Egypt. J. Chem. Environ. Health, 1 (1):274-290 (2015)

Experimental studies on whole bee venom inactivated Rift Valley fever vaccine candidate in vitro

[1]Samia Ahmed Kamal, [2]Haggag E. Bedair and Ragaa ASR Faisal

[1,3]Animal Health Research Institute (AHRI), Egypt.&[2] Plant Protection Institute, (ARC)

Abstract

In regard to the safety concerns about RVF vaccines, this work aim to use the safest natural product to inactivate the Rift Valley fever virus (RVFV). Also, our study on the effect of honeybee (Apis mellifera L) venom on some types of cell lines cultures showed it cause apoptosis at lower concentrations and digestion of the cells at relatively higher concentration. In this study, honey bee venom is used as inactivant of Rift Valley fever virus (RVFV). Beside in vitro study on this inactivated RVFV it was injected in rabbits and mice to investigate its behavior in vivo. The results showed that rabbits and mice immunized with this RVFV inactivated vaccine exhibited satisfied antibodies response. Pathological investigation of the immunized mice showed that bee venom inactivated RVF vaccine didn't cause any side effects in liver, kidneys, brain and spleen. Therefore, honey bee (Apis mellifera) venom as a natural product can be used as RVFV inactivant and recommended for animal's vaccines.

Introduction

Rift Valley fever virus (RVFV, genus *Phlebovirus* and family *Bunyaviridae*), was firstly isolated by **Daubney et al., 1930 (Murphy et al, 1995)**. RVFV has the ability to persist in nature through mosquito's eggs **(Linthicum et al, 1985)** . It is enveloped virus, with single strand RNA genome which consisting of three segments; small (S), medium (M) and large (L). The S segment is of ambisense polarity and encodes for two proteins; nucleocapsid protein (N) that coats the viral genome in the virion and a nonstructural protein (NSs). The NSs protein is a filamentous nuclear protein, expressed by a virus that replicates and assembles in the cytoplasm of infected cells. The NSs protein is a virulence factor and known to be involved in altering the host immune system. The M segment of the genome encodes for two viral glycoproteins (Gn and Gc) that are on the surface of the virion, as well as a nonstructural protein (NSm). However, L segment encodes the viral RNA-dependent RNA polymerase **(Yadani et al, 1999; Billecocq et al, 2004; Bouloy et al, 2001, Vialat et al, 2000)**. RVFV causes high morbidity and mortality in humans and livestock **(McElroy et al, 2009)**.

Honeybee venom (BV) from the sting of Honeybee (*Apis mellifera* L) is traditionally used in China, Korea, and Japan for arthritis, tendinitis, bursitis and other chronic conditions. It is reported to have pro-inflammatory **(Sumikura et al, 2003)** and anti-inflammatory effects **(Nam et al, 2003; Park et al, 2004; Jang et al 2005; Amin et al, 2008; Yoon et al, 2008)**. BV contains various peptides, including mellitin,

apamin, adolapin and enzymes as well as non-peptide components such as histamine, lipid, and carbohydrates **(Habermann, 1971; Banks & Shipolini, 1986; Lee, 2001)**.

Also, some components of bee venom can cause inflammation by inducing interleukin (IL)-1β via p38 mitogen activated protein kinase (MAPK) while others act as anti-inflammatory by suppressing inducible nitric oxide synthase (iNOS) and cyclooxigenase (COX)-2 via nuclear factor (NF)-kB. The naturally occurring peptides of whole bee venom have various pharmacological potencies to produce local inflammation, nociception and pain hypersensitivity in mammals. Over 50 % of whole bee venom plays a central role in the production of local inflammation; however, these components exhibit anti-inflammatory activity in inflammatory cells **(Pham et al, 2010; Chen et al, 2005)**.

The major components of honeybee venom are mellitin, phospholipase A2, and hyaluronidase **(Schumacher, 1993)**. The peptide mellitin, comprises approximately 50% of bee venom **(McKenna, 1993)**. However, bee venom has antibacterial, anti-parasitic, and antiviral properties **(Guillaume et al, 2006)**. The phospholipases A2 (PLA2) of honey bee and snake venoms have potent anti-human immunodeficiency virus (HIV) activities. These PLA2s block HIV-1 entry into host cells through a mechanism linked to PLA2 binding to cells **(Fenard, 2001)**.

