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1     **Asian hornet *Vespa velutina nigrithorax* venom: Evaluation and identification**  
2     **of the bioactive compound responsible for Human Keratinocyte protection**  
3                     **against oxidative stress**

4  
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25

26 **Abstract**

27 The present study aimed to explore the potential use of antioxidant molecules of the  
28 Asian hornet venom (*Vespa velutina nigrithorax*) to prevent or reduce skin aging. We  
29 developed a first technical platform that combined a DPPH radical scavenging  
30 chemical assay and cytotoxicity and ROS (reactive oxygen species) production in  
31 HaCaT keratinocyte cells exposed to UVB to evaluate the antioxidant property of *V.*  
32 *velutina* venom. We further employed Thin Layer Chromatography (TLC) combined  
33 with the DPPH assay as a targeted separation approach to isolate the antioxidant  
34 compounds responsible for the free radical scavenging property of *V. velutina* venom.  
35 In parallel, the latter was fractionated by a HPLC-DAD non-targeted separation  
36 approach. From this experiment, nine fractions were generated which were again  
37 evaluated separately for their antioxidant properties using DPPH assays. Results  
38 showed that only one fraction exhibited significant antioxidant activity in which  
39 serotonin was identified as the major compound by a UHPLC-ESI-QTOF HRMS/MS  
40 approach. We finally demonstrated, using purified serotonin molecule, that this  
41 bioactive structure is mostly responsible for the free radical scavenging property of  
42 the crude venom as evidenced by DPPH and ROS assays in HaCaT cells exposed to  
43 UVB.

44

45 **Key words: Antioxidant activity; Keratinocyte; Mass spectrometry; Reactive**  
46 **oxygen species; Thin Layer Chromatography; *Vespa Velutina* Venom.**

47

48

49 **Abbreviations**

50	DPPH	2,2-Diphenyl-1-picrylhydrazyl
51	HPLC-DAD	High-performance liquid chromatography–Diode
52		Array Detector
53	LC-MS	Liquid chromatography-mass spectrometry
54	ROS	Reactive Oxygen Species
55	RSA	Radical scavenging activity
56	TLC	Thin layer Chromatography
57	UHPLC-ESI-QTOF HRMS/MS	Ultra-high performance liquid chromatography-
58		electrospray ionization–quadrupole-time of flight–
59		high resolution mass spectrometry-tandem mass
60		spectrometry
61	UVB	Ultraviolet B
62	<i>V. velutina</i>	<i>Vespa velutina</i>
63		

## 64 1. Introduction

65 Skin aging can seriously alter a person's physical appearance, which in some cases  
66 can have a negative impact on the person's social life. There are two main pathways  
67 responsible for the aging process in human skin: firstly, intrinsic aging or natural  
68 aging caused by the passage of time or by the deleterious effect of skin matrix  
69 metalloproteinases (MMP-1) that degrade structural skin proteins such as collagen  
70 fiber (Quan and Fisher, 2015); **secondly**, extrinsic aging or photoaging that is mostly  
71 caused by skin surface exposure to ultraviolet (UV) rays (Kusumawati et al., 2018).  
72 UV irradiation results in the formation of reactive oxygen species (ROS) in cells, in  
73 which ions and free radicals derived from oxygen such as hydrogen peroxide ( $H_2O_2$ ),  
74 hydroxyl radical ( $\cdot OH$ ), oxygen ( $O_2$ ), and superoxide ( $O_2^{\cdot -}$ ) (Sharma et al., 2012) are  
75 known to be toxic in cells. Free radicals are molecules with unpaired electrons that  
76 are highly unstable and tend to react with enzymes, receptors or the cellular lipid  
77 membrane. They remove electrons from any molecule in their path and create new  
78 free radicals. These reactions **occur** continuously, inducing skin structure damage or  
79 cell function disorders. To deal with this impact, antioxidants have been used for  
80 many years in cosmetics to improve the skin defense (Kusumawati and Indrayanto,  
81 2013).

82 The use of natural substances from plants has been shown to have outstanding  
83 advantages, not only in traditional but also in modern cosmetic treatments, **and** these  
84 substances are now considered as a natural reservoir for antioxidant and anti-aging  
85 molecules. Bioactive compounds from animals have also been exploited in the  
86 search for therapeutic and cosmetic ingredients. In a recent review on cosmetic  
87 applications of animal sources, products from the honey bee (*Apis mellifera L.*) such  
88 as royal jelly, bee propolis and bee venom were documented as a reliable source of

89 novel compounds with skin wound healing properties in mice and anti-inflammatory  
90 properties (Garraud et al., 2017). There is some evidence that a secretion of the  
91 mollusk *Cryptomphalus aspersa* possesses an antioxidant property *in vitro* and  
92 induces fibroblast proliferation by promoting the extracellular matrix assembly that  
93 can be applied for wound healing (Brieva et al., 2008). Spider venom (*Argiope*  
94 *lobate*) was recently studied as a potential inhibitor of melanogenesis in skin aging  
95 and a polyamine ArgTX-636 was identified as the novel class of melanogenesis  
96 inhibitors both *in vitro* and in B16F10 cells (Verdoni et al., 2016). In other studies,  
97 venoms from Hymenoptera were recently reported as a potential source for  
98 pharmacology and cosmetic applications while bee venom was found to possess  
99 significant antimicrobial activity on different bacterial species (Morais et al., 2011;  
100 Surendra et al., 2011). Moreover, cosmetics containing crude honeybee venom that  
101 was obtained by electric shock had a positive effect on acne vulgaris both *in vitro* and  
102 *in vivo* (Han et al., 2013). A research of active compounds in venom showed that a  
103 low molecular weight protein (6.7 kDa) isolated from Indian *Naja naja* snake venom  
104 can increase the antioxidant enzymes of Ehrlich ascites carcinoma mice (Das et al.,  
105 2011). Also, the *Chrysaora quinquecirrha* (sea nettle) nematocyst venom peptide  
106 fraction as proved to possess a significant antioxidant property against Ehrlich  
107 ascites carcinoma in Swiss Albino mice (Balamurugan et al., 2010). Moreover, crude  
108 bee venom and phospholipase A2-free bee venom were also reported to exhibit an  
109 inhibitory property on matrix metalloproteinase (MMP-1) in HaCaT and HDF (Human  
110 dermal fibroblasts) human skin cells (Lee et al., 2015), while other studies have  
111 reported the potential antioxidant activity of hornet venom (Yang et al., 2011) and  
112 bee venom (Sobral et al., 2016; Somwongin et al., 2018).

113 *Vespa velutina nigrithorax* is an alien species from Asia that was accidentally  
114 introduced into Europe, first in southwest France (Monceau et al., 2014). Generally,  
115 *Vespid* venom comprises multiple potential bioactive compounds: low molecular  
116 weight compounds (bioamines such as histamine, tyramine and catecholamine (Piek,  
117 2013) or pheromones such as aliphatic ketones and alcohols (Cheng et al., 2017)),  
118 peptides (occupying up to 70% of the dried venom, the most popular peptides being  
119 mastoparan and wasp kinins), and proteins (neurotoxins, proteases, phospholipases)  
120 (Piek, 2013). Surprisingly, up to now the venom of the *Vespa velutina nigrithorax*  
121 species has been generally ignored as a potential natural source of bioactive  
122 compounds, and it was only recently that a study was carried out on *Vespa velutina*  
123 venom to investigate its **toxin composition**. From this analysis, 293 putative toxin-  
124 encoding sequences were identified **in which** the main groups of interest were  
125 hemostasis-impairing toxins, neurotoxins, proteases, antimicrobial proteins and  
126 peptides (Liu et al., 2015). In two other papers, *V. velutina* venom was reported to  
127 contain apamine and melittin with structures similar to those found in the honeybee  
128 (Shi et al., 2003; Su- fang et al., 2003).

129 To our knowledge, **the antioxidant property** has not been studied in *V. velutina*  
130 venom. In this study, we demonstrated that *V. velutina* venom possesses a strong  
131 antioxidant potential for the protection of keratinocytes against ROS. Our objective in  
132 this study was to identify the compounds responsible for this property in *V. velutina*  
133 venom. To deal with this challenge, an analytical methodology combined with  
134 biological **assays** was developed to determine **and purify the bioactive compounds**.  
135 Traditionally, HPLC and free radical scavenging DPPH assays are used to isolate  
136 and identify the antioxidants from a complex mixture (Zhu et al., 2013). However,  
137 these approaches have disadvantages such as **non-targeted sample collection**,

138 **significant sample consumption** and, in the case of HPLC-DPPH online, the  
139 installation of specific and costly equipment (Bandoniené and Murkovic, 2002). Thin  
140 layer chromatography (TLC) is a **simple, rapid and targeted method** that is  
141 compatible with direct derivatization by DPPH solution and thus can help to visibly  
142 locate the antioxidants on the plate surface (Lam et al., 2016). Therefore, in our study  
143 we conducted TLC and HPLC separations in parallel to collect active antioxidant  
144 compounds in *V. velutina* venom. Then high performance liquid chromatography  
145 coupled with high-resolution mass spectrometry was used for structural identification  
146 of the active compounds. In order to confirm the involvement of these molecules in  
147 the antioxidant mechanism, the identified compounds were tested in DPPH and ROS  
148 assays in HaCaT cells exposed **to UVB**.

## 149 **2. Material and methods**

### 150 **2.1. Materials.**

151 *Vespa velutina nigrithorax* workers were provided by the Institute of Research on  
152 Insect Biology (IRBI) located in Tours, France. The hornets were collected in the  
153 Indre-et-Loire (France) in October (2017) and were stocked at -80°C before use. 2,2-  
154 Diphenyl-1-picrylhydrazyl (DPPH), serotonin, ascorbic acid, and ninhydrin were  
155 obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Butanol, methanol,  
156 acetonitrile, formic acid, and acetic acid were HPLC grade and were purchased from  
157 VWR (Fontenay-sous-Bois, France). The Bradford reagents were purchased from  
158 Bio-Rad. Ultra-pure water was obtained from the Purelab water system of Elga  
159 LabWater.

