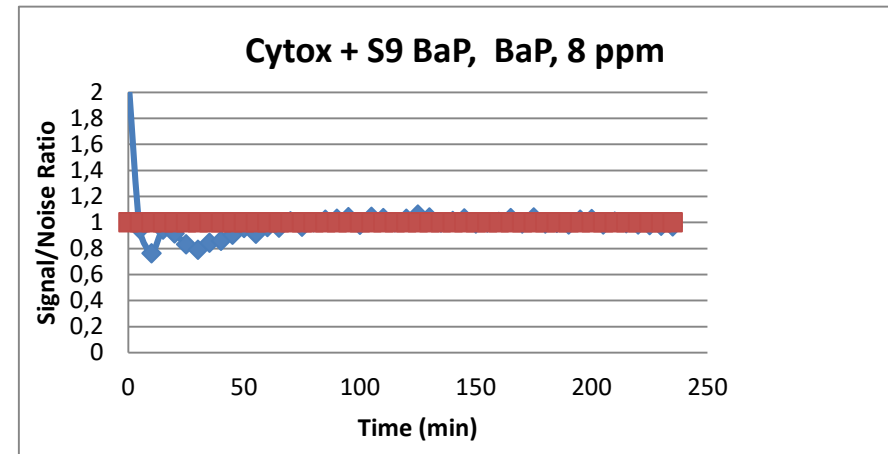
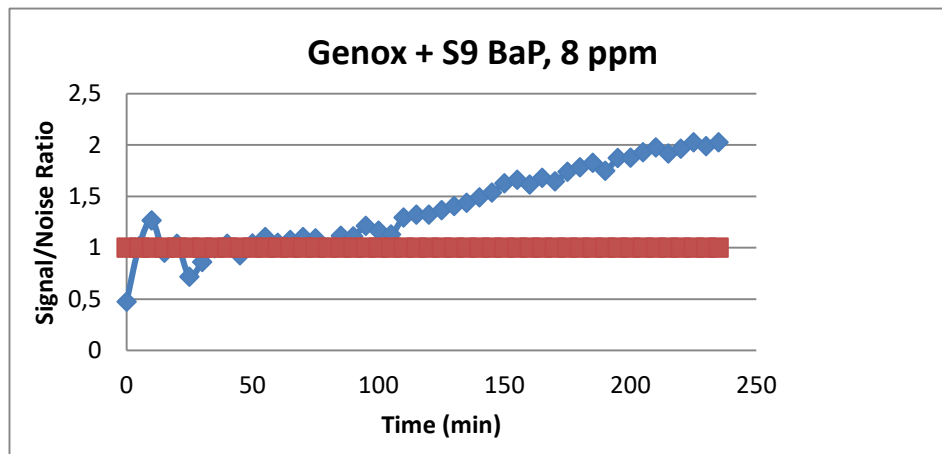
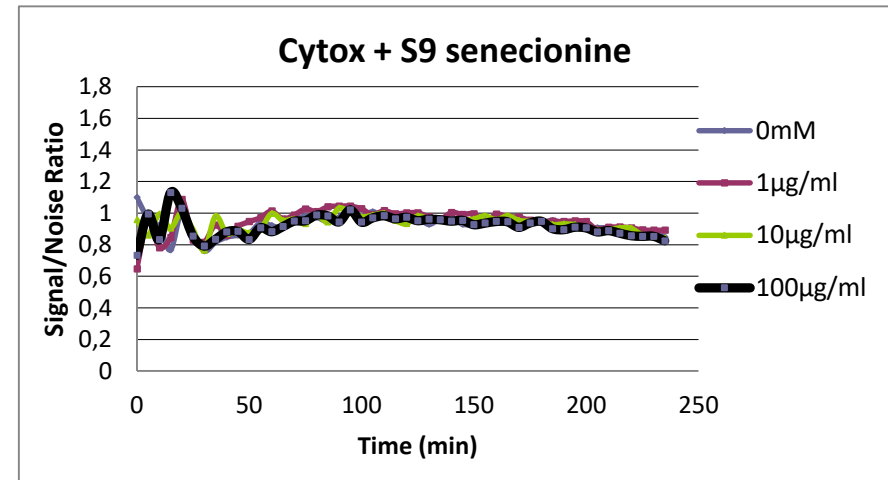
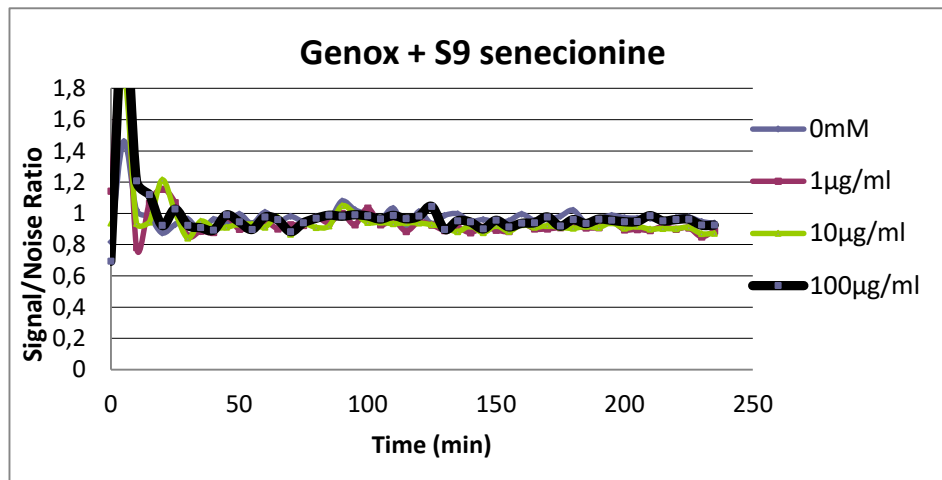
567