In regard to the safety concerns about RVF vaccines, this work aim to use the safest natural product to inactivate the Rift Valley fever virus (RVFV). Also, study the effect of honey bee (Apis mellifera) venom on some types of cell lines cultures.

Materials & Methods

1- Colonization of honeybee

The colonization is conducted at the apiaries of Plants Protection Institute (PPI), Beekeeping department, Agriculture Research Center (ARC), Egypt.

2- Extraction of honeybee venom and SDS-Polyacrylamide Gel Electrophoresis of Proteins:

The electrical shock method has been used to stimulate the bees to sting. The collector frame placed at the entrance of the hive and connected to a device which supplies electrical impulses, when bees receive a mild electric shock; they sting the surface of the collector sheet as they see this to be the source of danger. The deposited venom between the glass and the protective material was dried then later scrapped off by a razor blade and collected in a dark bottle in a powder form. These procedures were performed According to **Mraz, 1983**. Gel electrophoresis was performed by protocol

adapted from "commonly used techniques in molecular cloning" **(Sambrook and Russel, 2001)**.

3- Cell lines: VERO cell line, HEP 2 Cell Line were prepared and provided by VACSERA Egypt**.**

4- Laboratory animals: The number of animals (mice) used in this study was forty (40). The animals were obtained from VACSERA in Cairo. They were classified into four groups. Each group was ten (1 month old), the animals were housed in a clean and mosquitoes free rooms and fed on ration and water ad libitum for one week. Meanwhile, the animals were tested serologically against RVF virus to prove that they were completely free from infection and did not contain neutralizing antibodies against RVF virus.

5- Samples: The collected samples were chicken embryos, whole blood, serum samples, frozen tissues specimens, formalin fixed tissues specimens, frozen tissues specimen for Agar Gel Precipitation Test (A.G.P.T).

5- Preparation of hyperimmune Serum: Three healthy rabbits were injected subcutaneously with 1 ml / animal RVF virus mixed with bee venom; then challenged after 2 weeks by another dose. Serum collected after three weeks from first injection and examined by Agar Gel Precipitation Test.

3- Preparation of Stock BV solution:

Preparation of bee venom (BV) solution was performed according to Abdeldayem (2008). Bee venom powder (crude) of dose 2 mg was dissolved in 2ml media (MEME) then filtered by 0.22 micron syring filter. That final concentration of the stock bee venom become 1000 ug /ml (i.e. 1ul=1 ug), and kept at -20◦C.

1mg (dried BV) ⟹ 1ml (media)

Final concentration (1ug/1 ul)

That means concentration of 5ul of solution is 5 ug; For preparation of 20ug of bee venom: Add 980ul of media to 20ul of stalk solution

4- RVFV titration and determination of infectious dose (ID50):

VERO cell lines were used for determination of virus' infectivity titer. 50% tissue culture infectious dose of a virus (TCID50%), was carries out by traditional methods of virus quantification. After discarding of the growth media (MEM EARLES medium supplemented with 10 % fetal calf serum FCS, GEPCO, USA, 100 µg/ml penicillin and 10 µg/ml streptomycin) from the 96 well tissue culture plates, inoculated using 10 fold dilution of virus by 100 µl/ well and sealed with sterile sealer, then

incubated at 37°C. These plates were incubated for 7 days with daily microscopic examination for the detection of the cytopathic effect (CPE). Viral infectivity titer was evaluated according to the method adopted by **Reed and Muench, (1938)**.

5- Incubation of RVFV with Bee Venom: The stock mixture of inactivated RVFV was prepared by adding bee venom to RVFV and left for 8 hours at room

temperature. Virucidal activity of bee venom was conducted according to **Aoki and Messiha, (1999)** where 0.5 ml of the virus was mixed with 0.5 ml of bee venom solution (500 µg/ml) virus control set was included where the virus was mixed with bee venom free medium, both sets were incubated at room temperature (24°C) for 8 hours. The mean virus titers were determined in a duplicate post time intervals using cell culture inoculation assay (CCIA) or the CCID50 as previous. The inhibitory effect of bee venom was determined by using the probability associated with a Student's t-Test. The virus titer depletion was expressed as X log (10)/0.1ml.