160



## 161 **2.2. Extraction of venom and preparation**

162 A venom sample was pooled from 183 frozen hornets. The extraction protocol was  
163 based on existing methods (Dias et al., 2014; Peiren et al., 2005; Sookrung et al.,  
164 2014) with some modifications to adapt it to our case. The stinger apparatus was  
165 gently pulled from the tip of the animal abdomen by forceps. The venom reservoir  
166 (about 1 mm in length, white and transparent) was collected and separated from the  
167 stinger apparatus, then washed with water to eliminate contaminants from the  
168 intestinal tract. Each venom reservoir was pressed against the Eppendorf tube wall to  
169 collect the venom inside. The empty reservoir was then removed with forceps from  
170 the tube. 100  $\mu$ L of ultra-pure water was added and the tube was then homogenized  
171 in vortex, followed by a centrifugation step at 10 000 xg for 15 min at 4°C. The  
172 supernatant was then collected, filtered through a 0.22- $\mu$ m syringe filter (Merck,  
173 Germany) and stored at -20°C. This venom solution was named Vv (Venom *Vespa*  
174 *velutina*) in our study. The venom protein concentration was determined by Bradford  
175 assay (Bradford, 1976) (Bio-Rad Protein Assay protocol for microtiter plate) and  
176 found to contain a concentration of protein of 18.75 mg protein/mL.

177

## 178 **2.3. Thin layer chromatography (TLC) collection: “targeted method”**

179 The 10x20 cm TLC silica gel 60 F<sub>254</sub> glass plate (Merck) used for this separation  
180 method was pre-washed with methanol to eliminate contaminants. Different  
181 development solvent systems for TLC separation were screened and the optimized  
182 elution solvent system was selected (supplementary data, figure 1.S) and used in the  
183 present study. Then 7  $\mu$ L of venom (Vv) was diluted in 20  $\mu$ L of water and spotted in  
184 four bands (5 $\mu$ L/band) to collect a significant amount of active spots and two control  
185 bands (1 $\mu$ L/band) for antioxidant detection and revelation with ninhydrin on the plate

186 by a TLC autosampler (Linomat IV, CAMAG Muttenz, Switzerland). The instrumental  
187 parameters were as follows: a band 10 mm in length at the constant application rate  
188 of 5s/ $\mu$ L, a space of 8 mm between each band and a 20 mm start position. The plate  
189 was developed with the selected development solvent (1-butanol/acetic acid/water,  
190 4:1:2, v/v/v) without the chamber pre-condition until the migration line reached 7 cm.  
191 The parts of the TLC plate containing each control band after elution were separated  
192 from the rest of the plate. The control band for antioxidant detection was revealed by  
193 manually dipping the plate in the 0.05% DPPH methanolic solution (Badarinath et al.,  
194 2010). The other control band was used for peptide-amino acid detection and was  
195 sprayed with a 0.1% ninhydrin ethanolic solution. The samples were then heated in a  
196 drying oven at 110°C for 5 min. In the part of the plate containing bands for venom  
197 collection, the antioxidant spot was obtained by scraping the band at the same  $R_f$  as  
198 the antioxidant band observed in the control band. The extraction of active  
199 compounds was carried out according to the protocol of Zhang et al. (Zhang et al.,  
200 2015) with the following modifications. Briefly, the silica powder was extracted with  
201 200  $\mu$ L of ultra-pure water and incubated in ice for 20 min (vortex for 2 min was  
202 performed twice). The extraction procedure was repeated once to recover as much  
203 active compound as possible. Then, the mixture was centrifuged twice at 12000 xg at  
204 4°C for 20 min to collect the pre-cleared supernatant, the supernatant was lyophilized  
205 and stored at -20°C.

206

#### 207 **2.4. RP-HPLC-DAD collection: “non-targeted method”**

208 10  $\mu$ L of venom (Vv) was precipitated with 40  $\mu$ L of ice cold methanol (Want et al.,  
209 2006) (solvent screening result not shown) for 20 min at -20°C to eliminate proteins.  
210 The supernatant was then recovered after a centrifugation step at 10 000 xg for 10

211 min at 4°C. The precipitation process was repeated twice and the supernatants were  
212 pooled and named Vv0. 15µL of Vv0 supernatant was injected into the HPLC Agilent  
213 1100-DAD system through the 2x125 mm LiChroCART Purosphere Star RP-18  
214 endcapped 5 µm column (Sigma Aldrich, France). The column was maintained at  
215 30°C and eluted using a linear gradient with solvent A containing 0.1% formic acid in  
216 water and solvent B containing 0.1% formic acid in acetonitrile: 2-5% solvent B in 5  
217 min and 5-50% B from 5 to 15 min, then an increase to 60% B in 5 min at a constant  
218 flow rate of 0.3 mL/min. The fractions were manually collected following the peak  
219 detection at 214 nm, 254 nm and 280 nm and lyophilized and stored at -20°C.

220

## 221 **2.5. DPPH assay for antioxidant activity**

222 The lyophilized fractions acquired from TLC-DPPH or RP-HPLC-DAD separation  
223 were re-dissolved in the initial volume of crude venom used in each separation  
224 method. The crude venom Vv was then diluted with ultra-pure water to obtain a  
225 concentration of 28 µg protein/mL. The fractions were also diluted with the same  
226 dilution factor as the crude venom to ensure that the percentage of each compound  
227 in the fractions was equivalent to that in the crude venom. The DPPH assay was  
228 subsequently performed in a 96-well microplate with these fractions.

229 The radical scavenging activity (RSA) of venom was evaluated by the DPPH assay,  
230 following a previously described method (Lu et al., 2014). 100µL of solution of Vv  
231 crude venom, Vv0 precipitated venom or fraction from TLC and HPLC were added in  
232 triplicate to the 96-well microplate. For IC<sub>50</sub> determination of crude venom, various  
233 concentrations in ultra-pure water were prepared between 0.24 to 31.25 µg  
234 protein/mL. 100 µL of 0.2 mM DPPH methanolic solution was then added to each  
235 well. The plate was shaken manually, kept in darkness at 37°C for 30 min before

236 reading the absorbance at 515 nm using a Thermo scientific Multiskan GO UV/Vis  
237 microplate spectrophotometer. Ultra-pure water and ascorbic acid were used for  
238 blank and positive control, respectively. Radical scavenging activity (RSA) was  
239 determined as the percent of DPPH inhibition. The IC<sub>50</sub> of these compounds was  
240 graphically determined by **Graphpad Prism** 6.0 (USA) and used for antioxidant  
241 activity comparison.

242

## 243 **2.6. Identification of antioxidant compounds by high resolution mass** 244 **spectrometry**

245 The fractions showing the highest % RSA in both approaches TLC and HPLC-DAD  
246 were analyzed with RP-HPLC-MS using the Dionex Ultimate 3000 UHPLC  
247 (Darmstadt, Germany) coupled with a Bruker Daltonik ESI-QTOF-HRMS system  
248 (Bremen, Germany). 1 µL of the fraction was injected into a Acquity Waters 2.1x50  
249 mm BEH C18 1.7µm column (Saint-Quentin-en-Yvelines, France) maintained at 42°C  
250 and eluted at a flow rate of 0.5 mL/min with a gradient starting at 2% of solvent B,  
251 maintained for 0.2 min and increased to 50% of solvent B from 0.2 to 3 min (solvent  
252 A consisted of 0.1% formic acid in water and solvent B was 0.08% formic acid in  
253 acetonitrile). The eluate was then loaded into an ESI-QTOF-MS mass spectrometer  
254 system with the following parameters: 4500V of capillary voltage; 2.0 bar of nebulizer  
255 gas; 9.0 L/min at 200°C of dry gas; 1 Hz of frequency and the spectrum was acquired  
256 in positive mode over the range 50-1660 *m/z*. The MS/MS scans were carried out  
257 with the peak of interest obtained at the collision energies of 10 eV, 20 eV, and 30  
258 eV. The data were handled by Compass DataAnalysis software (Bruker) and  
259 submitted to the online MassBank database (<https://massbank.eu/MassBank/>) for  
260 identification.

261

## 262 **2.7. Human keratinocyte cell culture and treatments**

263 The immortalized human keratinocyte cell line HaCaT was obtained from ATCC,  
264 cultured in high glucose Dulbecco's modified Eagle's media (DMEM) supplemented  
265 with 10% heat-inactivated fetal calf serum, 50 IU mL<sup>-1</sup> penicillin, 50 mg mL<sup>-1</sup>  
266 streptomycin and 2 mM glutamine and maintained in a humidified atmosphere of 5 %  
267 CO<sub>2</sub> at 37°C. Before treatment, the cells were plated at a density of 2x10<sup>4</sup> cells/well  
268 in 96-well plates overnight to reach about 90% of confluence the following day and  
269 then treated with venom extracts or purified molecules in serum-free media. For the  
270 cytotoxic assay, the Cell Proliferation kit II (XTT- Roche) was employed to evaluate  
271 the potential toxicity of extracts according to the manufacturer's instructions. Briefly,  
272 at the indicated time post-treatment, the XTT solution at a final concentration 0.3  
273 mg/ml was added to each well and incubated for 4 hours at 37°C to form orange-  
274 colored formazan produced by viable cells. The intensity of coloration was then  
275 quantified by measuring the absorbance with a specific absorbance filter at 450nm  
276 using a Victor spectrophotometer (PerkinElmer, Waltham, MA, USA). The data were  
277 expressed as percentage of viability by normalizing the absorbance value detected in  
278 non-treated cells to the arbitral value of 100 %. For the ROS protection assay, the  
279 cells were also plated in 96-well plates as indicated above but treated with the  
280 compounds for **six** hours at 5 % CO<sub>2</sub> at 37°C before loading the 200 µL of 2',7'-  
281 Dichlorofluorescein diacetate at 10 µM in PBS (DCF-DA Sigma Aldrich, France) for 20  
282 min. After a quick wash in PBS, the plate was irradiated at 25 mJ/cm<sup>2</sup> and then  
283 incubated for 30 min at 37°C at 5% CO<sub>2</sub>. Then, the fluorescence values at 485-535  
284 nm were read with the same spectrophotometer. Non-UVB irradiated cells were used  
285 as internal control to measure the basal level of ROS content in cells. The final data

286 were expressed as the percentage of ROS production by normalizing the absorbance  
287 value detected in non-treated cells to the arbitral value of 100 %. All experiments  
288 were repeated at least three times in triplicate.

