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Short Comunication: Proposal Standardize Method for Characterization and Quantification of Bee Venom in Honeybee *Apis mellifera* by High Performance Liquid Chromatography (HPLC)

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Despite the well-known chemical characterization of bee venom that enable to identify the three major peptides such as apamin, phospholipase A2, and melittin through HPLC test its chromatographic condition use to be diverse (mobile and stationary phases). The main purpose of this manuscript is to summarize the suitable HPLC condition for characterization and quantification of bee venom in Colombia honeybee (*Apis mellifera*) based on a literature review. Bee venom is often collected using the electrostimulation method because this cutting-edge technology prevents bee deaths as the bee venom is efficiently extracted. The bee venom samples normally are stored under suitable conditions to prevent degradation such as autohydrolysis (frozen conditions). According to scientific evidence gathered from published article High-Performance Liquid Chromatographic method is usually used to assay the major venom compounds as melittin, apamin, and phospholipase A2. However, its running conditions seem to be diverse. In the present manuscript we aim to review the optimal HPLC conditions to separate the mains bee venom compounds. Therefore, according to literature review the best Chromatographic separation was performed using the following mobile phases: A - 0.1% trifluoroacetic acid (TFA) in water, B - 0.1% TFA in acetonitrile: water (80:20). The assays of the separated venom compounds were made using a UV detector at 220 nm wavelength. The stationary phase is carried out using a chromatographic column with C18 packing materials. The proposal HPLC running condition allows an adequate separation of the major HPLC peaks. As it has been previously observed in *Apis Mellifera syriaca* and *Apis mellifera meda* the bee venom samples showed up the expected chromatographic profile that allows to identify the three major protein fraction compounds: melittin, phospholipase A2 and apamine. Therefore, those chromatographic conditions could be widely used to perform routine bee venom assessments in the pharmaceutical industry. Efficacy of apitoxin extraction through the electrostimulation method has been shown to work well since it allows to identify the three major peptides of the bee venom as follows apamin, phospholipase A2, and melittin using HPLC.

Keywords – Apis mellifera, HPLC, bee venom, Apitoxin, melittin, apamin, phospholipase A2.

I. Introduction

Apis mellifera to protect themselves against a wide range of potential biological predators [1-10]. Apitoxin contains a broad battery of biological molecules such as carbohydrates, organic acid, nucleotides, peptides, and other molecules of low weight still unidentified [2,4,10]. However, according to several studies based on high performance liquid chromatography (HPLC), there are three major expected peptides in the chromatography profile of apitoxin known as apamine, phospholipase A2, and melittin [9,11,12]. In *Apis mellifera* L, it has been proven that Apamine and phospolipase A2 represented the 10-15% of the whole content of the dry weight [8,10,11]. Otherwise, melittin is the main toxic peptide of honey bee venom with various biological and pharmacological activities and represents around 60-70% of the whole dry weight of the apitoxin molecule [8,10,11]. Chromatographic tests have been widely used to confirm apitoxin purity through a quantitative identification of these three peptides [10,12].
It is widely accepted that the environment condition and diet play a key role on the chemical composition of domestic bee derivatives such as apitoxine [13,14-19]. Several ecological factors such as temperature and flowering stage have a strong impact on the weight and protein profile of apitoxin collected from a *Corymbia calophylla* ecosystem, Southwestern Australia, suggesting that research using a combination of proteomics, and bio-ecological approaches is recommended to further understand causes of bee venom variation in order to standardize and improve the harvest practice and product quality attributes [13,14-19].

In the last decade, a burst of scientific evidence has shown that apitoxin has a wide range of biological attributes that allows to mitigate a broad spectrum of diseases such as diabetes, endocrine issues, obesity, inflammation, arthritis, neurodegenerative diseases, sclerosis, kidney issues, amyotrophic lateral sclerosis, thyroid gland disease, wounds, immune problems, and cancer [2,3,5,16-20]. Indeed, bee venom has also shown successful cosmetic application and currently apitoxin is already included in several cosmetic formulations intended as anti-age creams (Peña-Romero Romel, data not publish). Furthermore, antimicrobial activity of apitoxin has been proven against several oral pathogens such as *Salmonella enterica*, *Listeria monocytogenes*, *Streptococcus salivarius*, *S. sobrinus*, *S. mutans*, *S. mitis*, *S. sanguinis*, *Lactobacillus casei*, and *Enterococcus faecalis*, being melittin and phospholipase A2 the main active principle for antimicrobial activity and oral biofilm eradication, showing its potential application against oral pathogens [16,20].