8-Determination of bee venom minimum safe dose: The minimum safe dose of bee venom was determined by testing different doses on cell lines. It was 0.5ug/ml. The safe doses were used in inoculation of laboratory animals and the embryonated chicken eggs. Antiviral activity of bee venom on rift valley fever virus (RVFV) was determined to evaluate the infectivity titer in Vero cells According to **Aoki and Messiha, (1999)** where confluent monolayer of Vero cells in 96–well tissue culture plates (TPP-Swiss), were divided into two sets, the first was treated with the safe concentration of bee venom (adding 100 µl/well) then incubated at 37°C at the following intervals; 3, 6 and 24 hours and the other set was treated with bee venom free media and incubated for the same intervals as a negative cell control, then media of the two sets were discarded after each incubation interval and inoculated at 37oC with the virus.

7- Inoculation of embryonated chicken eggs (yolk sac route):

Embryonated Chicken eggs were used in the experiment (60 eggs).

1- Candle 6-day-old eggs to establish their viability.
2- Using the Candler, locate the yolk sac membrane (end of egg opposite air sac).
3- Disinfect the eggshell in the area over the air sac using 70% alcohol. With a sterile needle puncture the shell over the air sac.
4- Using a 1 ml syringe (needle 22G-1 1/2) inoculate 0.25 to 0.50 ml of specimen into the egg side opposite of air sac.
5- Seal both extremities with surgical tape.
6- Incubate at the optimal temperature for the replication of the virus under study. Examine daily and discard embryos that die prematurely (within 24 h).

7- At the end of the incubation period, harvest the yolk sac fluid. Use a 5 ml syring with an 18 G-1 1/2 needle inserted into the yolk sac (opening on the opposite side to the air chamber) and collect 2-5 ml **(Pelczar et al, 1998)**.

8-Sterility testing

In cases of contamination, the naked eye may identify the presence of bacteria and fungi. However, it is necessary to detect low level of contamination by incubation of culture cells and/or their products in microbiological sterility testing broth media. The tests performed according to **European pharmacopoeia, biological tests, 1980**.

Results and Discussion

1-SDS-Polyacrylamide Gel Electrophoresis of Proteins:

SDS is used with a reducing agent and heat to dissociate the proteins. SDS-polyacrylamide complexes form and migrate through the gels according to the size of the polypeptide. By using markers of known molecular weight, the molecular weight of the polypeptide chain (s) can be estimated. Honeybee venom in concentration 1/1000 was dissolved in distilled water. However, 10ul from this solution was run in gel to separate its contents of proteins. The results showed that three bands were formed in spite of the lower concentration of bee venom (column-1). This result indicates the high protein content of bee venom. Our result is in accordance with **(Banks and Shipolini, 1986)** who mentioned that bee venom is essentially protein.

2-Antiviral activity of bee venom

Bee venom showed apparent activity against viruses that tested in this study (RVFV). The in vitro tests were performed on tissue culture cell line (Vero) which infected with RVFV. In the present work, the sets of tissue culture plates were inoculated with RVFV, the control set was left without adding bee venom. The test groups were inoculated with RVFV and bee venom the results showed sterile flasks in the test groups which showed absence of CPE and absence of contamination in fluids of test flasks. The test flasks were further investigated for the presence of RVFV live particles by using another tissue culture cell lines (VERO), and by laboratory animals (baby mice), intracerebral inoculation.

In comparison with control set of tissue culture flasks, it was approved that bee venom kill RVFV, and able to prevent it from causing any growths on VERO cell lines. The absence of CPE on VERO in test groups gives us presumptive conclusion about bee venom antiviral effects on virus (RVFV) and diminishes any doubts about its antiviral effects. This antiviral activity also indicating that the mechanisms of action against virus are not specific and simple. The rapid action that lasts for seconds which not needed

complicated biological reactions refers to the direct action on virus's constituents and destruction effects.