289

## 290 **2.8. Statistical analysis**

291 The tests were carried out in triplicate or in quintuplicate and performed at least  
292 twice. The significant difference between bioactivity values was evaluated by an  
293 ANOVA test. These statistical analyses were done with Rstudio software  
294 (version 1.0.153) and statistical significance was set at  $p < 0.05$ . Graphpad Prism 6.0  
295 was used to construct  $IC_{50}$  and graphics in inhibition activity.

296

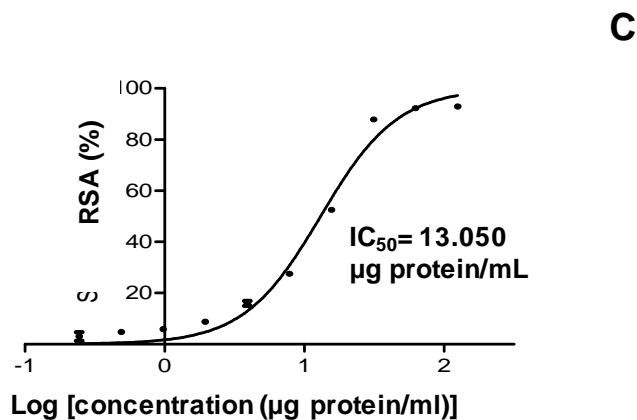
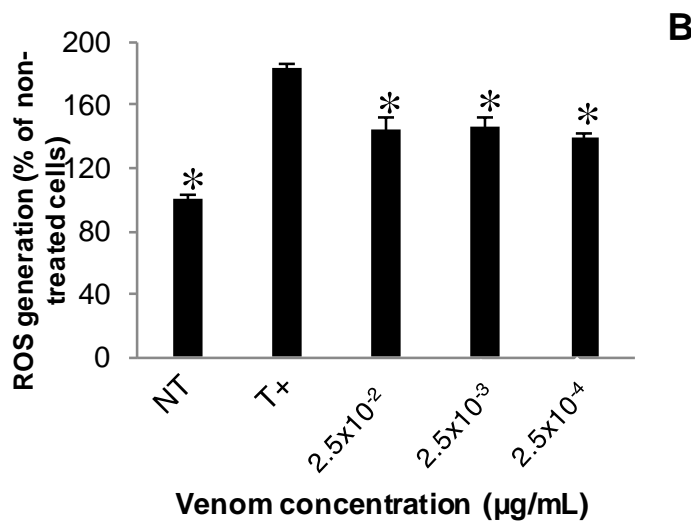
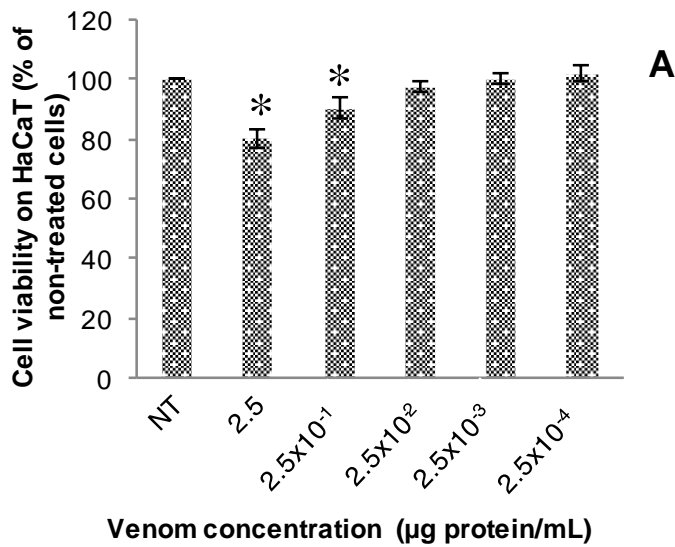
## 297 **3. Results and discussion**

### 298 **3.1. Antioxidant activity of *Vespa velutina* crude venom**

299 The antioxidant activity of *V. velutina* venom was tested in HaCaT immortalized  
300 human keratinocytes exposed to UVB as a source of ROS induction. This assay is  
301 considered as a crucial criterion for the screening of antioxidant activity in cosmetics  
302 (Dunaway et al., 2018). **We first performed a cytotoxicity assay to determine the**  
303 **concentration of venom that was well tolerated by the cells, as the relative toxicity of**  
304 **compounds at low concentration is a key point when considering a potential human**  
305 **application.** Our results from the XTT assay, shown on Fig.1.A, demonstrated a slight  
306 toxicity of the venom at concentrations of  $2.5 \times 10^{-1}$  and  $2.5 \mu\text{g protein/mL}$ . No  
307 significant toxicity was observed when the concentration of venom tested ranged  
308 from  $2.5 \times 10^{-4}$  to  $2.5 \times 10^{-2} \mu\text{g protein/mL}$  after 48 hours of incubation. It has been  
309 recently reported that bee venom (*Apis mellifera L.*) induces a strong toxicity (60%  
310 loss of cell viability compared with the control) in HaCaT cells at  $3 \mu\text{g/mL}$  (Lee et al.,

311 2015). Our results demonstrate therefore that *V. velutina* venom is better tolerated by  
312 HaCaT cells than the bee venom. Non-toxic concentrations were then employed to  
313 evaluate the reduction effect of venom in ROS generation. According to figure 1.B,  
314 irradiation of HaCaT cells with UVB (named T+) resulted in a substantial production  
315 of ROS. Remarkably, when the cells were pre-incubated with *V. velutina* venom for  
316 48 hours, a significant reduction in ROS production was detected in the tested  
317 concentration range. However, no dose-response effect was observed in this range.  
318 The crude venom extract appears to reach its maximum activity even with the low  
319 concentration applied, indicating that it has a strong anti-oxidant property against the  
320 production of ROS induced by UVB.

321 These initial results prompted us to identify the molecules responsible for this activity.  
322 First, a simple assay was applied to quickly screen for compounds possessing  
323 antioxidant activities. The DPPH assay is an easy, sensitive and rapid method for  
324 investigation of the free radical scavenging activity of a compound by  
325 spectrophotometry (Kedare and Singh, 2011). The results of this assay shown in  
326 figure 1.C demonstrated that *V. velutina* venom (Vv) has a free radical scavenging  
327 property. Remarkable significant inhibition of DPPH was observed in a dose-  
328 response manner. The IC<sub>50</sub> of *V. velutina* venom Vv was graphically calculated as  
329 13.050 µg protein/mL.



330

331 **Figure 1. Antioxidant activity evaluation of *Vespa velutina* venom (Vv).** (A) XTT  
 332 cytotoxicity assay of *V. velutina* venom in HaCaT cells for 48h. The value is  
 333 expressed as Mean ± SD, n=3. NT: Non-treated cells. \*: significant discrimination  
 334 versus non-treated group at p<0.05 in ANOVA test (R software). (B) Reactive oxygen



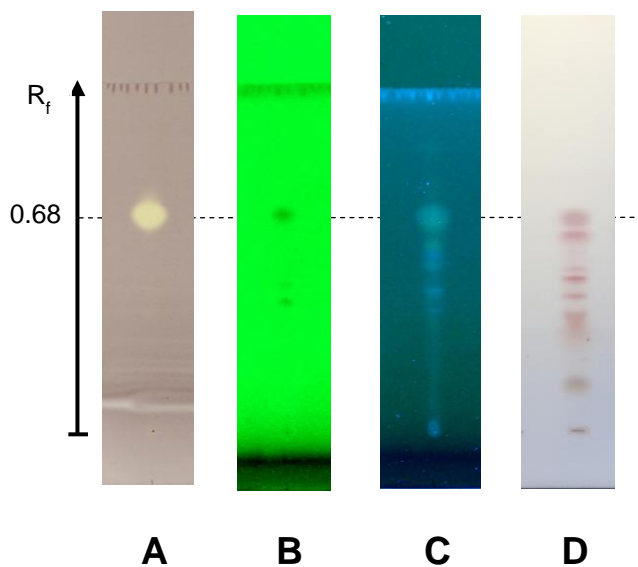
335 species (ROS) reduction effect of *V. velutina* venom in HaCaT cells irradiated by  
336 UVB, the cells were cultured for 48h. NT: Non-treated cells, T+: HaCaT cell irradiated  
337 with UVB without adding venom, \*: significant discrimination versus T+ group at  
338  $p < 0.05$  in ANOVA test (R software). (C) Dose-response curve of *V. velutina* venom  
339 on DPPH radical scavenging activity assay measured at 515 nm. These results are  
340 representative of three independent experiments, the value is expressed as Mean  $\pm$   
341 SD,  $n=3$ . RSA: Radical scavenging activity.  $IC_{50}$  was calculated by Graphpad Prism  
342 6.0.

343

### 344 3.2. Detection and isolation of antioxidant compound in TLC plate

345 The advantage of the DPPH assay is that antioxidant spots can be directly detected  
346 on the plate and the active fractions visible on the surface can then be collected by  
347 scraping. It is known from the literature that peptides and amino acids can be  
348 separated by thin layer chromatography (Bhusan et al., 1989; Neda et al., 2012). In a  
349 second step, we therefore conducted TLC separation of *V. velutina* venom (Vv)  
350 whose composition is rich in proteins, peptides and amino acids. As shown in figure  
351 2.A, one intense yellow spot was detected with DPPH 0.05% methanolic solution at  
352 the  $R_f = 0.68$  while no yellow spots were detected in the elution zone. It was therefore  
353 deduced that the compounds responsible for the antioxidant activity of the venom  
354 were mainly located in this yellow spot. To obtain more information on the property of  
355 this spot, different detection methods were applied. As shown in figure 2.B and 2.C,  
356 this spot can be detected under a UV lamp at 254 nm or 366 nm. It was assumed to  
357 possess conjugated C=C double bonds in the structure. The ninhydrin reagent is  
358 used to detect ammonia and primary amines by generating a purple Schiff base  
359 compound, or a yellow-orange iminium salt in the case of secondary amines

360 (Friedman and Williams, 1974). Figure 2.D shows that there are many purple bands,  
361 indicative of the amine-rich profile of *V. velutina* venom (Vv). The spot at  $R_f = 0.68$   
362 reacted with ninhydrin solution and produced a purple spot. Therefore, we  
363 hypothesized that molecules present in the yellow spot possessed structures  
364 containing the amine group. This spot (named S-TLC) was then scraped for in-plate  
365 antioxidant activity and structure identification (Zhang et al., 2015).



