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

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Abstract

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

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

Abbreviations

DPPH	2,2-Diphenyl-1-picrylhydrazyl
HPLC-DAD	High-performance liquid chromatography–Diode Array Detector
LC-MS	Liquid chromatography-mass spectrometry
ROS	Reactive Oxygen Species
RSA	Radical scavenging activity
TLC	Thin layer Chromatography
UHPLC-ESI-QTOF HRMS/MS	Ultra-high performance liquid chromatography-electrospray ionization–quadrupole-time of flight–high resolution mass spectrometry-tandem mass spectrometry
UVB	Ultraviolet B
V. velutina	*Vespa velutina*

1. Introduction

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

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

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

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

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

2. Material and methods

2.1. Materials.

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

2.2. Extraction of venom and preparation

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

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

The 10x20 cm TLC silica gel 60 F_{254} glass plate (Merck) used for this separation method was pre-washed with methanol to eliminate contaminants. Different development solvent systems for TLC separation were screened and the optimized elution solvent system was selected (supplementary data, figure 1.S) and used in the present study. Then 7 µL of venom (Vv) was diluted in 20 µL of water and spotted in four bands (5µL/band) to collect a significant amount of active spots and two control bands (1µL/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/µL, 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 µl 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 µL of venom (Vv) was precipitated with 40 µl 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

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

2.5. DPPH assay for antioxidant activity

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

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

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

2.6. Identification of antioxidant compounds by high resolution mass spectrometry

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

2.7. Human keratinocyte cell culture and treatments

The immortalized human keratinocyte cell line HaCaT was obtained from ATCC, cultured in high glucose Dulbecco's modified Eagle's media (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 50 IU mL^{-1} penicillin, 50 mg mL^{-1} streptomycin and 2 mM glutamine and maintained in a humidified atmosphere of 5 % CO_2 at 37°C. Before treatment, the cells were plated at a density of $2x10^4$ cells/well in 96-well plates overnight to reach about 90% of confluence the following day and then treated with venom extracts or purified molecules in serum-free media. For the cytotoxic assay, the Cell Proliferation kit II (XTT- Roche) was employed to evaluate the potential toxicity of extracts according to the manufacturer's instructions. Briefly, at the indicated time post-treatment, the XTT solution at a final concentration 0.3 mg/ml was added to each well and incubated for 4 hours at 37°C to form orange-colored formazan produced by viable cells. The intensity of coloration was then quantified by measuring the absorbance with a specific absorbance filter at 450nm using a Victor spectrophotometer (PerkinElmer, Waltham, MA, USA). The data were expressed as percentage of viability by normalizing the absorbance value detected in non-treated cells to the arbitral value of 100 %. For the ROS protection assay, the cells were also plated in 96-well plates as indicated above but treated with the compounds for six hours at 5 % CO_2 at 37°C before loading the 200 µL of 2'7' 2′,7′-Dichlorofluorescin diacetate at 10 µM in PBS (DCF-DA Sigma Aldrich, France) for 20 min. After a quick wash in PBS, the plate was irradiated at 25 mJ/cm^2 and then incubated for 30 min at 37°C at 5% CO_2. Then, the fluorescence values at 485-535 nm were read with the same spectrophotometer. Non-UVB irradiated cells were used as internal control to measure the basal level of ROS content in cells. The final data

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

2.8. Statistical analysis

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

3. Results and discussion

3.1. Antioxidant activity of *Vespa velutina* crude venom

The antioxidant activity of *V. velutina* venom was tested in HaCaT immortalized human keratinocytes exposed to UVB as a source of ROS induction. This assay is considered as a crucial criterion for the screening of antioxidant activity in cosmetics (Dunaway et al., 2018). We first performed a cytotoxicity assay to determine the concentration of venom that was well tolerated by the cells, as the relative toxicity of compounds at low concentration is a key point when considering a potential human application. Our results from the XTT assay, shown on Fig.1.A, demonstrated a slight toxicity of the venom at concentrations of $2.5x10^{-1}$ and 2.5 µg protein/mL. No significant toxicity was observed when the concentration of venom tested ranged from $2.5x10^{-4}$ to $2.5x10^{-2}$ µg protein/mL after 48 hours of incubation. It has been recently reported that bee venom (*Apis mellifera L.*) induces a strong toxicity (60% loss of cell viability compared with the control) in HaCaT cells at 3 µg/mL (Lee et al.,

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

These initial results prompted us to identify the molecules responsible for this activity. First, a simple assay was applied to quickly screen for compounds possessing antioxidant activities. The DPPH assay is an easy, sensitive and rapid method for investigation of the free radical scavenging activity of a compound by spectrophotometry (Kedare and Singh, 2011). The results of this assay shown in figure 1.C demonstrated that *V. velutina* venom (Vv) has a free radical scavenging property. Remarkable significant inhibition of DPPH was observed in a dose-response manner. The IC50 of *V. velutina* venom Vv was graphically calculated as 13.050 µg protein/mL.