3-Antibacterial activity of bee venom

The antibacterial activity was approved by repeating tests on contaminated tissue culture flasks. The flasks' fluids decontaminated by adding bee venom. Firstly we are observing the disappearance of turbidity from tested fluids, then culturing from flasks on blood agar and flasks of tissue culture cell line (Vero) inoculated by contaminated fluids only. The blood agar plates showed bacterial growth, indicating the presence of contaminating microorganism in control position group.

The blood agar plate inoculated by contaminated fluids added to it bee venom, showed sterile culture without any bacterial growths.
The Antibacterial activity of bee venom has been mentioned by (Fennel et al, 1968) who reported that Apis mellibora venom (bee venom) and extract mellittin are effective against PKSA (Penicillin Resistant Strain of Staph. Aureus), the present study showed that bee venom activity against bacteria is apparently has broad spectrum activity against different types of bacteria.

4-Sterility testing

Generally, bacterial contamination is visible to the naked eye and detected by a sudden increase in turbidity and the changes in color of the culture medium as the result of the change in pH. So that in the present study, sterility testing of used cell lines showed no turbidity was detected in test broth tubes 14 days post inoculation. The control positive groups showed evidence of micro-organism growths with time of test (14 days). The negative control groups showed no evidence of bacterial or fungal growths.

5-HEp2 Cell lines treated with Bee venom:

Microscopic examination of cell lines:

None treated HEp2 cell line showed intact and confluent growth**.** The cell lines of HEp2 were treated with different concentrations of bee venom and examined after 24 hours post-inoculation. The cell lines treated with various concentrations of bee venom in a descending manner (25,20, 15, 10, 5, and 0.5 ug/ml of bee venom) **(figures; 2-9)**.

Morphological changes of unstained HEp2 cell lines according to doses: Figure (2-9): Inverted microscopy examination of unstained **HEp2** cells treated with different concentrations of BV: (**2)** Non treated cells showed complete and intact sheet of cells, (**3**) Cells treated with 20 µg / mL showed incomplete sheet with destructed cells, **(4)** Cells treated with 15 µg / mL showed destructed sheet, (**5**) Cells treated with 10 µg / mL showed incomplete sheet with some normal parts (**6)** Cells treated with

5µg/mL showed moderate changes in the form of slowing cell growth, **(7)** Cells treated with 2.5 µg /mL, showed slight changes in the form of slowing in the cell growth (**8)** Cells treated with 1 µg /mL, showed complete sheet with slight changes (**9)** Cells treated with 0.5 µg /mL. showed complete sheet with slight changes (unstained X 20).

6-Virus quantification: Traditional methods:

Groups of at least 6 mice should be used in order to determine statistical significance: Following the protocol for inoculation of mice. However, inject groups of at least 6 mice with serial dilutions of inocula (i.e, 10-fold, 5-fold, 2-fold) that are estimated to flank the number of viruses particles required to infect 50% of the mice in a single group. At least 4 different doses should be used for accurate calculation of the

LD 50%. Determine the number of infected mice and calculate the LD50, according to the methods of **Reed and Muench (1938)**.

7-Chicken embryo inoculation:

Eggs inoculation: Inoculation of the RVFV mixed with BV into chicken embryonated eggs: Sample-1: Bee venom, 0.5 ml of bee venom concentration 0.5 ug/ml added to 1 ml saline, all eggs embryo died after less than 24 hrs. Sample-2: RVFV plus bee venom, 20 eggs embryos died after 2 days and appear pale and completely free from contaminations; negative with AGPT; 20 egg embryos stay alive till day 5 post-inoculation and the embryo is pale and nearly normal without hemorrhage and negative by AGPT **(figure-10)**. Sample-3 RVFV Menia strain, showed hemorrhages and positive by AGPT **(figures11&15)**, 10 died after 2 days and 10 stay alive till 5 days PI with necrotic foci in liver and severe hemorrhages **(figure-12)**.