366  
367 **Figure 2. DPPH scavenging activity detection and separation of *Vespa velutina***  
368 **venom (Vv) on TLC silica gel plate 60.** The elution solvent was 1-butanol : acetic  
369 acid : water = 4:1:2 (v:v:v) in 90 min. (A) Detection by immersing in DPPH 0.05%  
370 solution. The yellow zone on the purple background detected by DPPH presents an  
371 antioxidant activity. (B) UV detection at 254 nm. (C) UV detection at 366 nm. (D)  
372 Detection by spraying with ninhydrin 0.1% solution. The orange/yellow zone indicates  
373 the presence of primary or secondary amines in the molecule.

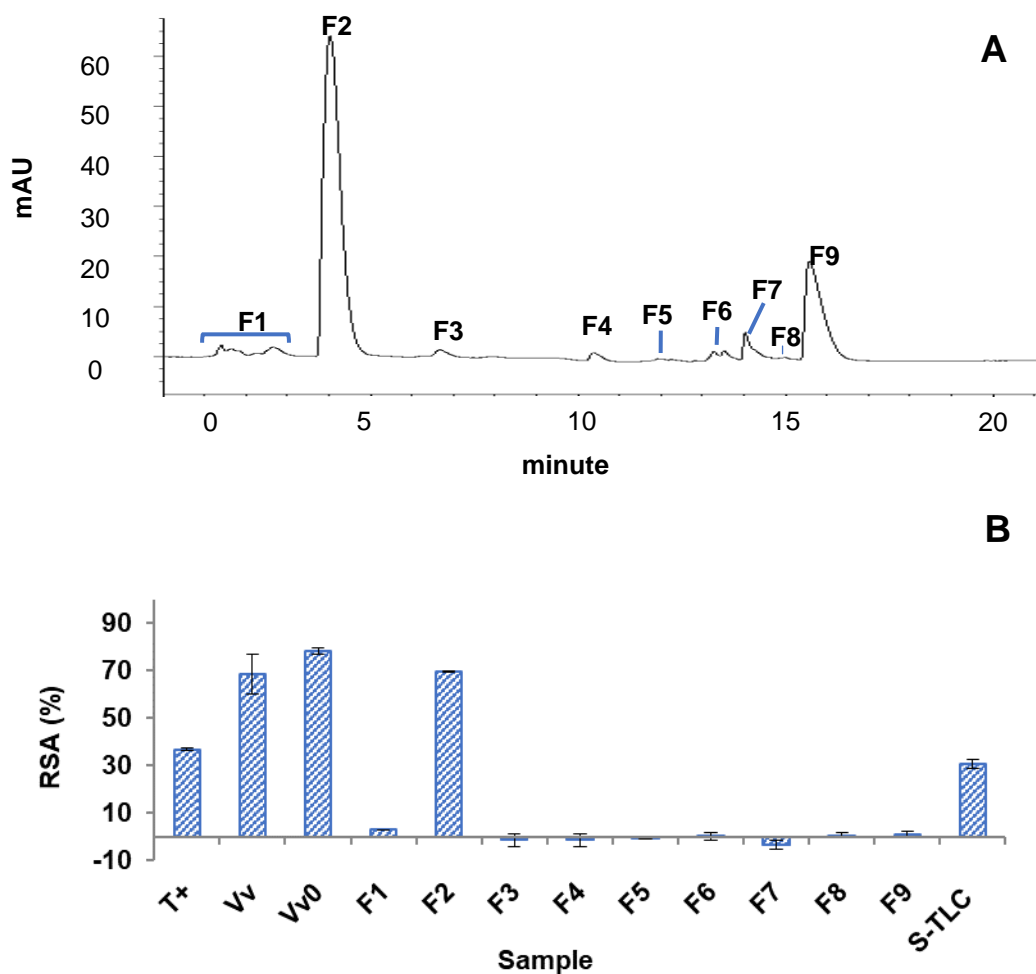
374

### 375 3.3. Isolation of the compound of interest by RP-HPLC

376 Although the TLC technique is simple and rapid, it can be contaminated by  
377 interferences from the migration system and it has a poor recovery for spot collection.

378 RP-HPLC was therefore carried out to enable comparison with antioxidant isolation  
379 on the TLC plate. Several fractions were collected to determine which of them had an  
380 antioxidant activity using a DPPH assay in a microtiter plate. The previous TLC  
381 results indicated that this main spot corresponds to a peptide or simple amino acids.  
382 Therefore, we eliminated the protein of *V. velutina* venom (Vv) by precipitation with  
383 the organic solvent method (Want et al., 2006) (methanol was chosen as the most  
384 suitable and effective solvent compared to acetone and acetonitrile, result not shown)  
385 before injection into the HPLC system.

386 As can be seen from figure 3.A, **nine** fractions were separated from the venom by  
387 RP-18 HPLC-DAD at 254 nm and their antioxidant activities were evaluated as  
388 shown in figure 3.B. To verify the influence of the protein precipitation step, the  
389 antioxidant activity of crude *V. velutina* venom (Vv) and precipitated venom (Vv0)  
390 were investigated. Figure 3.B shows that antioxidant activity was mostly retained  
391 after precipitation. Among the HPLC fractions, only fraction F2 collected at 4 min  
392 showed a marked DPPH scavenging activity (69.59%). It is thought that F2 might be  
393 the main fraction responsible for the antioxidant activity of *V. velutina* venom (Vv).  
394 The abundant fraction F9 (high absorbance at 254 nm) was used as a non-active  
395 reference for antioxidant identification. In parallel, the antioxidant activity of the active  
396 spot collected from the TLC approach was also confirmed with the DPPH assay.  
397 However, only mild inhibition was observed in comparison with that of F2, indicating  
398 that the quantity of active molecules that can be collected by TLC is much lower than  
399 that obtained by HPLC.



400

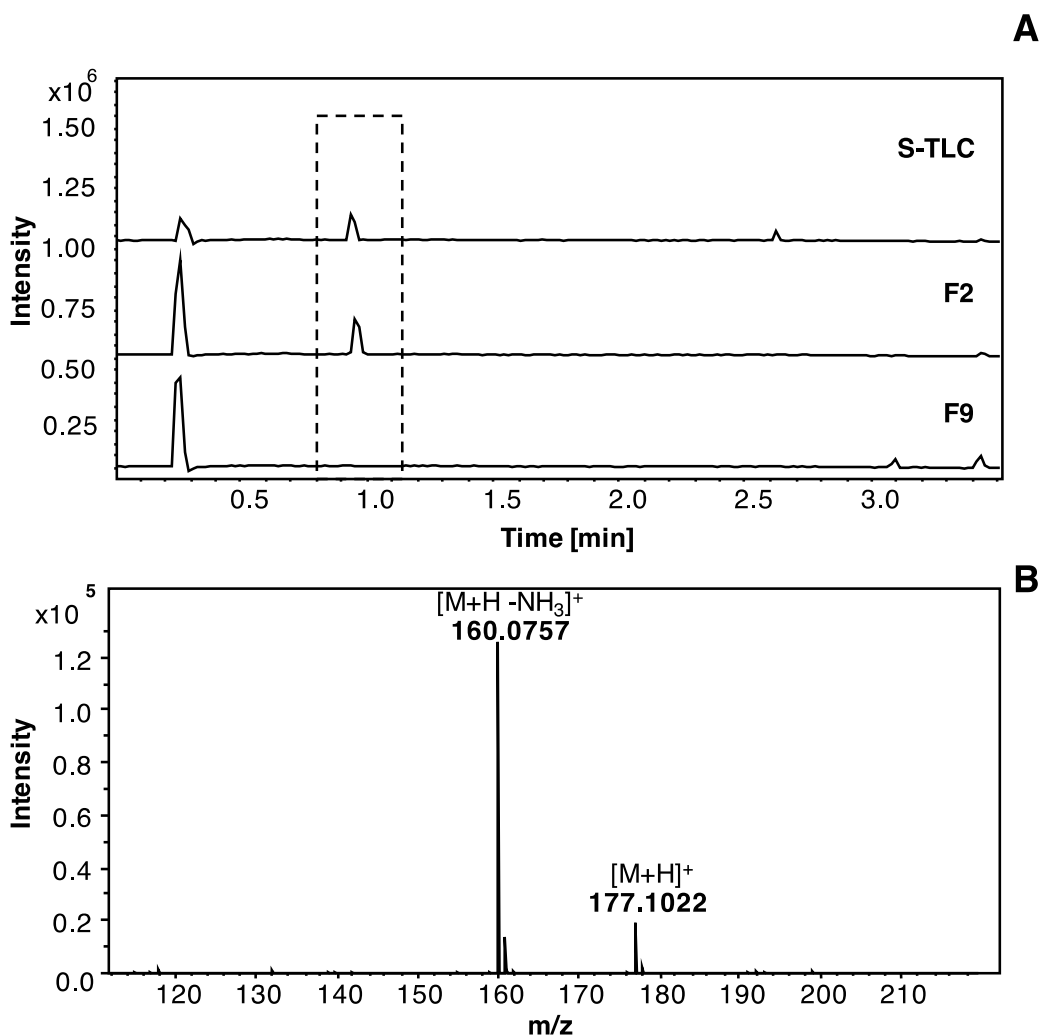
401 **Figure 3: RP-HPLC chromatogram of precipitated venom (Vv0) and active**  
 402 **antioxidant detection by DPPH scavenging activity assay (A) RP-HPLC-DAD**  
 403 profile of precipitated venom at 254 nm **(B) DPPH scavenging activity assay of RP-**  
 404 HPLC-DAD fractions and TLC-active spot.