These biological properties become bee venom as an active principle attractive to use apitoxin as a raw material, driving to open new avenues to designs of new pharmaceutical products that can provide an alleviation for all these high-impacting worldwide diseases [16,20].

Nonetheless in Colombia the beekeeping has been exclusively focused on the harvesting almost exclusively of honey, providing a scarce interest to other bee derivatives such as royal jelly, propoleo, and apitoxin, which are marginally produced in Colombia apiculture. Therefore, in Colombia there is not either a developed or standardized process that includes both a successful extraction and laboratory characterization of bee venom, that allow to carry out a quality assessment which involves organoleptic, microbiological and physicochemical assessment in the Colombian apitoxin collected, in order to safely include bee venom as an integral part of different pharmaceutical and cosmetic formulations. Thus, organoleptic assays should include smell, color, and appearance assessment. Meanwhile, microbiological rehearsal needs to test a total aerobic mesophyll count. Physicochemical tests will be carried out through HPLC assay.

Therefore, it has become a priority in the pharmaceutical industry the development of a collection method based on electric pulse followed by a validated method for quantitative determination of domestic local apitoxin. In this way, electrostimulation has been widely used to collect honeybee venom in large quantities. This procedure

provides an electric shock directly to the hive that makes it possible to collect pure venom from several thousand honey bees (*Apis mellifera*), utilizing a glass surface as a trap to collect apitoxin [17].

Regarding that the extraction and characterization of apitoxin from *Apis mellifera* is almost completely unexplored in Colombia country in the pharmaceutical industry, the main aim of this minireview is to lay down a methodology based on electrostimulation that permits an optimal collections of bee venom without causing any damage to bee and keeping safe the apitoxin original biological properties. Once the collection process has taken place a quantitative detection of the major constituents of honeybee venom is proposed by specific HLPC conditions based on literature overview.

II. Literature Review

2.1 Short comunication

Healthy hives of domestic local *Apis mellifera* need to be selected. The apitoxin collection need to be made following the standard electroshock method [17]. An electric pulse will trigger bees into a stress stage that led to localized massive bee attacks directly over the glass trap surface, allowing to make a pure bee venom collection without causing any fatal injuries to the bee [17]. The collection apparatus is placed over the main entrance of the hive and other entrances are obstructed in order to guarantee a massive attack on the glass surface trap placed at the entrance of the hive [17]. Overall, 30 to 40 volts may be used for apitoxin collection. Extraction may be made for 15–20 min on each colony and it could be repeated twice every 2 weeks. Once the apitoxin collection is completed the voltage will be turned off. Then the hive will be allowed to calm down, and the collection apparatus will be removed [17].

Secreted venom from bee sting dried rapidly when exposed to the air. Apitoxin sting is odorless, white, crystalline powder. Dried apitoxin will be scraped off with a sharp scalpel and placed in dark bottles before being transferred to the laboratory for further analysis.

Suitable stores condition will be used to keep bee venom original attribute unaffected. So, the apitoxin collection will be stored at a temperature of 20 C until further analysis. It is worth to take into account that apitoxin collected by this technique contains pollen, vegetative tissue and other kinds of organic detritus from the Hymenoptera.

The melittin content of the apitoxin samples will be determined according to the method developed by Lamas *et al* 2020 and Helena Rybak-Chmielewska, 2004 [11,12,16]. Thus, the freeze-dried bee venom samples will be mixed with 5 mL of ultrapure water thoroughly mixed by a vortex shaker for 2 min, and sonicated for 5 min, and the liquid was filtered through a 0.45 m polytetrafluoroethylene syringe filter and collected in an amber glass vial (Lamas et al 2020). The resultant solutions will be centrifuged at 10,000 rpm at 4 °C for 10 min. The supernatants will be diluted with ultrapure water, and it will be used for analysis.