Bee venom and RVFV mix injected in chick embryo at age 8 days showed 10 out of 20 died at the 3rd day PI without signs of hemorrhage or congestion, another 10 stay alive till the 6th day PI also without signs of hemorrhage or congestion, and the embryo growth looks more normal than RVFV injected egg embryo, all samples are negative for AGPT, which indicate that bee venom inactivate RVFV completely, and the death of embryo may be due to the effects of bee venom toxicity because the control are all died **(figure-13)**. RVFV injected eggs are gives positive for AGPT **(figure-14)** with signs of hemorrhages and necrotic foci in liver, where 10 eggs died at the 3rd day PI and 10 stay alive till day 6th PI. The embryo growth is retarded compared to the embryo injected with bee venom and RVFV mix.

Rift Valley fever virus (RVFV), like many other Bunyaviridae family members, is emerging pathogen. The bunyaviridae is family of human, animal and plant pathogens. Rift valley fever virus (RVFV) is medically and agriculturally important virus across the world **(Schmalijhon and Nichol, 2007)**. Human infected by bite from Aedes or various other species of mosquito, and by contact and aerosol with infected

livestock. The spread of Aedes mosquitos into Europe and the Americas raises concerns of further expansion of RVFV towards new free zones. However, recently discovered viruses belonging to the same phlepovirus genus as RVFV have been associated with cases of human diseases **(Swanpoel and Coetzer, 1994; Gargon et al,1988; Enserin, 2008; Stone, 2010; McMullan et al, 2012)**.

The efforts of control RVFV in endemic areas by vaccination with killed RVFV vaccines were success in preventing this virus from spreading across the world. However, RVFV causes severe illness in affected animal or human, so that many trials are directing to find treatment against Rift Valley fever disease (RVF). The present work aim to find treatment as well as test another tool to kill the virus. Killing RVFV in laboratory has many benefits and applications. One of these applications is using the product in vaccine preparation. Other benefits are using new methods in disinfecting

laboratory wastes by effective and safe substance like bee venom. Moreover, bee venom is natural products that has not any hazards on environment and have the possibilities of using honeybee venom in treatment of RVF illness in therapeutic doses. A variety of insect's species use venoms for defense and predation. These venoms, which consist of a complex mixture of toxic components, are usually delivered into their victims via bites or stings. The targeting of mammalian species by venomous animals has presumably introduced evolutionarily relevant selective pressures to either avoid or minimize the toxic effects of envenomation. **(Fry,etal.2009; Vonk, et al, 2008; Palm, 2012)**. Venoms contain various components that are responsible for their toxicity and for triggering the inflammatory response to envenomation **(Fry et al, 2009; Fry et al,.2009)**. A large class of cytotoxic venom components consists of proteins with direct cytolytic activities that are dependent on the ability of these compounds to bind and disrupt cellular membranes either through direct pore formation or cleavage of membrane phopholipids **(Raghuramon and Chattopadhyay, 2007)**. This class of toxins includes various cytolytic cationic peptides, such as melittin in bee venom, scorpion toxins in scorpion venom, lycotoxin in wolf spider venom **(Van den Bogaart et al, 2008)**. Furthermore, nearly all insect venoms contain phospholipase A2s (PLA2s), which can lead to cell lysis by cleaving plasma membrane phospholipids. Venoms induce a variety of immune response, including both acute inflammatory responses, such as mast cell degranulation, and adaptive immune responses, such as T helper type 2 responses and IgE production **(Kourie and Shorthouse, 2000)**. Stings by hymenoptera species, including the European honey bee (Apes mellifera) are a common environmental cause of inflammatory and allergic responses **(Muller, 2010)**. Bee venoms typically cause an immediate local inflammatory reaction consisting of redness, pain, heat, and swelling, but can also lead to an allergic response in a subset of individuals-approximately 3% of the population is allergic to hymenoptera stings and

100 people die each year from hymenoptera sting-induced anaphylaxis in the USA **(Brown and Tankersley, 2011)**. Large, multiprotein complexes responsible for the activation of caspase-1, termed inflammasomes, are activated in response to various infectious and noninfectious stimuli **(Strowig et al, 2012)**.