405

### 406 3.4. Identification of antioxidant compound

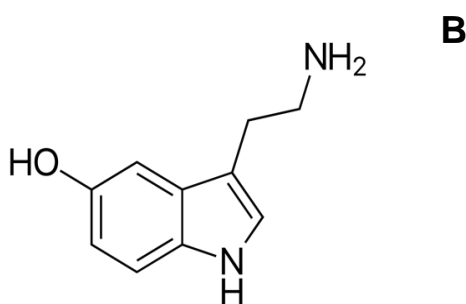
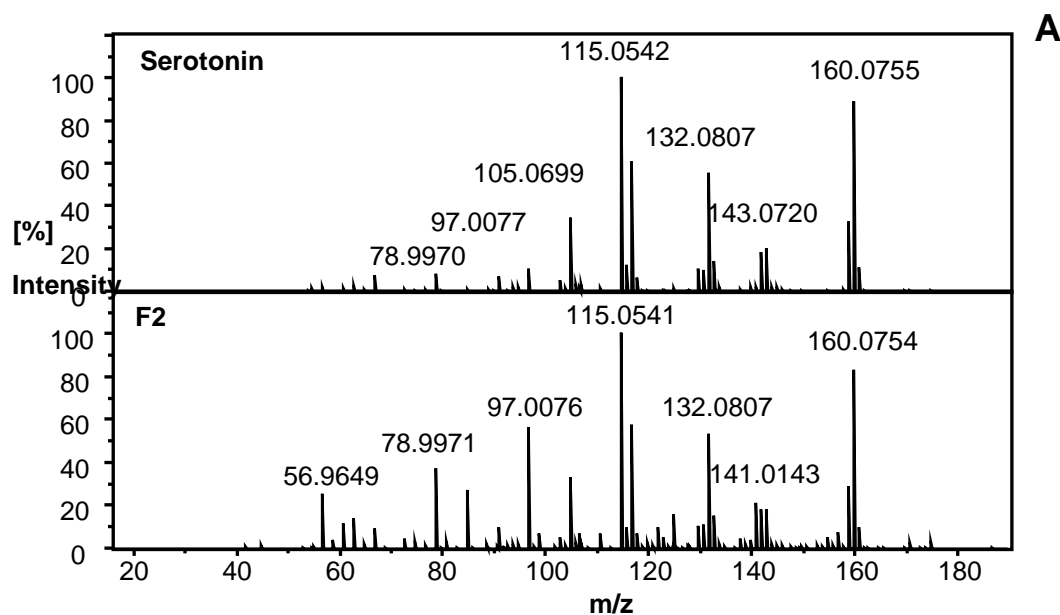
407 The active spot  $R_f = 0.68$  (S-TLC) from the TLC method and the active fraction F2  
 408 from the RP-HPLC method were analyzed by LC-MS. The non-active fraction (F9)  
 409 was used as negative control. Their LC-MS profiles were studied to compare the  
 410 targeted and non-targeted approaches and identify the antioxidant compound. As  
 411 can be seen from figure 4.A, the S-TLC and F2 fractions contain a common peak at  $t_r$

412 =0.95 min which was not present in the non-active fraction F9, indicating that this  
413 peak is the main molecule responsible for the antioxidant activity.



414  
415 **Figure 4: LC-MS profile of active fraction obtained from TLC and RP-HPLC**  
416 **separation. (A)** Comparison of LC-MS profiles among active spots scraped from  
417 TLC glass plate, RP-HPLC active fraction and RP-HPLC non-active fraction. The  
418 dotted line indicates the coincident peak of two active fractions obtained from two  
419 distinct collection methods (S-TLC and F2). This peak is not present in the non-active  
420 fraction (F9). (B) Mass spectrum of coincident peak at the  $t_r = 0.95$  min.  
421

422 The mass spectrum corresponding to the peak at  $t_r = 0.95$  min reveals a single-  
423 charge ion  $[M+H]^+$  at  $m/z$  177.1022 with a major fragment  $[M+H-NH_3]^+$   $m/z$  160.0757.  
424 Using the Smart formula tool (Compass DataAnalysis software, Bruker), we deduced  
425 that this ion corresponds to a compound with a molecular weight of 176.0943 Da and  
426 with a molecular formula  $C_{10}H_{12}N_2O$  (the mass error of ion  $[M+H]^+$  is 0.1 ppm,  
427  $m\sigma = 8.5$ , theoretical mass  $[M+H]^+ = 177.1023$ ). Additionally, the MS/MS profile  
428 of ion  $[M+H]^+$  at  $m/z$  177.1022 was acquired by FIA-ESI-QTOF-HRMS/MS at different  
429 collision energies (10 eV, 20 eV, 30eV) from the **two** active fractions. On applying the  
430 MS/MS profiles to the online database library (<https://massbank.eu/MassBank/>), the  
431 active compound present in the F2 and S-TLC fractions **was** determined as  
432 serotonin. The standard serotonin analyzed in MS/MS showed a similar mass  
433 fingerprint (Fig 5.A and 5.B).



434

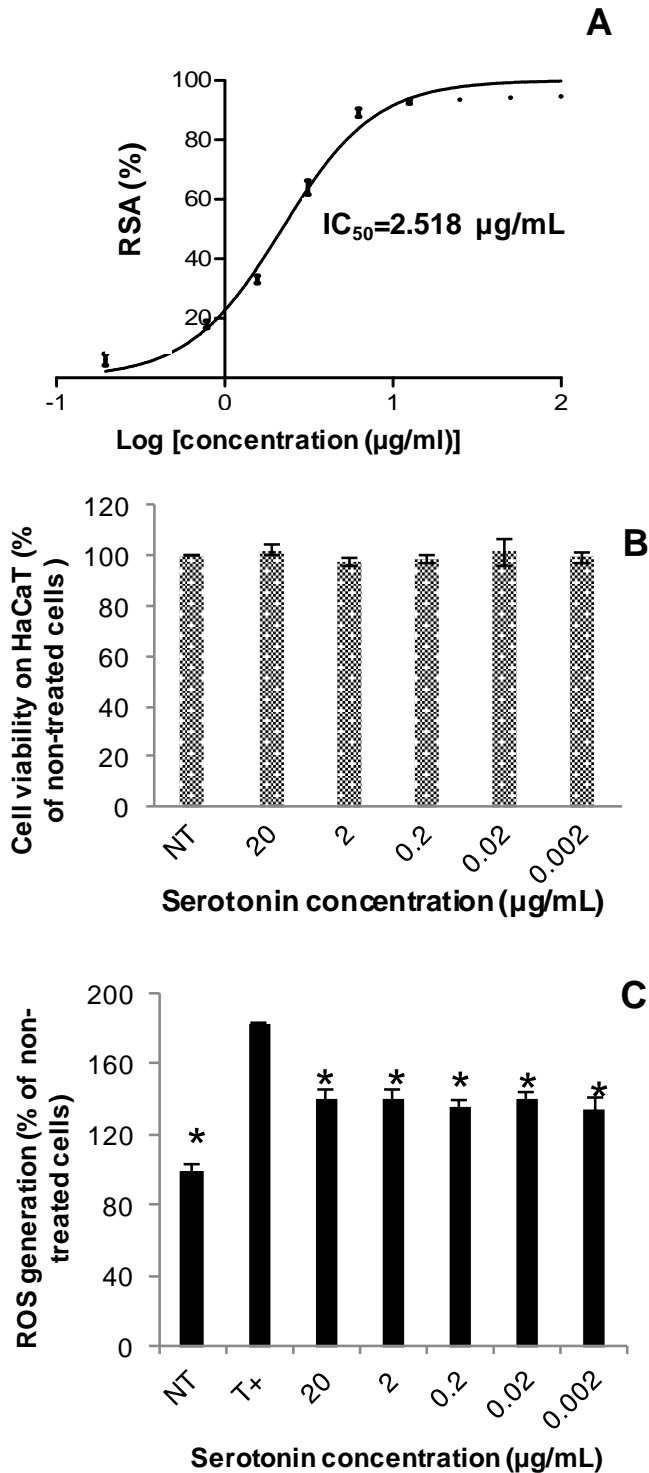
435 **Figure 5: Coincident peak characterization. (A)** MS/MS profile at the collision  
 436 energy 30 eV of *m/z* 177.1022 of serotonin (upper spectrum) and active fraction F2  
 437 (lower spectrum). **(B)** Primary chemical structure of serotonin.

438

### 439 3.5. Serotonin antioxidant activity evaluation

440 To confirm serotonin as the compound responsible for antioxidant activity in *V.*  
 441 *velutina* venom (Vv), a serotonin standard was tested for its antioxidant property by  
 442 DPPH assay on a TLC plate (supplementary data figure S.2). Results of this  
 443 experiment indicate that serotonin presents a strong antioxidant activity as  
 444 demonstrated by the presence of a yellow spot at the same  $R_f = 0.68$  as that of crude  
 445 venom. Moreover, in the detection with ninhydrin 0.1%, serotonin also displays a

446 purple-orange spot at  $R_f = 0.68$ , which can be explained by its amine structure  
447 containing one primary amine and one secondary amine (see figure 5.B).  
448



449

450 **Figure 6: Antioxidant activity of serotonin. (A)** Dose-response curve of serotonin  
451 in DPPH scavenging activity assay measured at 515 nm, results are expressed as



452 Mean  $\pm$  SD, n=3. **RSA: Radical scavenging activity.**  $IC_{50}$  was calculated by Graphpad  
453 **Prism 6.0. (B)** XTT cytotoxicity assay of serotonin in HaCaT cells for 48h. Results are  
454 expressed as Mean  $\pm$  SD, n=3. NT: Non-treated cells \*: significant discrimination  
455 versus non-treated group at  $p < 0.05$  in ANOVA test (R software). **(C)** Reactive oxygen  
456 species (ROS) reduction effect of serotonin in HaCaT cells irradiated by UVB.  
457 Results are expressed as Mean  $\pm$  SD, n=3. T+: HaCaT cell irradiated by UVB without  
458 adding venom. NT: Non-treated cells \*: significant discrimination versus T+ group at  
459  $p < 0.05$  in ANOVA test (R-software).