III. Proposal HPLC testing conditions based on scientific literature

Recent efforts in apitoxin research have focused on its therapeutic and cosmetic applications, becoming worthy of knowledge about Colombian local bee venom. Several previous studies have been carried out in *Apis mellifera* apitoxin from different parts of Europe, Australia, and middle east region [11,12,16]. As it has been previously shown the efficacy of electrostimulation to perform an optimal recovery of bee venom without causing either physiological or physical harmless effects to the bees.

For the mobile phase, polar solutions such as Acetonitrile (ACN), purified water (PW), formic acid (FA), and trifluoroacetic acid (TFA) were identified to have an excellent performance to separate the different main bee venom compounds. Therefore, according to literature review the best Chromatographic separation was performed using the following mobile phases: A - 0.1% trifluoroacetic acid (TFA) in water, B - 0.1% TFA in acetonitrile: water (80:20). The assays of the separated venom compounds were made using a UV detector at 220 nm wavelength. The stationary phase will be carried out using a Reversed-phase high-performance liquid chromatography (C18) packing materials. Those chromatographic running conditions could be used as a routine

characterization in the pharmaceutical industry in order to used Apitoxin as a raw material for the manufacturing of a wide range of either personal care products or pharmaceuticals products.

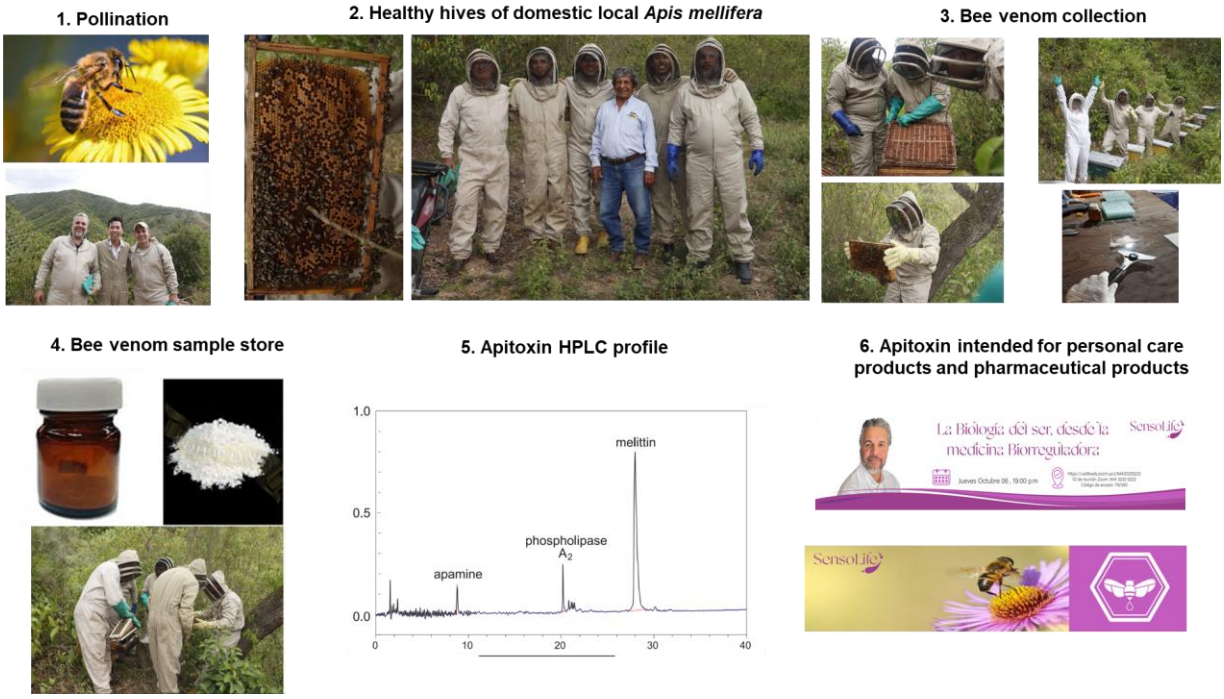

Diagram. (1) Pollination. (2) Harvesting healthy hives. (3) Bee venom collection by electrostimulation. (4) Bee venom sample store. (5) Apitoxin HPLC profile. (6) Apitoxin as a raw material for pharmaceutical and personal care products.

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