Conclusions

In vitro study showed that Rift Valley fever virus (RVFV) has been inactivated by the honeybee (Apis mellifera L.) venom (0.5 ug / ml). Fertilized eggs of age 8 days chick embryo was inoculated with dose 0.1 ml/egg of this mixture in yolk sac. Ten out of twenty died at 3rd day PI, and ten out of twenty still alive 7th days PI. The embryos survived this dose showed normal growth without any signs of congestions or hemorrhage. The internal organs of these embryos appear normal without necrotic foci in liver. This indicated the failure of infecting the egg embryo and the inactivation of RVFV by bee venom. AGPT performed in tissues homogenates was negative,

indicating the absence of RVFV antigens in the mixture and the absence of live virus contaminants with complete inactivation of RVFV by bee venom. Beside RVFV inactivation, the antigenic characteristic remains intact and strong as the mixture showed positive Immunoflurescent and strong line of precipitate (ppt) by testing it in vitro by AGPT against hyperimmune serum of RVFV which prepared in rabbits (figures-14&15). Ten out of twenty eggs embryo died PI of RVFV Menia strain at dose 0.1 ml/egg with signs of hemorrhage and congestion, and other ten still alive till 7th days PI, all embryos gives strong line of ppt by AGPT against hyperimmune serum of RVFV. Necrotic foci were seen in liver of these embryos. Also, RVFV / bee venom mixture has tested in rabbits and mice to investigate its behavior in vivo. Mice treated with this mixture at 0.1 ml/animal still alive till 7th day PI and exhibited high antibodies response. No pathological lesions were observed in mice, which indicated the absence of live virus particles and that the mixture is safe. However, the humeral immune response in rabbits following treatment with this mixture resembles that of RVFV whole antigen; in addition RVFV/bee venom didn't cause any side effects in liver, kidneys, brain and spleen. We can conclude that, the potential of using bee venom to inactivate viruses is strong and promising for its special advantages over other known substances regarding the safety and innocuous characteristics. Moreover it can be used as vaccine carrier (adjuvant) and much recommended for human vaccines.

Biosafety recommendations

The present study showed the apparent effects of bee venom on bacteria and RVFV **(Banks and Shipolini, 1986)**. However, the effects were rapid and strong and detected by gross observations. So that we recommend the use of bee venom as biosafety treatment for highly biological environmental health hazards materials and biological contaminants that produced by laboratories such as virology departments and

vaccine production institutions and also for decontamination of bacterial cultures before discarding it in bags of high threats biological wastes. In country like Egypt, these suggestions are strongly recommended regardless the presence of company responsible for dealing with highly dangerous biological wastes.

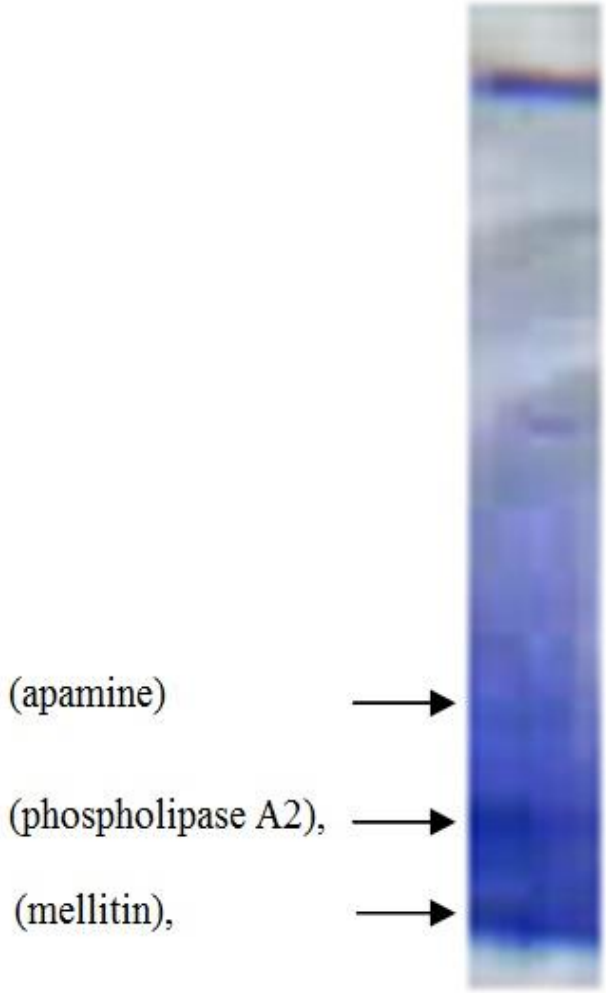

Figure -1: showed SDS electrophoresis column with three bands, first band near base is the heaviest (mellitin), then second band (phospholipase A2), and the third band is the lighter one (apamine) (Banks and Shipolini , 1986).