460

461 The cytotoxicity assay revealed that serotonin is not toxic for the HaCaT cells in the  
462 concentration ranging from 0.002 to 20  $\mu\text{g/mL}$  (equivalent to 0.01136-113.6  $\mu\text{M}$ ) (Fig.  
463 6.B). Serotonin was then tested at the same concentration range in the ROS cellular  
464 assay to **evaluate the strength of its antioxidant property in keratinocytes.** As shown  
465 in Fig. 6.C, serotonin decreases the production of ROS in HaCaT cells exposed to  
466 UVB in comparison with the positive control T+ ( $\approx$  40-48%) at all concentrations  
467 used. **However, no dose-response effect was observed, showing that these**  
468 **concentrations reached the maximum of capacity and therefore that lower**  
469 **concentrations of serotonin may also possess a significant antioxidant capability.**  
470 Consequently, we demonstrate that serotonin has a considerable protective property  
471 against oxidation in HaCaT cells exposed to UVB. In a recent paper, serotonin was  
472 demonstrated to decrease the viability of HaCaT cells when a concentration  $>10 \mu\text{M}$   
473 was used; the effect was even more pronounced when a concentration  $>100 \mu\text{M}$  was  
474 evaluated (Choi et al., 2010). Additionally, serotonin was not found to have any  
475 inhibition property against ROS produced in HaCaT cells exposed to  $\text{H}_2\text{O}_2$  at  
476 concentrations of 1  $\mu\text{M}$  and 10  $\mu\text{M}$ . In another review, serotonin was described as a

477 causative agent for increased ROS generation in diabetes mellitus (Yang et al.,  
478 2017). In our study, the results show that serotonin has a cytoprotective property in  
479 UVB-irradiated HaCaT cells at a low concentration and is not toxic at a higher  
480 concentration (e.g. 0.01136-113.6  $\mu$ M). These results were also supported by  
481 research into the mechanism involving serotonin as an antioxidant agent and its  
482 interaction with lipid membranes, protecting them from oxidation (Azouzi et al., 2017).  
483 Moreover, a conformational space study of serotonin recently indicated that the  
484 structure of the indole ring is a reactive site for free-radical scavenging activity  
485 (Lobayan and Schmit, 2018).

486 Although our study has succeeded in confirming the antioxidant property of serotonin  
487 found in Asian hornet venom on UVB-irradiated keratinocyte cells, it is necessary to  
488 discuss the role of serotonin in the venom and the applicability of this compound in  
489 cosmetics.

490 Serotonin is a well-known neurotransmitter with vasoactive, immunomodulatory, and  
491 growth factor properties. This compound produces pro-inflammation, vasodilation and  
492 pro-edema (Slominski et al., 2002). In some studies, serotonin is reported to be  
493 frequently well represented in Vespidae venom and to be derived from tryptophan in  
494 venom-producing cells. This bioactive amine is generally considered to be the major  
495 pain-producing factor in Vespidae venom for defense purposes, in particular toward  
496 vertebrate attackers (Piek, 2013; Weisel-Eichler and Libersat, 2004). In the case of  
497 marine snail venom, serotonin has been known to induce muscular paralysis or  
498 vasodilation in prey, thereby increasing the distribution of the other venom  
499 compounds (Weisel-Eichler and Libersat, 2004).

500 In human skin, the detection of serotonin and serotonergic machinery including its  
501 transporters and receptors in the epidermis have proved the serotonin production

502 capability of this organ. This expression may be involved in the regulatory function of  
503 the skin and serotonin may be part of this system (Sansone and Sansone, 2013;  
504 Slominski et al., 2002). Another study has shown that serotonin can enhance cell  
505 proliferation and cell migration in both keratinocytes and fibroblasts, making serotonin  
506 a potential therapeutic candidate for wound healing (Sadiq et al., 2018). Therefore, in  
507 an injection of Asian hornet venom into the human skin, serotonin will certainly react  
508 as a pain-producing molecule but will also simultaneously induce the effects related  
509 to its strong antioxidant property and its impact on the skin serotonergic system.  
510 However, in view of the strong influence of serotonin on human monoaminergic  
511 systems, the use of Asian hornet venom or serotonin as a cosmetic ingredient seems  
512 complicated.

513

#### 514 **4. Conclusions**

515 This study is the first to investigate the antioxidant activity of Asian hornet venom  
516 (*Vespa velutina nigithorax*). We demonstrate that this venom showed an inhibition  
517 capability against HaCaT intracellular Reactive Oxygen Species at a low  
518 concentration ( $2.5 \times 10^{-4}$  to  $2.5 \times 10^{-2}$   $\mu\text{g protein/mL}$ ) without cytotoxicity.

519 A biotechnologically-oriented strategy was developed using TLC-DPPH targeted and  
520 HPLC-DPPH non-targeted separation methods to purify the active fraction. Serotonin  
521 was identified by UHPLC-ESI-QTOF-HRMS/MS as the major active compound  
522 responsible for free radical scavenging activity in this venom.

523 Since this compound has a harmful effect on the human nervous system, the direct  
524 use of serotonin as a new cosmetic ingredient should be considered with caution.

525 Nevertheless, these results contribute to current knowledge in bioactivity research of

526 this insect and the methodology applied in this study can be exploited in the search  
527 for other biological targets.

528

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534 industry. Located in the heart of the cosmetic valley, this program led by University of  
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536

### 537 **Supporting Information**

538 **Figure S.1: Screening of elution solvent system for Vespa velutina venom (Vv)**  
539 **separation on TLC plate. Silica gel 60 plate: (A)** 1-butanol: acetic acid: water =  
540 **4:1:2 (v:v:v) (B)** 1-butanol: acetic acid: water = 4:1,5:3 (v:v:v) **(C)** Acetonitrile:  
541 methanol: water = 5:1:1 (v:v:v) **(D)** Chloroform: methanol: ammoniac 35% = 2:2:1  
542 (v:v:v). **RP-18 W plate: (E)** Methanol: water = 7:3 (v:v)

543

544 **Figure S.2:** TLC profile of crude venom (Vv) and serotonin on TLC silica gel 60 plate  
545 (Merck) by reagent: (1) DPPH/methanol (2) Ninhydrin/ethanol. The elution solvent  
546 was 1-butanol : acetic acid : water = 4:1:2 (v:v:v) for 90 min.

547

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712

### 713 **Figure captions**

714 **Figure 1. Antioxidant activity evaluation of *Vespa velutina* venom (Vv). (A)** XTT  
715 cytotoxicity assay of *V. velutina* venom in HaCaT cells for 48h. The value is  
716 expressed as Mean  $\pm$  SD, n=3. NT: Non-treated cells. \*: significant discrimination  
717 versus non-treated group at p<0.05 in ANOVA test (R software). (B) Reactive oxygen  
718 species (ROS) reduction effect of *V. velutina* venom in HaCaT cells irradiated by

719 UVB, the cells were cultured for 48h. NT: Non-treated cells, T+: HaCaT cell irradiated  
720 with UVB without adding venom, \*: significant discrimination versus T+ group at  
721  $p < 0.05$  in ANOVA test (R software). (C) Dose-response curve of *V. velutina* venom  
722 on DPPH radical scavenging activity assay measured at 515 nm. These results are  
723 representative of three independent experiments, the value is expressed as Mean  $\pm$   
724 SD,  $n=3$ . RSA: Radical scavenging activity. IC<sub>50</sub> was calculated by Graphpad Prism  
725 6.0.

726

727 **Figure 2. DPPH scavenging activity detection and separation of *Vespa velutina***  
728 **venom (Vv) on TLC silica gel plate 60.** The elution solvent was 1-butanol : acetic  
729 acid : water = 4:1:2 (v:v:v) in 90 min. (A) Detection by immersing in DPPH 0.05%  
730 solution. The yellow zone on the purple background detected by DPPH presents an  
731 antioxidant activity. (B) UV detection at 254 nm. (C) UV detection at 366 nm. (D)  
732 Detection by spraying with ninhydrin 0.1% solution. The orange/yellow zone indicates  
733 the presence of primary or secondary amines in the molecule

734

735 **Figure 3: RP-HPLC chromatogram of precipitated venom (Vv0) and active**  
736 **antioxidant detection by DPPH scavenging activity assay (A) RP-HPLC-DAD**  
737 **profile of precipitated venom at 254 nm (B) DPPH scavenging activity assay of RP-**  
738 **HPLC-DAD fractions and TLC-active spot.**

739

740 **Figure 4: LC-MS profile of active fraction obtained from TLC and RP-HPLC**  
741 **separation. (A) Comparison of LC-MS profiles among active spots scraped from**  
742 **TLC glass plate, RP-HPLC active fraction and RP-HPLC non-active fraction. The**  
743 **dotted line indicates the coincident peak of two active fractions obtained from two**

744 distinct collection methods (S-TLC and F2). This peak is not present in the non-active  
745 fraction (F9). (B) Mass spectrum of coincident peak at the  $t_r = 0.95$  min.

