

## ANTIVIRAL EFFECT OF THE EGYPTIAN BEE VENOM (BV) AND ITS FRACTION (PLA<sub>2</sub>) ON INFLUENZA VIRUS: IN-VITRO STUDY

By

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### Abstract

Influenza is a viral infection that attacks the respiratory system. For most people, the flu disappears on its own but sometimes the flu and its complications can be deadly. This study established a new trend to treat Influenza virus by using bee venom and its fraction compared with the traditional flu drug. It was proved that bee venom (BV) has an antiviral effect. The bee venom (BV), bee venom phospholipase A<sub>2</sub> (BV-PLA<sub>2</sub>) and ribovinal as a control drug were used. The cytotoxicity of the selected compounds was determined using MTT assay. Also, the cytopathic effect was measured after 24, 48 & 72 hours incubations and determination of gene expression of INF-gamma using SYBR Green. The IC<sub>50</sub>s of BV, BV-PLA<sub>2</sub> and ribovinal were 87.8, 17.3 & 137.5 µg/ml after 24 hours incubation, 36.9, 9.6 & 24.6 µg/ml after 48 hours incubation, and 9.7, 7.45 & 31.6 µg/ml after 72 hours incubation, respectively. In addition, the cytopathic effect of both BV and ribovinal was directly proportional to its concentration and incubation time. Whereas, BV-PLA<sub>2</sub> showed an increase in cytopathic effect after 48 hours in spite of its concentration in case of direct antiviral effect. However, the indirect antiviral effect for both BV and PLA<sub>2</sub> showed the same cytopathic effect in all concentrations and incubation times. So, the gene expression of INF-γ was significant increased after 48 hours incubation of either BV or ribovinal in direct and indirect effects. While the INF-γ expression was (14.8 & 16.4) direct and in indirect antiviral effects of BV-PLA<sub>2</sub>, respectively. These findings revealed the antiviral effect of both bee venom and BV-PLA<sub>2</sub> against influenza virus.

### Introduction

Influenza virus by virus classification is an enveloped the RNA virus composed of five strains of the family of intact mucosal viruses (Chertow and Kindrachuk, 2020). There are two fundamental sorts of flu (influenza) infection; Types A & B. The flu A & B infections that routinely spread in individuals (human flu infections) are in charge of the regular influenza scourges annually. The influenza (flu) is different from a cold usually comes on suddenly and can cause mild to severe illness, and at times can lead to death (CDC, 2019). People who have flu often feel some or all of these symptoms: fever or feeling feverish/chills, cough, sore throat, runny or stuffy nose, muscle or body aches, headaches, fatigue (tiredness), some people may

have vomiting and diarrhea, though this is more common in children than adults. But, it's important to note that not everyone with flu will have a fever (Sehatazadeh, 2012).

However, the problem of influenza is mainly related to its potential complications, which include: pneumonia, bronchitis, ear infections and CVS & CNS complications. Specific people are more likely to have influenza complications especially immunocompromised and its incubation period ranged from one to four days (Blut, 2009). During this period, the virus is present and grows in the body before the onset of symptoms. Also, you will not have any symptoms of the flu, but you may have recently been infected by others. A person infected with the virus can infect others before the onset of symptoms one to

two days and continued to be able to transmit the infection until one week from the onset of symptoms and may be able to transmit infection up to ten days (Carman *et al.*, 2019). The accessible investigations on the antiviral resistance of flowing influenza strains are rare, so this work embraced an examination of the sequences announced in public gene banks to play out a deliberate investigation of the antiviral resistance markers on both M2 & NA. Taking all facts together (sequences from 2000 to 2017 were considered), 284 M2 successions and 423 NA arrangements were recovered from three hereditary databases (Toledo-Rueda *et al.*, 2018). Bee venom (BV) contains in any event 18 pharmacologically dynamic parts including melittin (MLT), phospholipase A2 (PLA2), and apamin and so forth. BV is safe for human medicines portion conditionally and demonstrated to have distinctive mending properties including antibacterial, anti-parasitic and antiviral properties. In any case, the antiviral properties of BV have not all around examined (Toledo-Rueda, 2018). Consequently, the potential antiviral properties of BV and its part were involved in wide variety of infections. The use of bee venom for such purposes was not been fully established, as it was a must. The effect of bee venom on the allergies removal from hypersensitive individuals is still under investigation. Pure bee venom is not available in quantities; these extracts contain many proteins that have no chemical relationship with proteins found in the pure bee venom (Blank *et al.*, 2017). But, the substances injected by the sting machine are also believed to contain specialized bee proteins (allergen) that cause sensitivity. The allergists preferred to use the entire bee body extract due to its low toxin content.