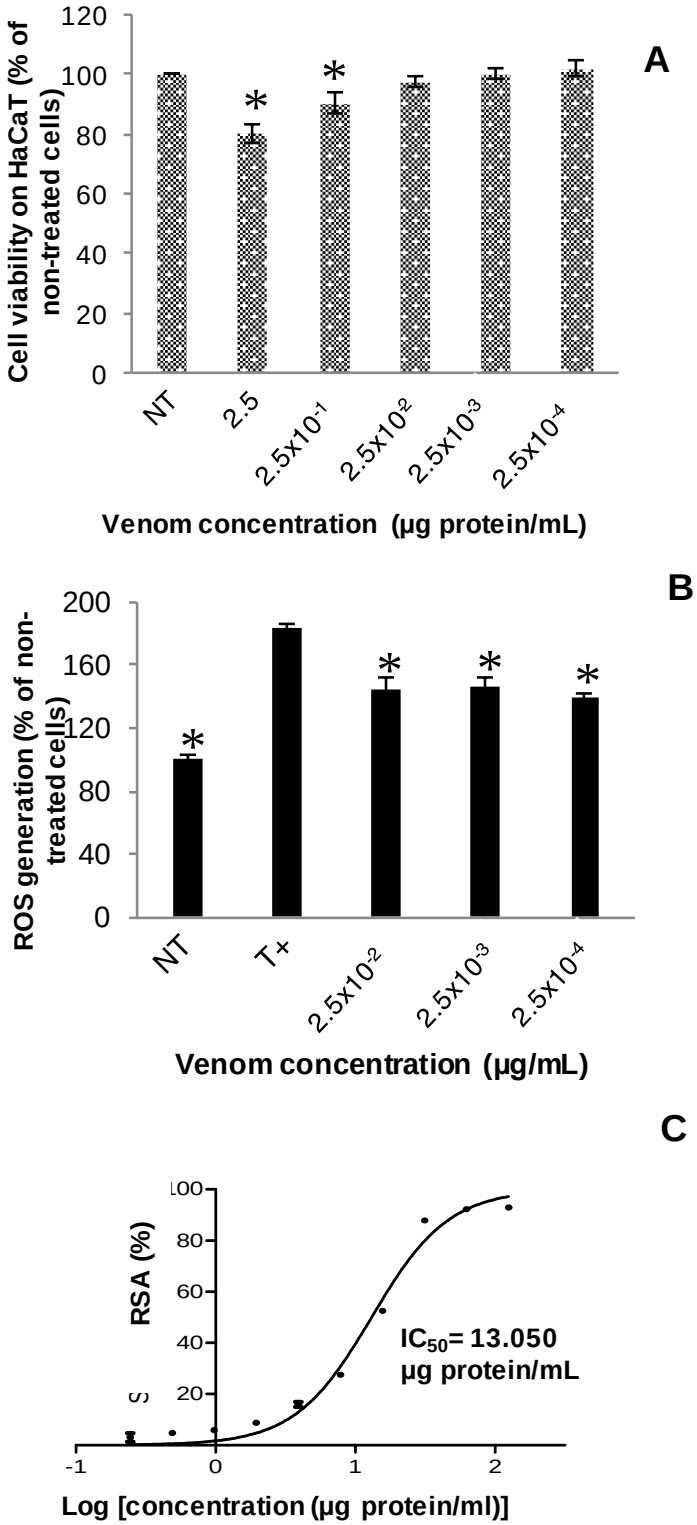


Figure 1. Antioxidant activity evaluation of *Vespa velutina* venom (Vv). (A) XTT cytotoxicity assay of *V. velutina* venom in HaCaT cells for 48h. The value is expressed as Mean ± SD, n=3. NT: Non-treated cells. *: significant discrimination versus non-treated group at p<0.05 in ANOVA test (R software).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

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

3.2. Detection and isolation of antioxidant compound in TLC plate

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

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

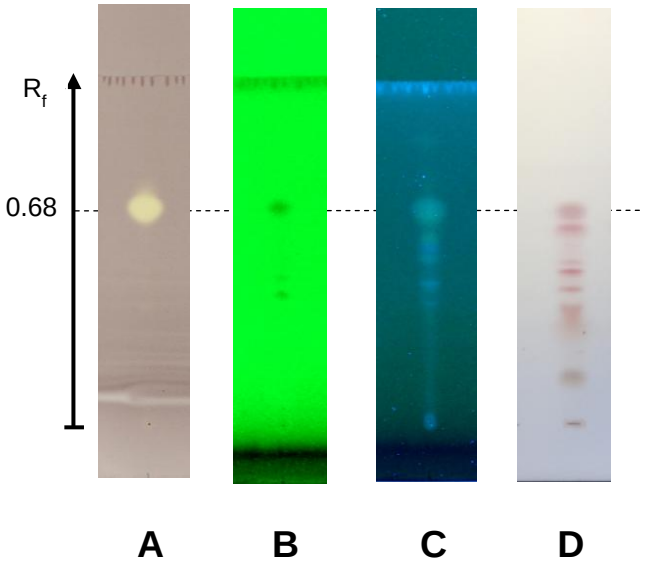


Figure 2. DPPH scavenging activity detection and separation of *Vespa velutina* venom (Vv) on TLC silica gel plate 60. The elution solvent was 1-butanol : acetic acid : water = 4:1:2 (v:v:v) in 90 min. (**A)** Detection by immersing in DPPH 0.05% solution. The yellow zone on the purple background detected 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.