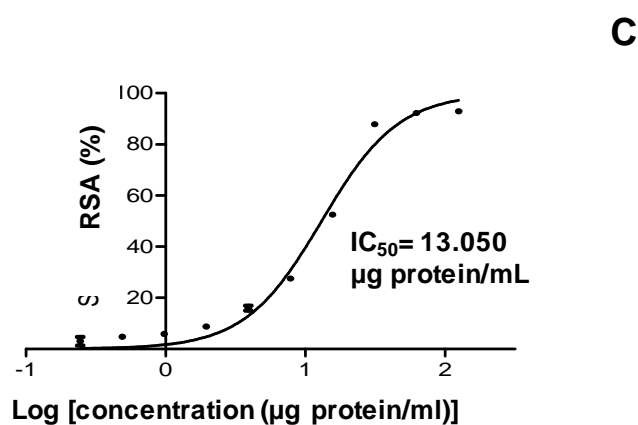
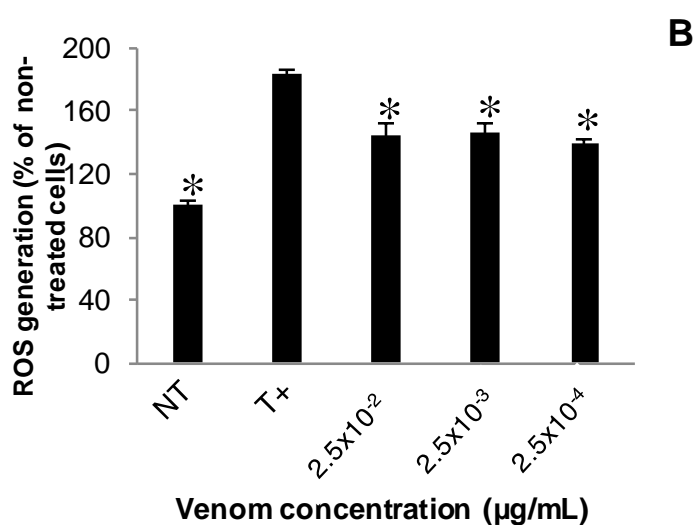
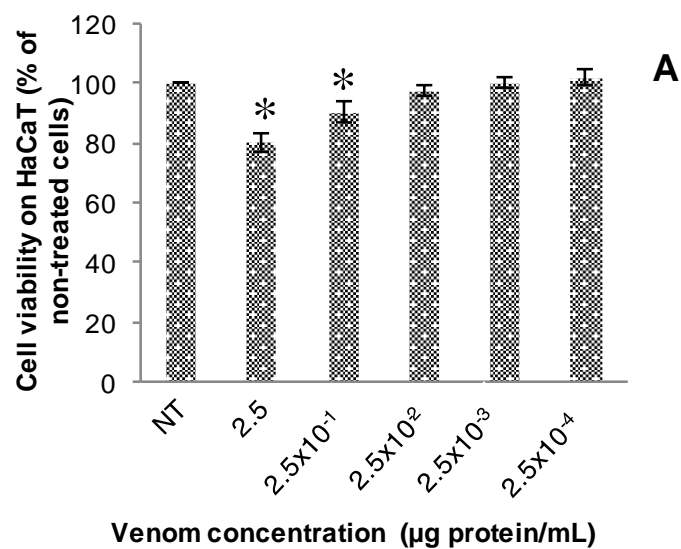
746 **Figure 5: Coincident peak characterization.** (A) MS/MS profile at the collision  
747 energy 30 eV of  $m/z$  177.1022 of serotonin (upper spectrum) and active fraction F2  
748 (lower spectrum). (B) Primary chemical structure of serotonin.

749

750 **Figure 6: Antioxidant activity of serotonin.** (A) Dose-response curve of serotonin  
751 in DPPH scavenging activity assay measured at 515 nm, results are expressed as  
752 Mean  $\pm$  SD, n=3. RSA: Radical scavenging activity.  $IC_{50}$  was calculated by Graphpad  
753 Prism 6.0. (B) XTT cytotoxicity assay of serotonin in HaCaT cells for 48h. Results are  
754 expressed as Mean  $\pm$  SD, n=3. NT: Non-treated cells \*: significant discrimination  
755 versus non-treated group at  $p < 0.05$  in ANOVA test (R software). (C) Reactive oxygen  
756 species (ROS) reduction effect of serotonin in HaCaT cells irradiated by UVB.  
757 Results are expressed as Mean  $\pm$  SD, n=3. T+: HaCaT cell irradiated by UVB without  
758 adding venom. NT: Non-treated cells \*: significant discrimination versus T+ group at  
759  $p < 0.05$  in ANOVA test (R-software).

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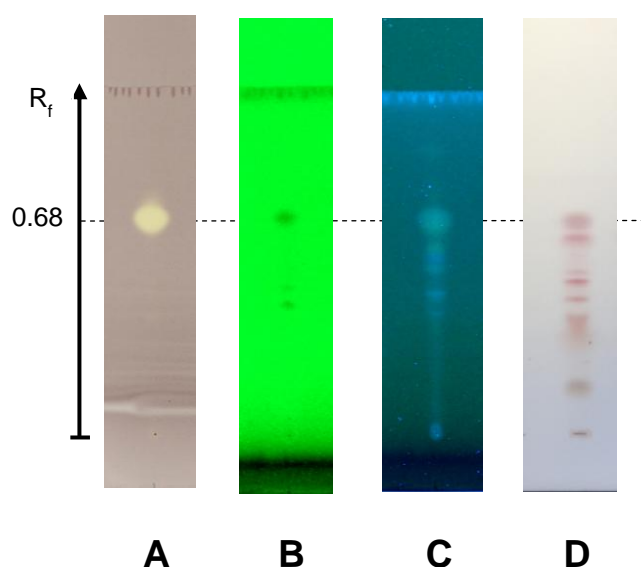


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Single column fitting image

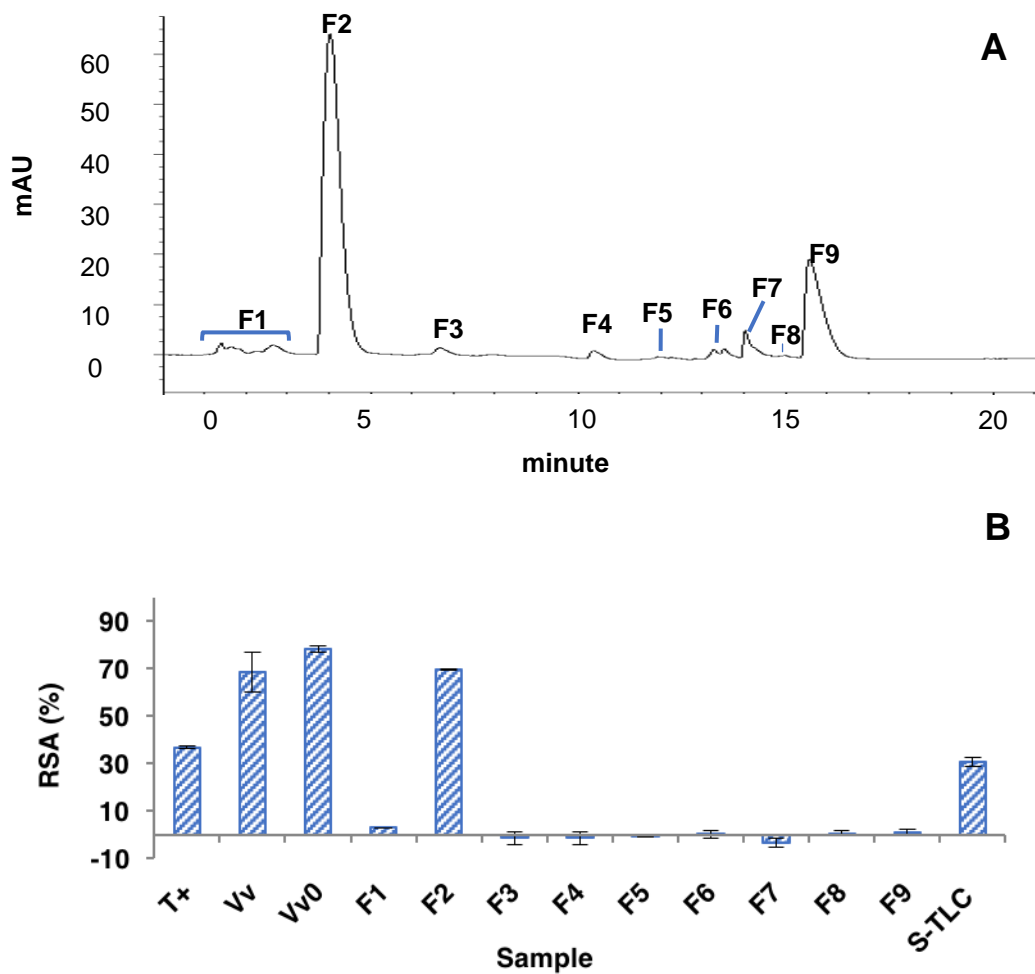


**Figure 2. DPPH scavenging activity detection and separation of *Vespa velutina* venom (Vv) on TLC silica gel plate 60.** The elution solvent was 1-butanol : acetic acid : water = 4:1:2 (v:v:v) in 90 min. **(A)** Detection by immersing in DPPH 0.05% solution. The yellow zone on the purple background detected by DPPH presents an antioxidant activity. **(B)** UV detection at 254 nm. **(C)** UV detection at 366 nm. **(D)** Detection by spraying with ninhydrin 0.1% solution. The orange/yellow zone indicates the presence of primary or secondary amines in the molecule.