568



569

Figure 3: Vitotox test results for senecionine in the absence of S9 and its corresponding positive control 4-nitroquinoline-oxide (4NQO).



570

Figure 4: Vitotox test results for senecionine in the presence of S9 and its corresponding positive control benzo(a)pyrene.

571

Table 1. *Gynura* species collected from different Chinese locations

No.	Sample	Species	Locations	Provinces	Sampling part	Harvesting time
1	GB-NJ	<i>G. bicolor</i>	Nanjing	Jiangsu	aerial part	June 2012
2	GB-NP	<i>G. bicolor</i>	Nanping	Fujian	aerial part	July 2012
3	GB-GZ	<i>G. bicolor</i>	Guangzhou	Guangdong	aerial part	June 2012
4	GB-HK	<i>G. bicolor</i>	Haikou	Hainan	aerial part	Nov. 2012
5	GB-NC	<i>G. bicolor</i>	Nanchang	Jiangxi	aerial part	May 2012
6	GD-NJ	<i>G. divaricata</i>	Nanjing	Jiangsu	aerial part	June 2012
7	GD-NP	<i>G. divaricata</i>	Nanping	Fujian	aerial part	July 2012
8	GD-GZ	<i>G. divaricata</i>	Guangzhou	Guangdong	aerial part	June 2012

572

573

574

575 **Table 2.** Hepatotoxic pyrrolizidine alkaloids determined by UPLC-MS/MS characteristic in *Gynura bicolor* and *G. divaricata* collected from different Chinese locations

Peak no.	Rt (min)	MW	[M+H] ⁺	Diagnostic ions (<i>m/z</i>)	Type	Assignment	Identification	Species							
								<i>G. bicolor</i> (GB)				<i>G. divaricata</i> (GD)			
								NJ	NP	GZ	HK	NC	NJ	NP	GZ
1	2.12	381	382	168, 150	OTO		MS		√		√			√	
2	2.48	253	254	138, 120	RET		MS							√	
3	3.23	367	368	138, 136, 120, 118	RETNO	retrorsine <i>N</i> -oxide ^a	MS, L	√							
4	3.38	367	368	138, 120	RET		MS	√							
5	3.60	337	338	138, 120	RET		MS	√							
6	3.63	349	350	138, 136, 120, 118	RETNO	seneciphylline <i>N</i> -oxide ^a	MS, L							√	
7	3.97	337	338	138, 120	RET		MS				√				
8	4.11	363	364	168, 150	OTO		MS							√	√
9	4.18	285	286	138, 120	RET		MS							√	
10	4.40	351	352	138, 136, 120, 118	RETNO	senecionine <i>N</i> -oxide ^a	MS, L	√						√	
11	4.46	351	352	138, 120	RET		MS	√						√	
12	4.53	351	352	138, 120	RET		MS		√		√	√	√	√	√
13	4.57	351	352	138, 120	RET		MS	√		√				√	
14	4.67	365	366	168, 150	OTO		MS	√	√	√				√	√
15	4.86	365	366	168, 150	OTO	senkirkine	MS, R	√	√		√			√	√
16	5.40	267	268	138, 120	RET		MS							√	
17	5.44	367	368	170, 152	Saturated-OTO		MS							√	
18	5.77	239	240	140, 122	Saturated-RET		MS	√		√	√	√	√	√	
19	5.96	385	386	138, 120	RET		MS								√
20	6.13	351	352	138, 120	RET	retrorsine	MS, R		√		√			√	√
21	6.29	351	352	138, 120	RET		MS				√				
22	6.52	333	334	138, 120	RET	spartioidine	MS, R				√	√	√		
23	6.60	321	322	138, 120	RET		MS				√				
24	6.65	333	334	138, 120	RET	seneciphylline	MS, R		√	√		√	√	√	
25	7.00	335	336	138, 120	RET	integerrimine	MS, R	√	√	√	√	√	√	√	√
26	7.10	335	336	138, 120	RET	senecionine	MS, R		√	√	√	√	√	√	√
27	8.06	375	376	138, 120	RET		MS				√				