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

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

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

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

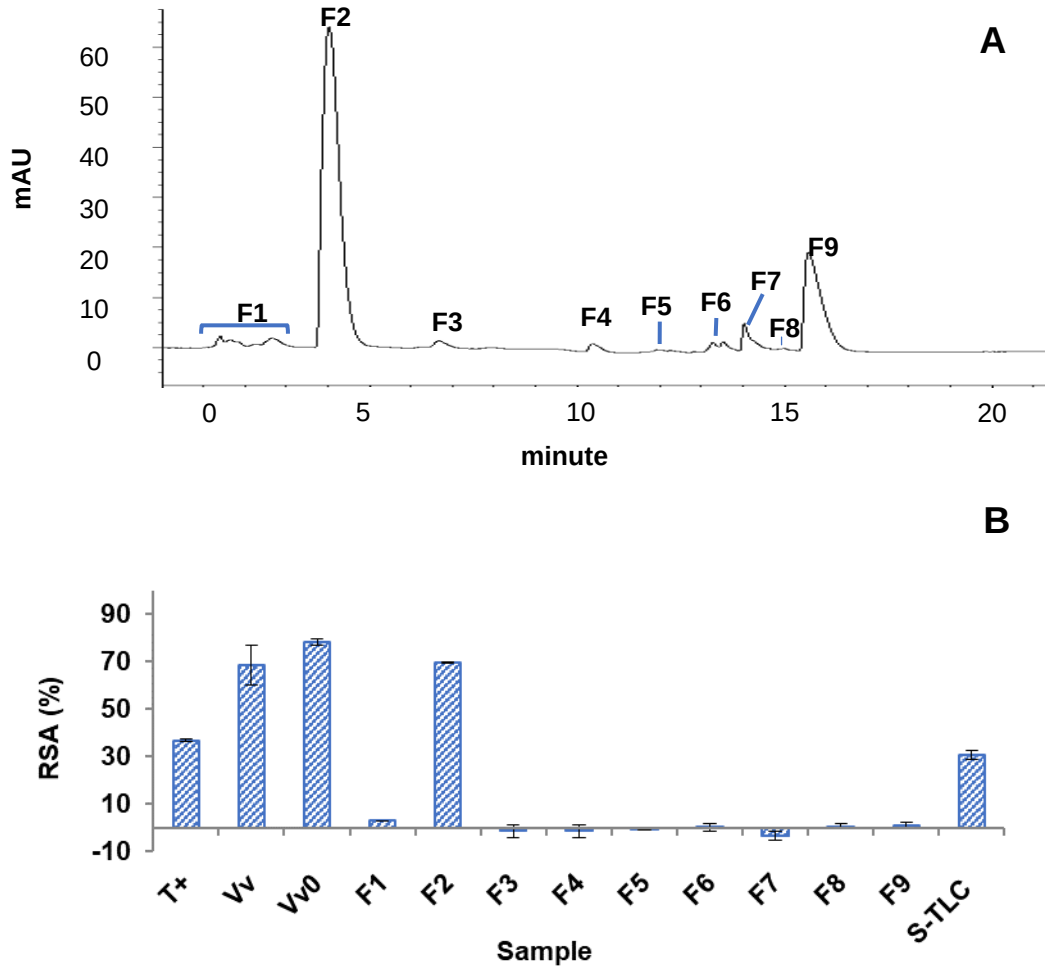
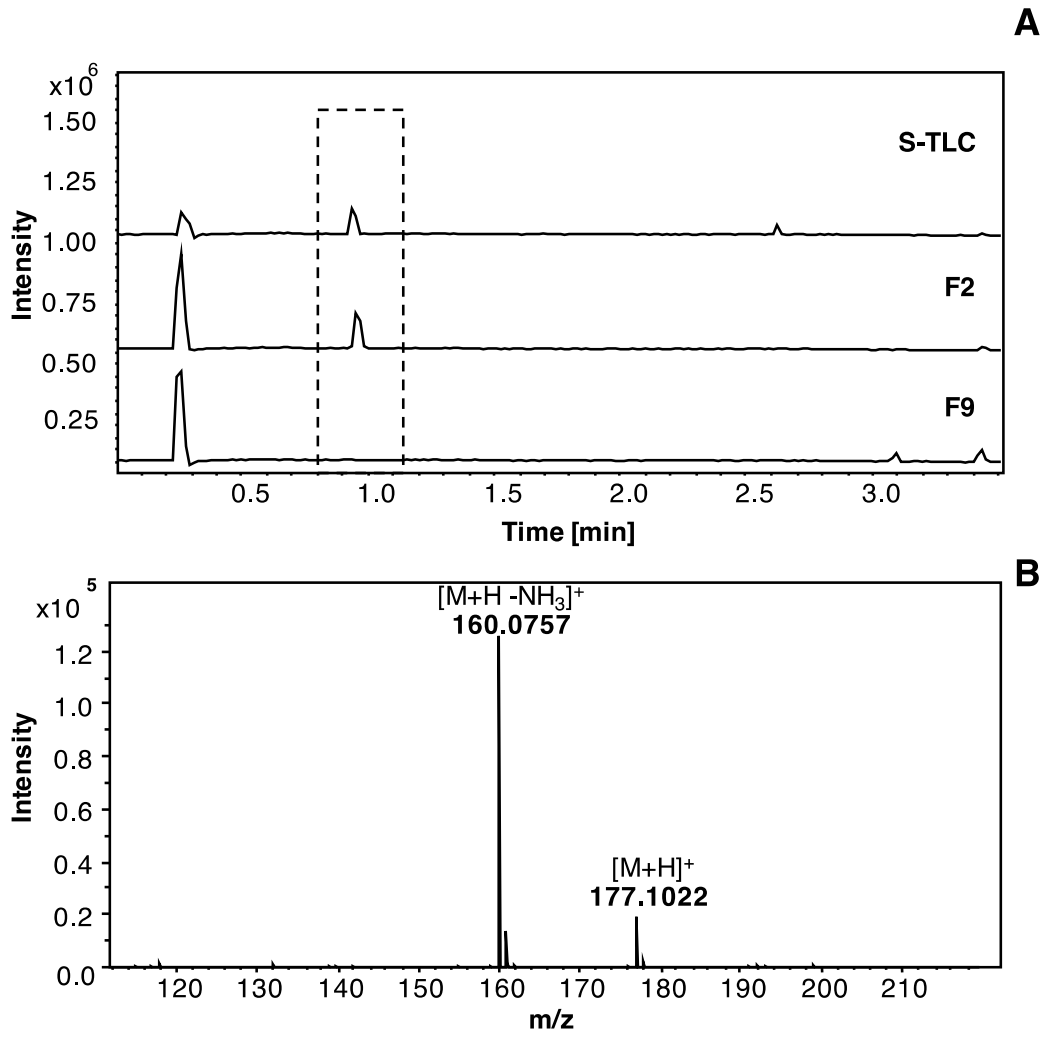


Figure 3: RP-HPLC chromatogram of precipitated venom (Vv0) and active antioxidant detection by DPPH scavenging activity assay (A) RP-HPLC-DAD profile of precipitated venom at 254 nm **(B)** DPPH scavenging activity assay of RP-HPLC-DAD fractions and TLC-active spot.