The present study aimed to through more light on the medical value of antiviral activity of Egyptian bee (*Apis mellifica*) venom.

#### **Materials and Methods**

Culture medium was removed to a centrifuge tube then the cell layer was rinsed

with 0.25% (w/v) trypsin 0.52 mM EDTA solution in order to remove all traces of serum contains trypsin inhibitor by the following step: 2.0 to 3.0ml of Trypsin EDTA solution were added to flask and observe cells under an inverted microscope until cell layer is dispersed (within 5 to 15 minutes) then 6.0 to 8.0mL of complete growth medium were added by pipetting after these steps the cell suspension was put into the centrifuge tube with the previous medium cells then the centrifuged at approximately 125xg for 5 to 10 minutes. The supernatant was discarded in fresh growth medium the cell was suspended, appropriate aliquots of cell suspension were added to new cultures vessels. The cultures were incubated at 37°C for 24hrs. Finally, after the cells treatment with serial concentrations of the compound to be stored using incubation for 48hrs at 37°C, and then the plates were examined under an inverted microscope and proceed for the MTT assay The cultures were removed from the incubator into laminar flow hood, each vial of MTT [M5655] was reconstituted to be used with 3ml of medium (without phenol red & serum) the reconstituted MTT was added in an amount equal to 10% of the culture medium volume the culture were incubated for 2-4hrs and then the cultures were removed. The resulted formazan crystal was removed by adding suitable amount of MTT solution (M-8910) equal to the original volume of culture medium. The gyratory shaker was used in mixing in order to enhance dissolution of the MTT formazan crystals. An appropriate type of plate reader was used or the contents of individual wells was transferred to appropriate size covets to be ready for measurement using the spectrophotometer absorbance at a wavelength of 690nm the result was subtracted from 570nm measurement. Determination of gene expression of all materials was done by using RT-PCR.

#### **Results**

The cytotoxicity (IC<sub>50</sub>) of all materials increased as the processing time increased. The cytotoxicity of bee venom (BV) was

87.8, 36.9 & 9.7 $\mu$ g/ml after 24 hours, 48 hours & 72 hours incubation, respectively. But, IC<sub>50</sub> of BV-PLA2 was 17.3 after 24 hours, 9.6, after 48 hours, & 7.45 after 72 hours incubation. Finally, in the case of ribovinal it was 137.5 after 24 hours, 24.6 after 48 hours, and 31.6 after 72 hours. The cytopathic effect determined in vero cells under the effect of BV, BV-PLA2 and ribovinal as compared with control and groups infected by influenza virus and not treated. Both BV and BV-PLA2 showed low cytopathic effects compared with the effect of ribovinal as a commercial drug. Gene expression represented gene expressions of interferon- gamma (INF $\gamma$ ) under the effect of BV, BV-PLA2 and ribovinal using RT-PCR. BV & ribovinal showed highly significant increase in INF $\gamma$  expression after 48h incubation in direct (35.6) and indirect (46.0) effects of BV, besides, 38.9, & 49.3 under the effect of ribovinal. But, BV-PLA2 showed an insignificant increase in both cases (14.8 & 16.4) direct and indirect effects, respectively. Details were given in figures (1, 2 & 3).

### Discussion

Egypt, is a Mediterranean country, locates in the Northeast region of Africa, spanning about 1 million square kilometers and considered as one of the highest populous country in the Middle East and North Africa (100 million inhabitants) mostly living near the Nile banks. For about ten years, Egypt became an endemic region for pathogenic influenza virus (El-Shesheny *et al*, 2020). At least, the Crimean-Congo hemorrhagic fever (El Bahnasawy *et al*, 2012), the West Nile virus (El Bahnasawy *et al*, 2013a), Rift Valley fever (El Bahnasawy *et al*, 2013b), dengue fever (Morsy, 2018) Antiviral drugs are a separate set of drugs prescribed for the management of a number of diseases caused by viruses (Vardanyan and Hruby, 2006). Do not assume that all antiviral drugs can help to prevent the development of common cold. The general idea behind the design of modern antiviral drugs was to identify viral