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1.5-column fitting image, in color

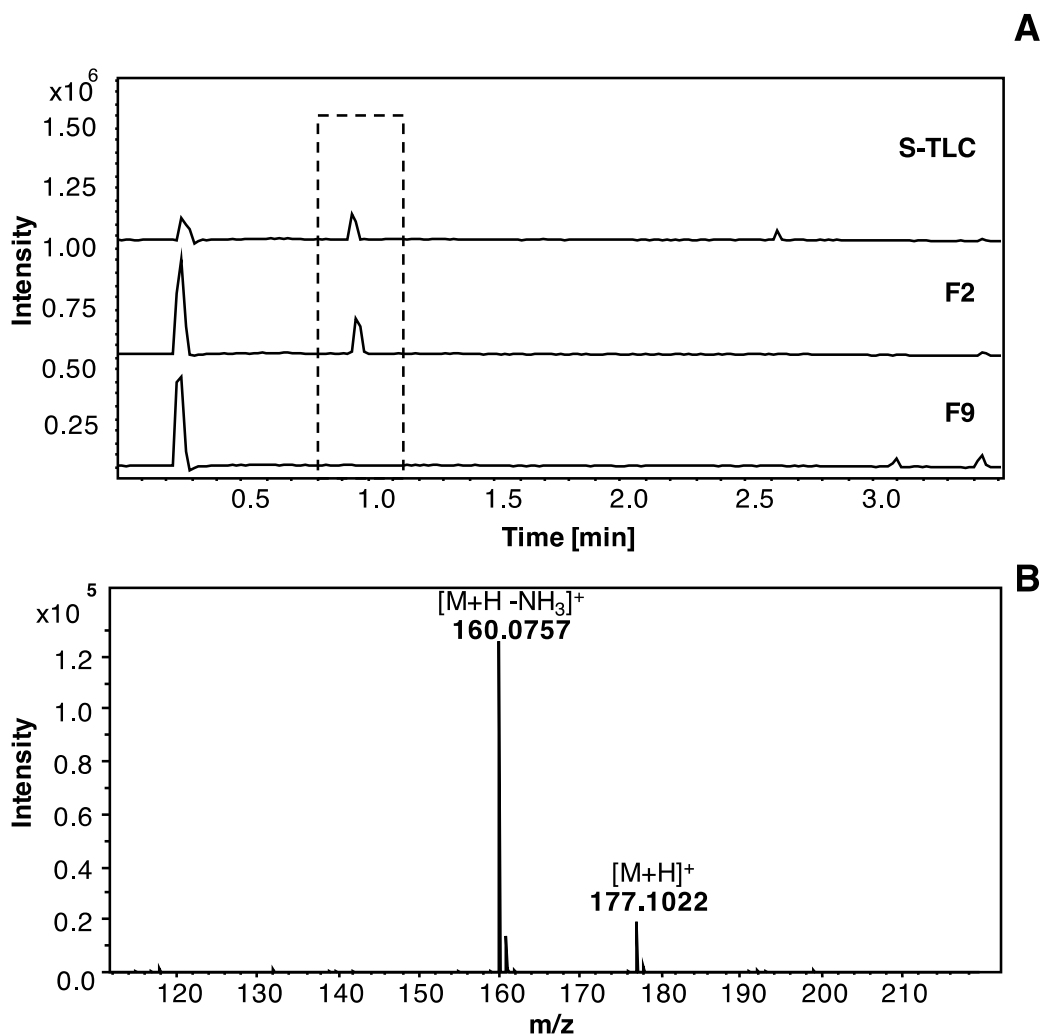




**Figure 3: RP-HPLC chromatogram of precipitated venom (Vv0) and active antioxidant detection by DPPH scavenging activity assay (A) RP-HPLC-DAD profile of precipitated venom at 254 nm (B) DPPH scavenging activity assay of RP-HPLC-DAD fractions and TLC-active spot.**

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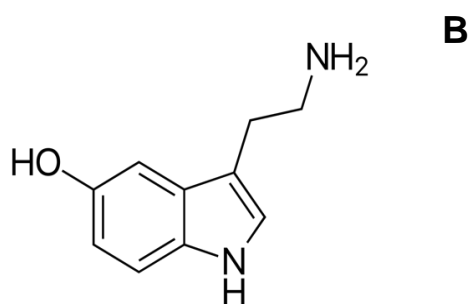
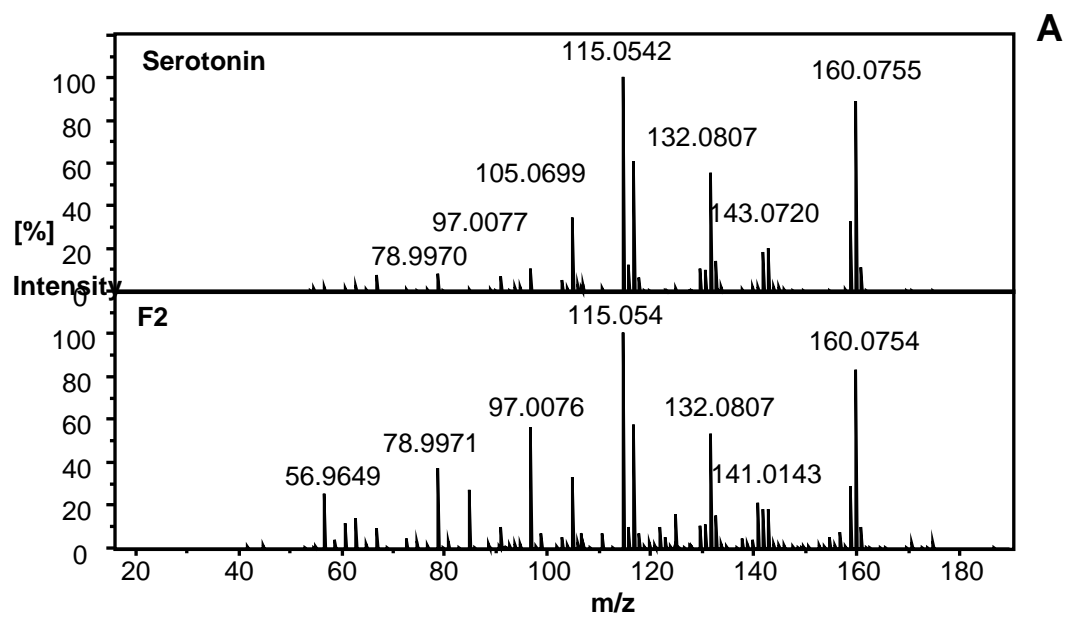
2-column fitting image



**Figure 4: LC-MS profile of active fraction obtained from TLC and RP-HPLC separation. (A)** Comparison of LC-MS profiles among active spots scraped from TLC glass plate, RP-HPLC active fraction and RP-HPLC non-active fraction. The dotted line indicates the coincident peak of two active fractions obtained from two distinct collection methods (**S-TLC** and **F2**). This peak is not present in the non-active fraction (**F9**). **(B)** Mass spectrum of coincident peak at the  $t_r = 0.95$  min.

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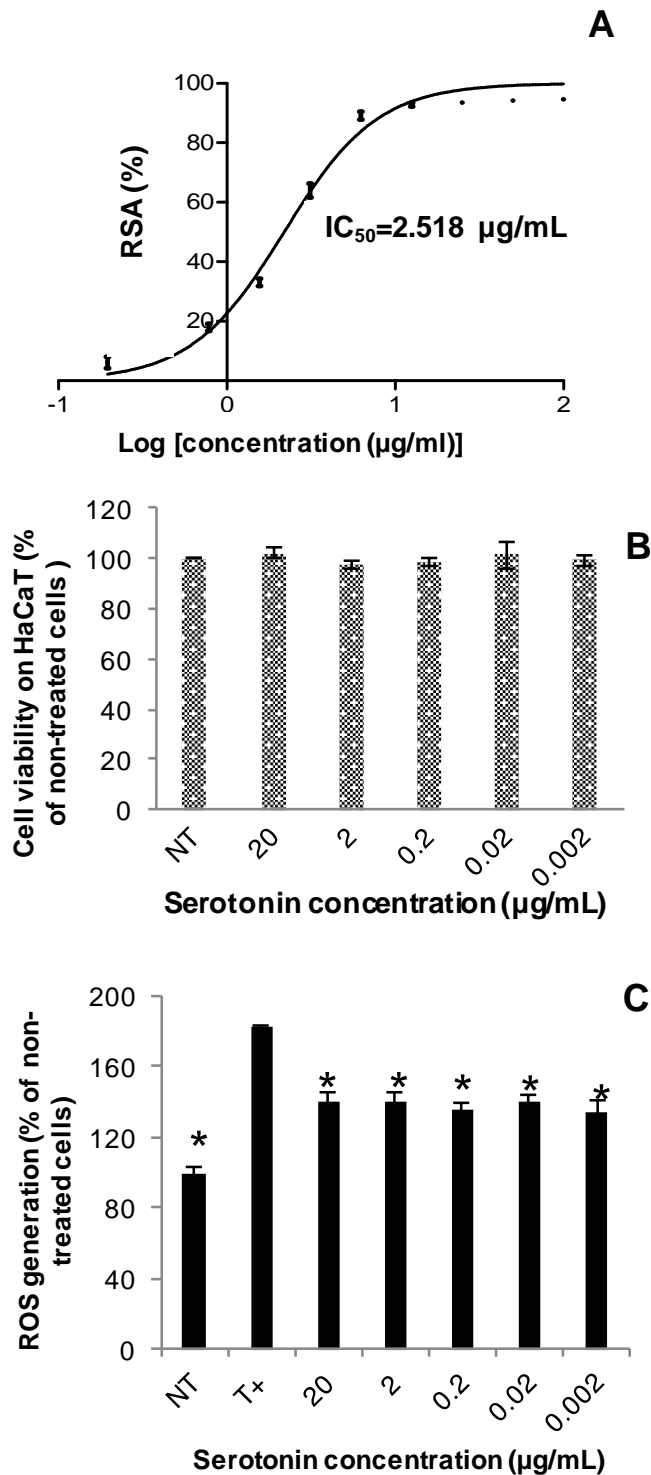
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**Figure 5: Coincident peak characterization. (A)** MS/MS profile at the collision energy 30 eV of  $m/z$  177.1022 of serotonin (upper spectrum) and active fraction F2 (lower spectrum). **(B)** Primary chemical structure of serotonin.

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2-column fitting image



**Figure 6: Antioxidant activity of serotonin. (A)** Dose-response curve of serotonin in DPPH scavenging activity assay measured at 515 nm, results are expressed as Mean  $\pm$  SD, n=3. RSA: Radical scavenging activity. IC<sub>50</sub> was calculated by Graphpad Prism 6.0. **(B)** XTT cytotoxicity assay of serotonin in HaCaT cells for 48h. Results are expressed as Mean  $\pm$  SD, n=3. NT: Non-treated cells \*: significant discrimination

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Single column fitting image

**E-component / supplementary material**

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