3.4. Identification of antioxidant compound

The active spot R_f = 0.68 (S-TLC) from the TLC method and the active fraction F2 from the RP-HPLC method were analyzed by LC-MS. The non-active fraction (F9) was used as negative control. Their LC-MS profiles were studied to compare the targeted and non-targeted approaches and identify the antioxidant compound. As 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

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

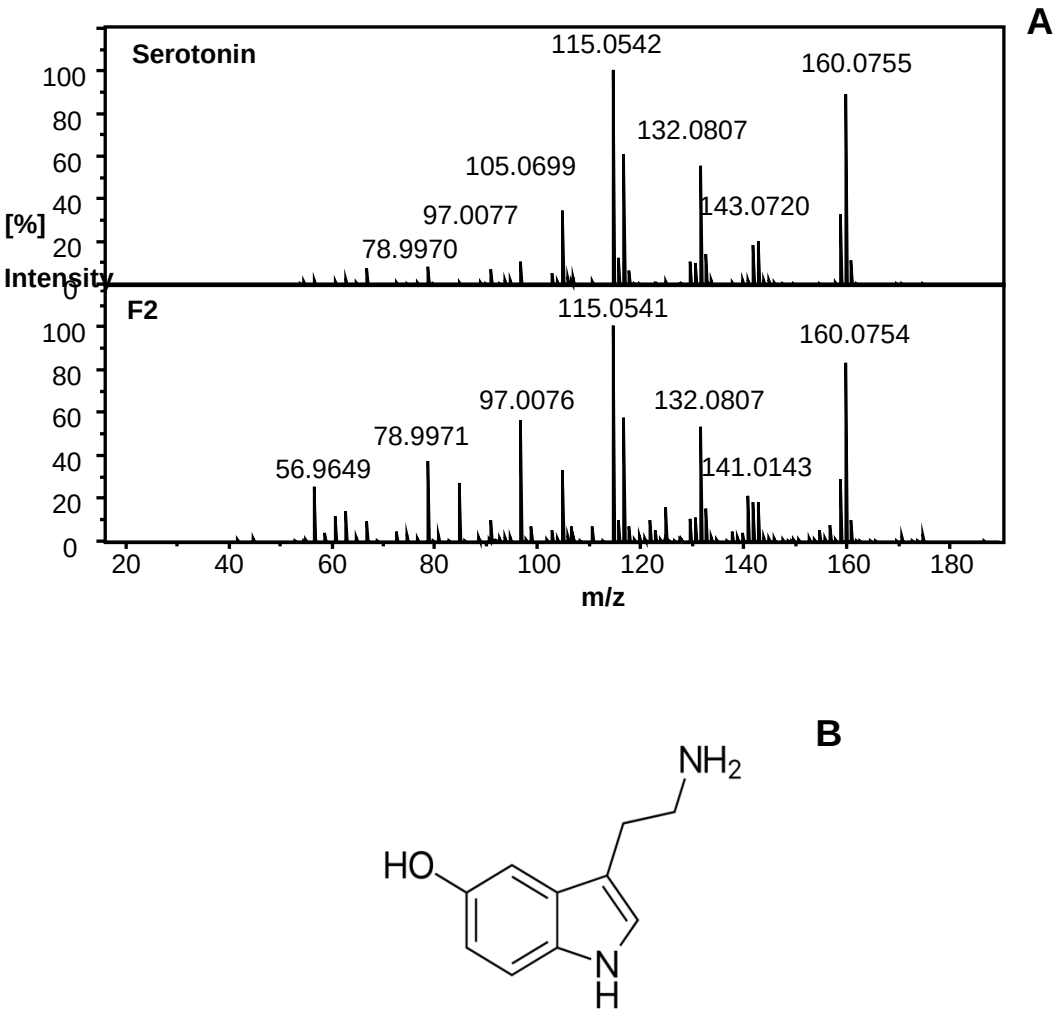


Figure 5: Coincident peak characterization. (A) MS/MS profile at the collision energy 30 eV of *m/z* 177.1022 of serotonin (upper spectrum) and active fraction F2 (lower spectrum). **(B)** Primary chemical structure of serotonin.