proteins or parts of these proteins that can be disabled. These "targets" in general should be different from any proteins or parts of human proteins, to reduce side effects. However, these proteins must be many strains of the virus, or even between different types of viruses belonging to one family, so that one drug has a widespread impact (Andersen *et al*, 2020). For example, a researcher may target an important enzyme, not the patient, to be involved in the strains of the virus, and then consider what can be done to stop it (Mifsud *et al*, 2019). Despite the importance of this set of drugs, it is considered as a child taking its first steps in terms of development and industry, most antiviral medications don't deliver a cure, yet rather permit control of the disease in addition to it has effected very few viruses, However, the limitations of antiviral treatment, including the high expenses in manufacturing these drugs, make the requirement for the counter-active action even increasingly pressing (De Clercq, 2004). This agreed with the present results of studying bee venom (BV) in vitro. But, the present result disagreed with Taky *et al*. (2017) who found that the decided IC<sub>50</sub> characteristics were prepared inside the request of EBV > PLA2 and the IC<sub>50</sub> became 13.7 & 25gm/ml. The tried items demonstrated the antiviral capability against DNA and RNA contamination models; HSV-1 & RVFV with an improvement 10% & 26.3% dilemma for PLA2 and EBV individually if there needed to stand up a prevalence of testing the direct impact of HSV-1.

In the present study, the inhibitory effect against to RVFV as a RNA contamination model of restraint changed into 7.7% & 27% for EBV & PLA2 respectively. Differences between both results might be due to difference in the incubation period. In the Rift Valley infection, bee venom and phospholipase gave low critical diminished ( $P \geq 0.05$ ) in both immediate and circuitous strategy. This outcome uncovered that the two have the direct activity on RVF infection agreed with Hassan *et al*. (2015). The past outcome

might be expected the immediate antiviral of melittin and phospholipase A2 on infection as observed (Kainulainen *et al*, 2016).

The honey bee venom acts as an antiviral restorative treatment either by fundamental or neighborhood application that it has enzymes and peptides influencing the infection. The antiviral impact of PLA2 depended basically on their capacity to hydrolyze phospholipids in host cell films. This study showed the same significant result about the antiviral effect of bee venom as previously established that discharged phospholipases A2 (sPLA2) from honey bee and snake venoms have an effective enemy of human immunodeficiency contamination (HIV) motion (Fenard *et al*, 2001). PLA2s square HIV-1 passage into host cells through a tool linked to sPLA2 authoritative to cells.

In present study, 12 manufactured peptides were given from honey bee venom sPLA2 (PLA2) had been attempted for restraint of HIV-1 infection. This agreed with Choi *et al*. (2005). The p3bv peptide (amino acids 21 to 35 of bvPLA2) turned into found to restrain replication of T-lymphotropic (T-tropic) HIV-1 separates (ID50 = 2 $\mu$ M) yet became without impact on monocytotropic (M-tropic) HIV-1 secludes. The P3BV was located ready for heading off the cellular-cellular mixture procedure interceded via T-tropic HIV-1 envelope. At long ultimate, p3bv can preclude the legitimate of the radiolabeled stromal cell-decided element (SDF)-1 $\alpha$ , the ordinary ligand of CXCR4, and the authoritative of 12G5, an enemy of CXCR4 monoclonal counter acting agent. Taken together, these consequences display that p3bv obstructs the replication of T-tropic HIV-1 strain via interfacing with CXCR4. Its gadget of a hobby anyway appears specific from that of BV PLA2 in mild of the truth that the closing represses replication of each T-tropic & M-tropic separates and did not contend with SDF-1 $\alpha$  & 12G5 authoritative to CXCR4 (Fenard *et al*, 2001).

Cytopathic effect: virus was cytotoxic and

Cytopathic effect: virus was cytotoxic and attacked tissues demonstrate morphological changes frequently termed cytopathic results which included mobile rounding along with change with redistribution of cell membrane. Genital herpes as RNA virus also brought by chemical changes including inhibition of interpretation along with transcribing (Ruiz-Sáenz *et al*, 2009). The antiviral effect of PLA2 depends fundamentally on ability to hydrolyze phospholipids in host cell films. PLA2 controls HIV contamination (as RNA infection) neither from virucidal effect nor cytotoxic effect, occurred by neutralizing activity of intracellular landing of viral capsid, and interfere viral entry into host cells (Fenard *et al*, 1999). Akdis *et al*. (1998) found that honey bee venom phospholipase A2 (PLA) instigated high IL-4, IL-5 & IL-13 generation infringe blood mononuclear cell societies. These highlights may empower new applications for more secure immunotherapy. This agreed with Ramadan *et al*. (2009) who assessed the virucidal activity of honey bee venom in opposition to RNA model virus of West Nile virus (WNV) revealed that there was a significant depletion rate of WNV infectivity titer post heat remedy brooding at 37°C for three, 6, 24 & 48hrs submit treatment with venom recording 5.1 log (10)/0.1 ml, 4.5 log (10)/0.1 ml, 2.1 log (10)/0.1 ml and 0.55 log (10)/0.1 ml for in part. The antiviral movement of honey bee venom of HSV-1 as a DNA model infection confirmed that there has been a non-noteworthy abatement in the infectivity titer that publishes warm treatment. In the same time, the antiviral motion of honey bee venom towards Adeno infection serotype 7 exposed that there was a non-essential abatement in Adeno 7 titer 3, 6 & 24 hours submit hatching at 37°C. Likewise, evaluation of antiviral movement of honey bee venom in opposition to RNA version contamination West There was a non-noteworthy lessening in West Nile Virus titer after brooding time of 3, 6 and 24 hours post remedy at 37°C. The antiviral action of honey bee venom