576 Abbreviations: RET = retronecine; OTO = otonecine; Saturated-RET = platynecine-type; Saturated-OTO = 1,2-saturated otonecine; RETNO = retronecine-*N*-oxide
577 R = reference compound; L = literature; MS = mass spectrum; √ = detected. For the sample abbreviations and details, see Table 1. ^a Tentative identification.

578

579

Table 3. The contents of total RET-PAs in *Gynura* species collected from different Chinese locations

No.	Sample	RET-PAs ($\mu\text{g/g}$)
1	GB-NJ	10.71
2	GB-NP	1.40
3	GB-GZ	8.23
4	GB-HK	14.14
5	GB-NC	14.38
6	GD-NJ	39.69
7	GD-NP	5.78
8	GD-GZ	26.25

580

For the sample abbreviations and details, see Table 1.

581

582

583

584

585

586

587

588

589

590

591

592

593

594

595

596

597

598

599

600

601 **Table 4.** Genotoxicity and cytotoxicity evaluation (Vitotox test) of total alkaloids extracts of two
 602 *Gynura* species from different Chinese locations in the absence and presence of S9.

No.	Sample	Genotoxicity*		Toxicity**	
		-S9	+S9	-S9	+S9
1	GB-NJ	NG (1-10 µg/mL)	NG (1-100 µg/mL)	T (100 µg/mL)	NT (1-100 µg/mL)
2	GB-NP	NG (1-10 µg/mL)	NG (1-100 µg/mL)	~T (100 µg/mL)	NT (1-100 µg/mL)
3	GB-GZ	NG (1-10 µg/mL)	NG (1-100 µg/mL)	~T (100 µg/mL)	NT (1-100 µg/mL)
4	GB-HK	NG (1-10 µg/mL)	NG (1-100 µg/mL)	T (100 µg/mL)	NT (1-100 µg/mL)
5	GB-NC	NG (1-10 µg/mL)	NG (1-100 µg/mL)	~T (100 µg/mL)	NT (1-100 µg/mL)
6	GD-NJ	NG (1-10 µg/mL)	NG (1-100 µg/mL)	T (100 µg/mL)	NT (1-100 µg/mL)
7	GD-NP	NG (1-10 µg/mL)	NG (1-100 µg/mL)	T (100 µg/mL)	NT (1-100 µg/mL)
8	GD-GZ	NG (1-10 µg/mL)	NG (1-100 µg/mL)	~T (100 µg/mL)	NT (1-100 µg/mL)
9	seneciphylline	NG (1-100 µg/mL)	NG (1-100 µg/mL)	NT (1-100 µg/mL)	NT (1-100 µg/mL)
10	senecionine	NG (1-100 µg/mL)	NG (1-100 µg/mL)	NT (1-100 µg/mL)	NT (1-100 µg/mL)

603 *NG = non-genotoxic at the given concentration range; **T = toxic; NT = non-toxic and ~T = border line toxic at the given
 604 concentration. For the sample abbreviations and details, see Table 1.

605

606

607

608

609

610

611

612

613

614

615

616

617

618

619

620

621 **Table 5.** Inhibitory effect of total alkaloids extracts from *G. species* on cell viability in HepG2 cells

Sample	Concentration	Inhibitory rate (% of solvent control)
GB-NJ	100 mg/mL	26.66
GD-NJ	100 mg/mL	52.92
Senecionine	0.335 mg/mL	58.76
Doxorubicin	0.543×10^{-3} mg/mL	67.00

622

For the sample abbreviations and details, see Table 1.

623

624

625

626

627

628

629

630

631

632

633

634

635

636

637

638

639

640

641

642

643

644