3.5. Serotonin antioxidant activity evaluation

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

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

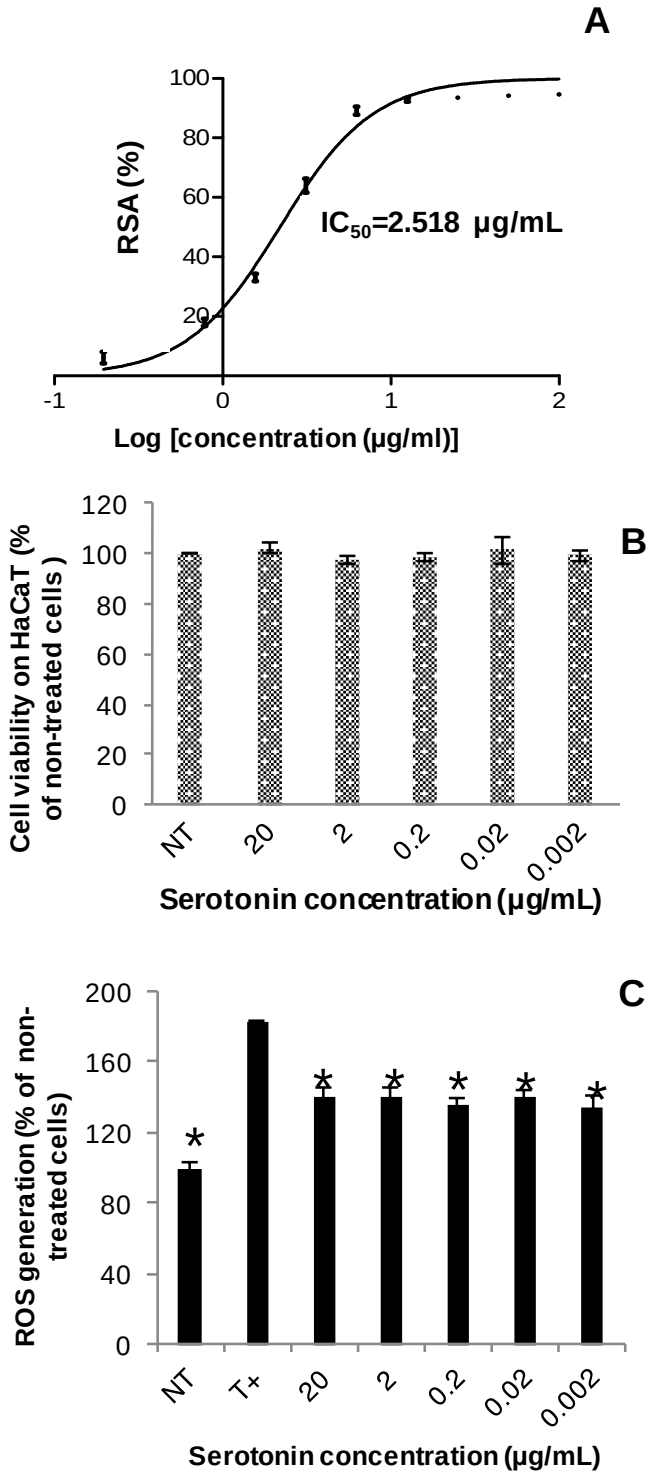


Figure 6: Antioxidant activity of serotonin. (A) Dose-response curve of serotonin in DPPH scavenging activity assay measured at 515 nm, results are expressed as

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

The cytotoxicity assay revealed that serotonin is not toxic for the HaCaT cells in the concentration ranging from 0.002 to 20 µg/mL (equivalent to 0.01136-113.6 µM) (Fig. 6.B). Serotonin was then tested at the same concentration range in the ROS cellular assay to evaluate the strength of its antioxidant property in keratinocytes. As shown in Fig. 6.C, serotonin decreases the production of ROS in HaCaT cells exposed to UVB in comparison with the positive control T+ (≈ 40-48%) at all concentrations used. However, no dose-response effect was observed, showing that these concentrations reached the maximum of capacity and therefore that lower concentrations of serotonin may also possess a significant antioxidant capability**.** Consequently, we demonstrate that serotonin has a considerable protective property against oxidation in HaCaT cells exposed to UVB. In a recent paper, serotonin was demonstrated to decrease the viability of HaCaT cells when a concentration >10 µM was used; the effect was even more pronounced when a concentration >100 µM was evaluated (Choi et al., 2010). Additionally, serotonin was not found to have any inhibition property against ROS produced in HaCaT cells exposed to H_2O_2 at concentrations of 1 µM and 10 µM. 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 µM). 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 Vespid 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 Vespid 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

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

4. Conclusions

This study is the first to investigate the antioxidant activity of Asian hornet venom (*Vespa velutina nigithorax*). We demonstrate that this venom showed an inhibition capability against HaCaT intracellular Reactive Oxygen Species at a low concentration ($2.5x10^{-4}$ to $2.5x10^{-2}$ µg protein/mL) without cytotoxicity.

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

Since this compound has a harmful effect on the human nervous system, the direct use of serotonin as a new cosmetic ingredient should be considered with caution. Nevertheless, these results contribute to current knowledge in bioactivity research of

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

Acknowledgment

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Supporting Information

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

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

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

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

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.

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

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
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759 $p<0.05$ in ANOVA test (R-software).
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Figure