became likewise contrasted with the same antiviral drug; Interferon-2a (IFN-2a). HSV-1 became irrelevantly faded account 5.5 log (10)/0.1ml inside the honey bee venom treated cells contrasted with large discount whilst treated with (IFN-2a) recording 3.2 log (10)/0.1 ml. In the meantime, the antiviral action in opposition to adenovirus-7 recorded a huge reduction in contamination infectivity titer recording 3.4 log (10)/0.1 ml submit honey bee venom contrasted and 3.25 log (10)/0.1ml post IFN treatment which implies that the *in vitro* investigations of the effect of standard IFN applied become equal to honey bee venom on the Adeno-7 infection. The RNA version infection; WNV become all around affected by honey bee venom than IFN where infection infectivity titer recorded 1 log (10)/0.1ml after honey bee venom treatment contrasted and 2.5 log (10)/0.1 ml contamination infectivity titer post IFN treatment. The different results obtained may be because of different viruses used in the two studies. This agreed with Uddin *et al.* (2016) who reported that co-incubation of non-cytotoxic quantities of BV and MLT, the principle element of BV, drastically inhibited the replication of enveloped viruses consisting of influenza an endemic (PR8), vesicular stomatitis virus (VSV), respiration syncytial virus (RSV), and Herpes simplex virus (HSV). Besides, the BV & MLT inhibited the replication of non-enveloped viruses consisted of Enterovirus-seventy-one (EV-71) and Coxsackie virus (H3). Such antiviral properties were mainly defined with the aid of virucidal mechanism. Besides, MLT covered mice that were challenged with lethal doses of pathogenic influenza A H1N1 viruses. Therefore, those effects offer the proof that BV and MLT can be a capability source as a promising antiviral agent, mainly to broaden as the vast spectrum antiviral agent. This was approved that BV & MLT derived from BV exhibited effective antiviral hobby in opposition to numerous enveloped and non-enveloped viruses *in vitro* and Influenza virus inside

the *in vivo* mouse version. Also, its antiviral mechanism was confirmed to involve direct interaction with the viral surface.

Apart from BV virucidal interest, BV can stimulate kind I IFN, which sooner or later ought to stimulate the antiviral country within the host cellular and also inhibit the viral replication. Taken collectively, the outcomes advise that BV or MLT has capability to be prophylactic or therapeutic agents for the infectious viral diseases.

Generally, the bee venom is a weapon that bees use to protect themselves against their enemies mainly man, and since the ancient times dates back to Hippocrates, and references to the treatment was found in ancient Egyptian and Greek Medical Writings as well as the skin aging application of honeybee venom (BV) was practiced and persisted until nowadays ( Han *et al.*, 2013).

Lui *et al.*, (1995) reported that bee venom immunotherapy has dual benefits: patients were protected from subsequent sting anaphylaxis and there was reduced psychological morbidity. But, to be effective, venom immunotherapy requires a prolonged period of carefully treatment and each venom injection caused local and systemic side effects. Genetic factors were present in patients who develop direct hypersensitivity to stings.

In humans, however, it has been used as an anti-inflammatory medicine to relieve pain and treat chronic inflammatory diseases such as rheumatoid arthritis and multiple sclerosis (Senel *et al.*, 2014). The use of bee venom as a therapeutic agent for the relief of joint pains Also, as apitherapy, the technique was widely used in Eastern Europe, Asia, and South America. The beneficial effects of bee stings can be attributed to mellitinin, an anti-inflammatory agent, hundred times active than cortisone (Alqutub *et al.*, 2011). While bee venom itself causes nociceptive and neurotoxic effects, several studies proved that its venom have a radio protective (Gajski and Garaj-Vrhovac, 2009), anti-mutagenic (Varanda *et al.*, 1999), anti-inflammatory (Choi *et al.*, 2012; Kim *et al.*, 2013; Park