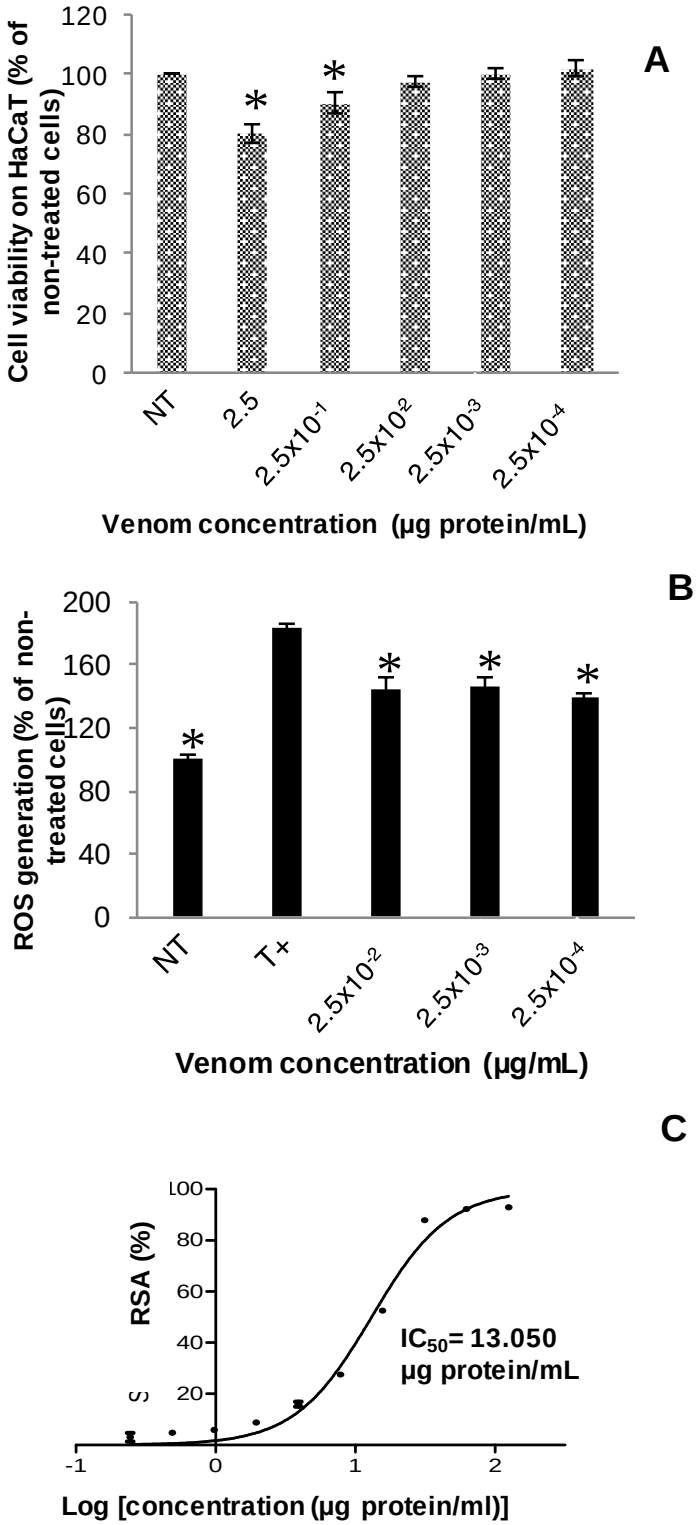

Figure 1. Antioxidant activity evaluation of *Vespa velutina* venom (Vv). (A) XTT cytotoxicity assay of *V. velutina* venom in HaCaT cells for 48h. The value is expressed as Mean ± SD, n=3. NT: Non-treated cells. *: significant discrimination versus non-treated group at p<0.05 in ANOVA test (R software).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

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

Single column fitting image

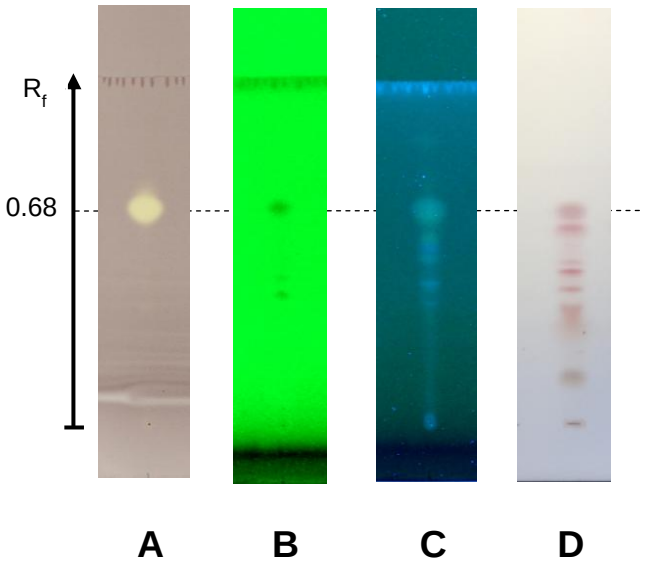

Figure 2. DPPH scavenging activity detection and separation of *Vespa velutina* venom (Vv) on TLC silica gel plate 60. The elution solvent was 1-butanol : acetic acid : water = 4:1:2 (v:v:v) in 90 min. (**A)** Detection by immersing in DPPH 0.05% solution. The yellow zone on the purple background detected 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.

1.5-column fitting image, in color

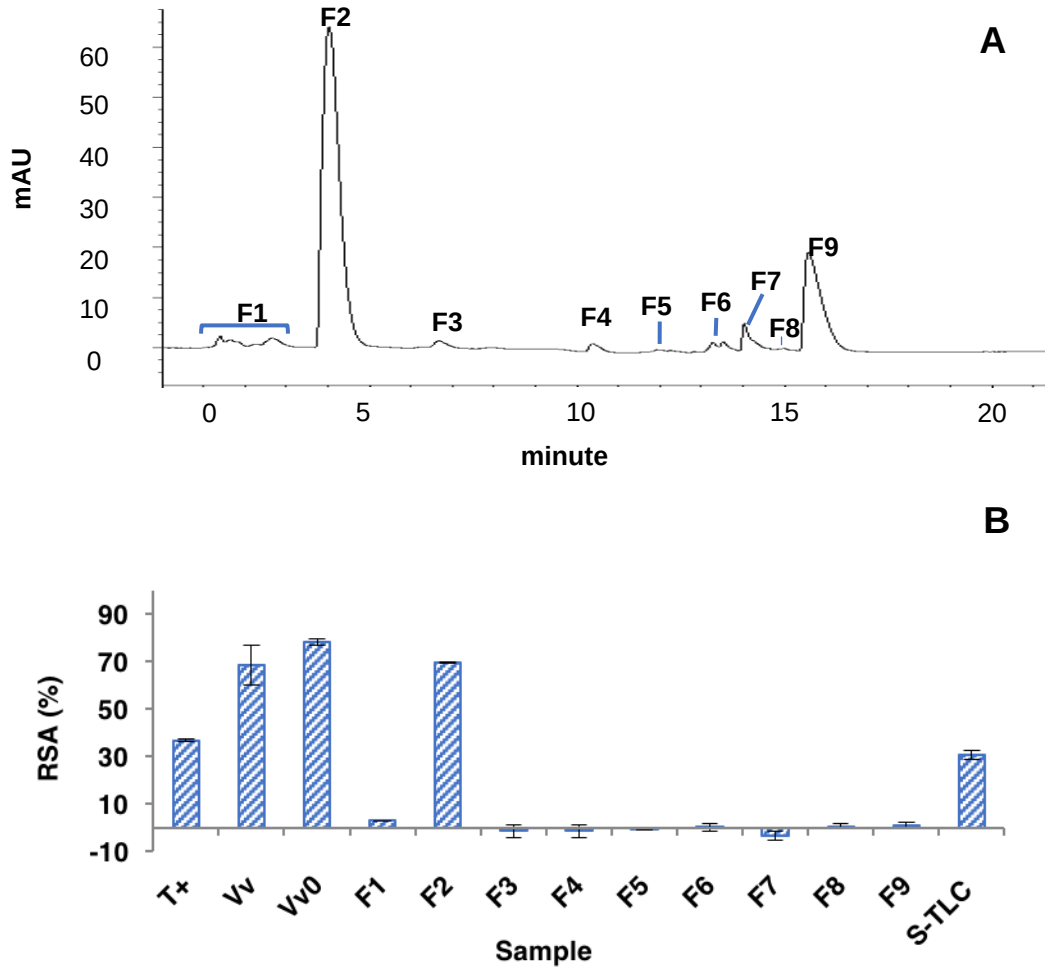

Figure 3: RP-HPLC chromatogram of precipitated venom (Vv0) and active antioxidant detection by DPPH scavenging activity assay (A) RP-HPLC-DAD profile of precipitated venom at 254 nm **(B)** DPPH scavenging activity assay of RP-HPLC-DAD fractions and TLC-active spot.