et al, 2014), anti-nociceptive (Baek et al, 2006; Lim et al, 2013; Yoon et al, 2015) and anticancer (Orsolich et al, 2003a,b; Moon et al, 2006; Huh et al, 2009) activities. Bee venom was given in the form of a direct bee sting or else by injection of a venom extract. The treatment is usually given twice a week. Some reports have shown beneficial effects of bee venom in post herpetic neuralgia (Jani et al, 2007), swine flu (Singla and Bhat, 2010), fibromyalgia, multiple sclerosis and others (Janik et al, 2007). Besides, Leandro et al. (2015) in Brazil found that BV was successfully used as antibacterial, antifungal and antiviral agent. Tawfik (2018) in Egypt reported that bee venom in hydatidosis not only affected the cellular structure of proto-scolices but, also triggered apoptosis. Also, An et al. (2018) in Korea suggested that bee venom and melittin would be suitable for epicutaneous application, as topical administration was often appropriate for treatment of atopic dermatitis. Wehbe et al. (2019) in Lebanon reported that the several active molecules in bee venom such as peptides and enzymes that have advantageous potential in central nervous system diseases, such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis

Moreover, the non-alcoholic fatty liver diseases (NAFLD) encompassed a spectrum of liver abnormalities, ranged from simple steatosis to steatohepatitis (non-alcoholic steatohepatitis; NASH) and fibrosis that may progress to cirrhosis and possibly hepatocarcinoma. NAFLD was associated with insulin resistance, obesity, diabetes mellitus, hyperlipidemia, visceral adiposity and other cardio metabolic alterations (Neuschwander-Tetri and Caldwell, 2003). Hanafi et al. (2018) represented an advance in biomedical science because it showed the antioxidant potential of BV treatment and its normalization effect on adipocytokines that made it a tempting therapeutic agent for NAFLD. But, unfortunately, the bee stings caused severe reactions and have caused many victims in the last years. Allergic reactions can be trig-

gered by a single sting and the greater the number of stings, the worse the prognosis. The poisoning effects can be systemic and can eventually cause death. The poison components were melitin, apamin, peptide 401, phospholipase A2, hyaluronidase, histamine, dopamine, and norepinephrine, with melitin being the main lethal component. Acute kidney injury (AKI) was observed in the patients suffered from the bee stings that was due to multiple factors, such as the intravascular hemolysis, rhabdomyolysis, hypotension and direct toxicity of venom components to the renal tubules. The arterial hypotension played the important role in this type of AKI, and led to the ischemic renal lesion (Silva et al, 2017). They concluded that the severity of AKI was associated with the stings number, since creatinine levels were higher, in most cases, when there were more than 1,000 stings. But, Cherniack and Govorushko (2018) in USA bee venom consists of multiple anti-inflammatory compounds such as melittin, adolapin, apamin, and phospholipase A2 could be anti-inflammatory in low concentrations and pro-inflammatory in others. The venom contained pro-inflammatory substances, melittin, mast cell degranulation on peptide 401, and histamine. They added that in small studies, bee venom acupuncture was used in man to successfully treat a number of muscuro-skeletal diseases such as lumbar disc disease, osteoarthritis of knee, rheumatoid arthritis, adhesive capsulitis, and lateral epicondylitis. Bee venom acupuncture can also alleviate neurological diseases, as peripheral neuropathies, stroke, Parkinson's disease, and such treatment was even piloted in one series to alleviate depression. They concluded that bee venom caused anaphylaxis, and even deaths in patients who successfully received the therapy prior to the adverse event. While the incidence of adverse events was unknown, the number of published reports of toxicity is small. Refining the bee venom to remove harmful substances may potentially limit its toxicity.

## Conclusion

The outcome data showed that bee venom (BV) and ribovinal have antiviral effects mainly by direct effect, while BV-pLA2 has mainly indirect antiviral effect against the influenza virus. Bee venom and ribovinal have antiviral effect mainly direct while pLA2 have mainly indirect antiviral effect against influenza virus.

Bee venom therapy is the specialized form of apitherapy, and required the expertise of a well-trained physician. Usually, a course of treatment starts with testing the patient for the bee venom allergy, which is known to occur in 1% of the general population

## Recommendations

The utilizing honey bee venom (BV) and BV-PLA2 as the infusion or the topical application as elective drugs in veterinary and human practice as it is sheltered and viable medicine against infectious disease of RNA.

In this way, the antiviral concentrating action of past tried material in creature model (*in vivo*) was prescribed.

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#### Explanation of figures

Fig. 1: IC<sub>50</sub> of BV, PLA2 and Ribovinal with time.

Fig. 2: Cytopathic effect of BV, PLA2 and Ribovinal with time.

Fig. 3: Direct and indirect expression of BV, BV-PLA and ribovinal