2-column fitting image

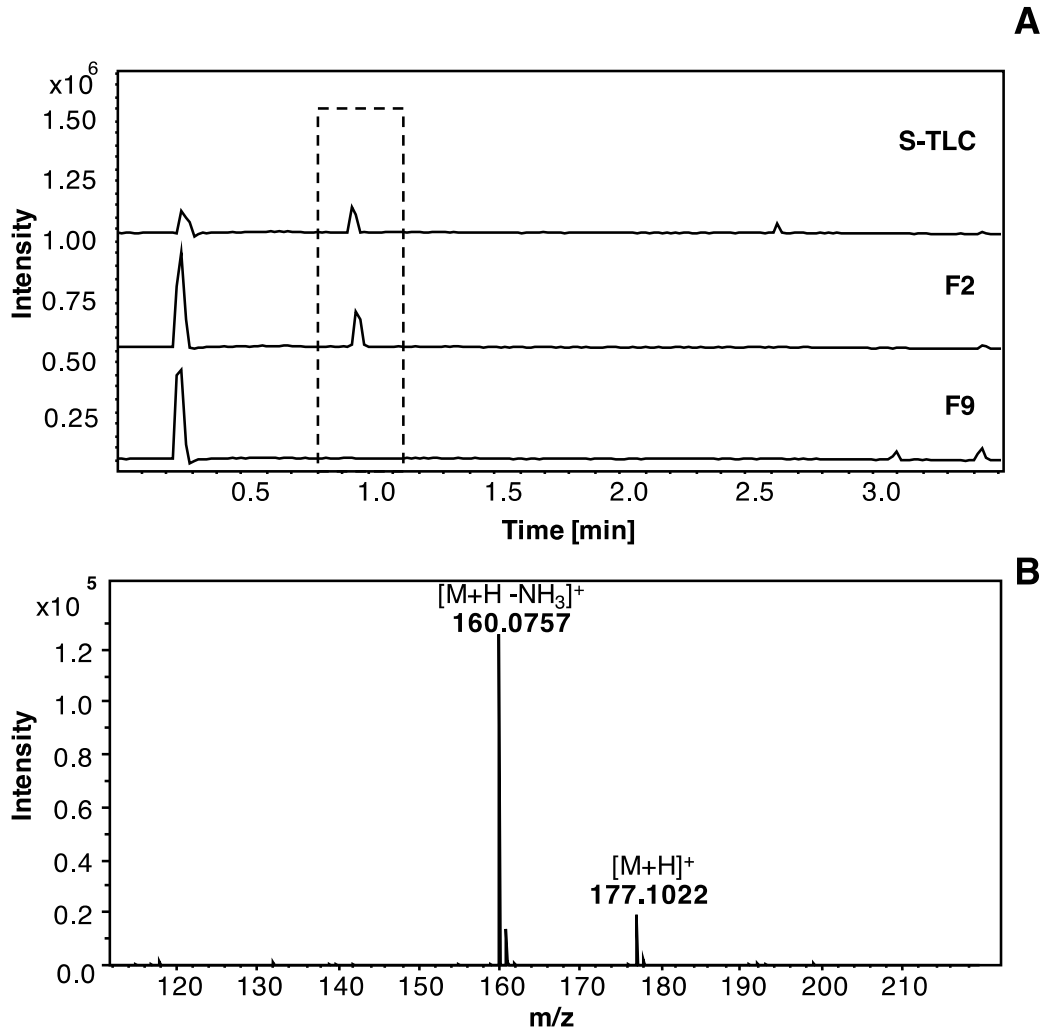

Figure 4: LC-MS profile of active fraction obtained from TLC and RP-HPLC separation. (A) Comparison of LC-MS profiles among active spots scraped from TLC glass plate, RP-HPLC active fraction and RP-HPLC non-active fraction. The dotted line indicates the coincident peak of two active fractions obtained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

2-column fitting image

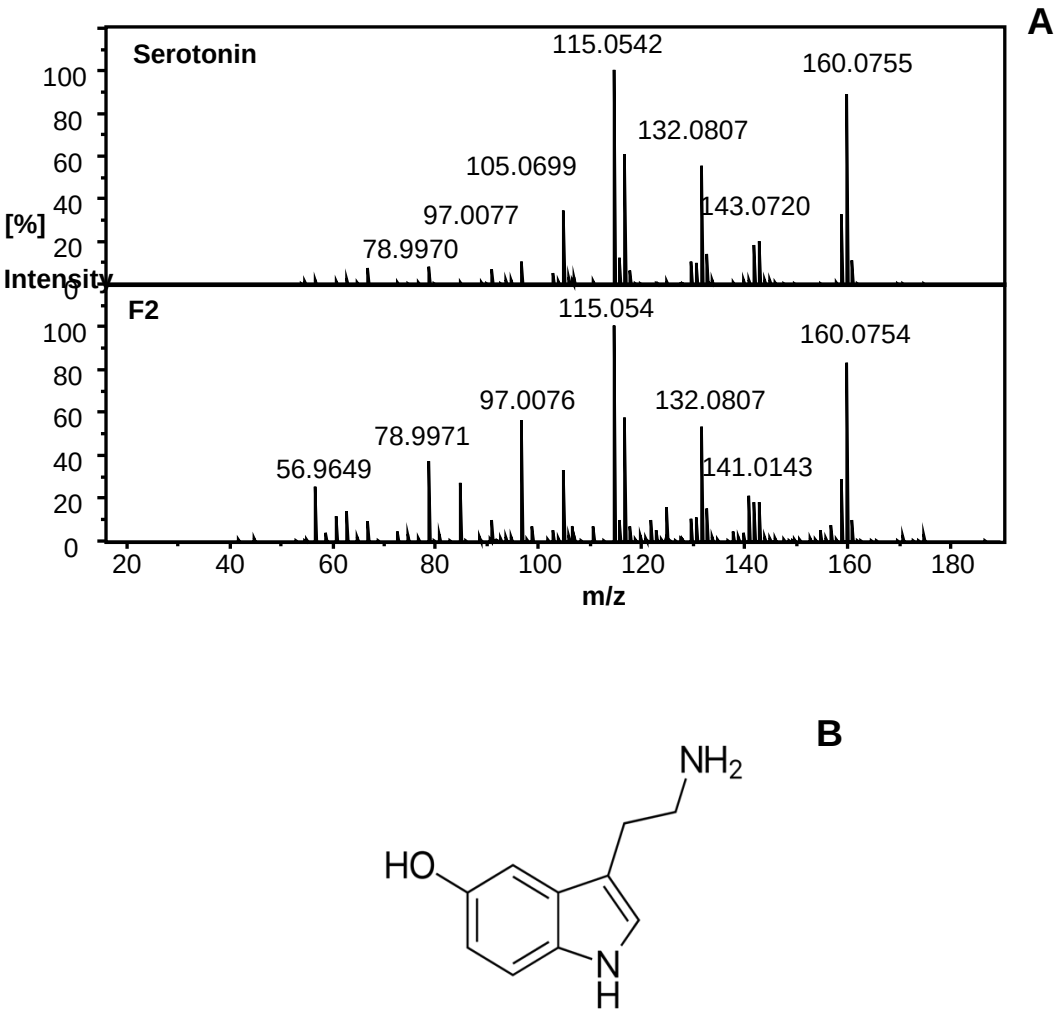

Figure 5: Coincident peak characterization. (A) MS/MS profile at the collision energy 30 eV of m/z 177.1022 of serotonin (upper spectrum) and active fraction F2 (lower spectrum). **(B)** Primary chemical structure of serotonin.

2-column fitting image

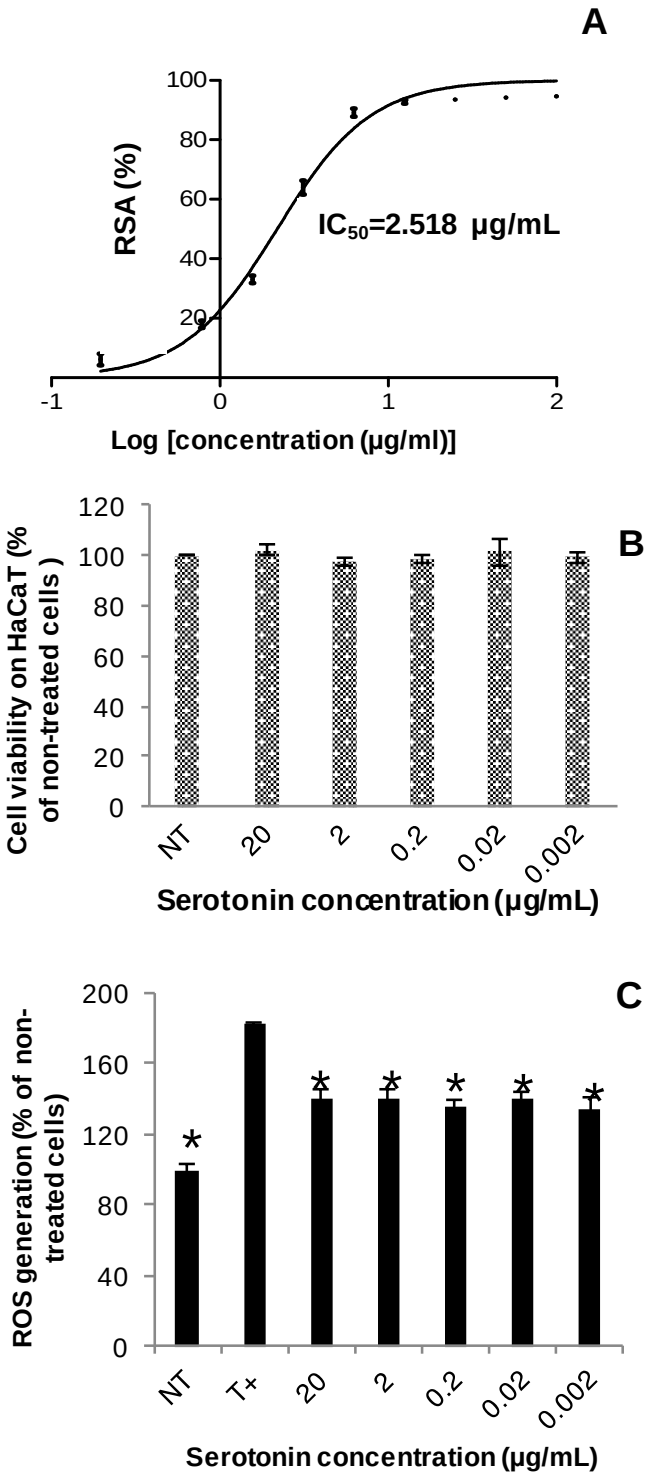

Figure 6: Antioxidant activity of serotonin. (A) Dose-response curve of serotonin in DPPH scavenging activity assay measured at 515 nm, results are expressed as Mean ± SD, n=3. RSA: Radical scavenging activity. IC_{50} was calculated by Graphpad Prism 6.0. **(B)** XTT cytotoxicity assay of serotonin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

versus non-treated group at $p<0.05$ in ANOVA test (R software). **(C)** Reactive oxygen species (ROS) reduction effect of serotonin in HaCaT cells irradiated by UVB. Results are expressed as Mean ± SD, n=3. T+: HaCaT cell irradiated by UVB without adding venom. NT: Non-treated cells *: significant discrimination versus T+ group at $p<0.05$ in ANOVA test (R-software).

Single column fitting image

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