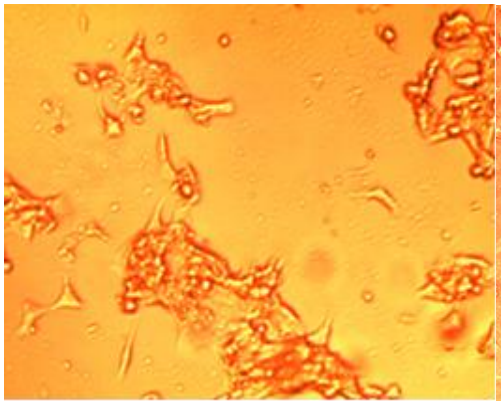

Fig-2

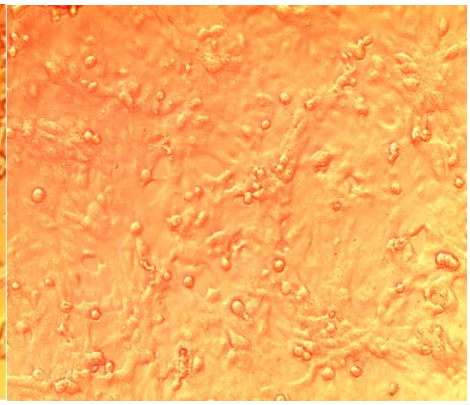

Fig-3

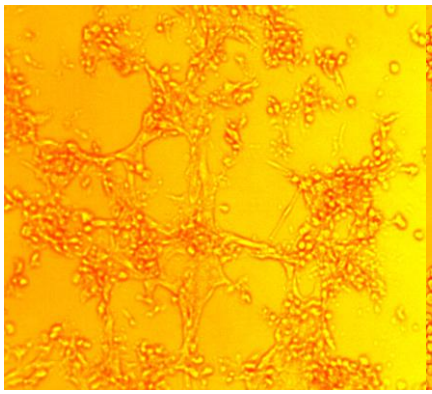

Fig-4

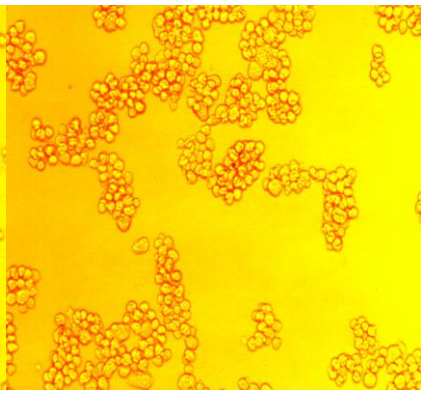

Fig-5

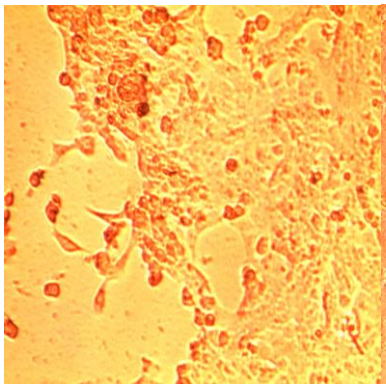

Fig-6

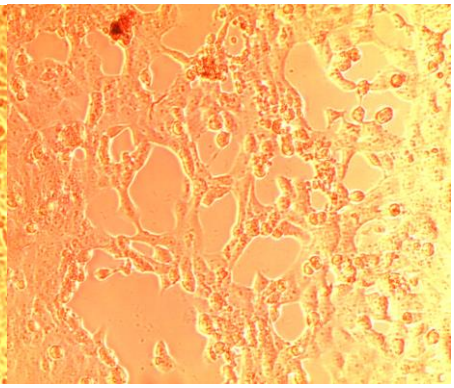

Fig-7

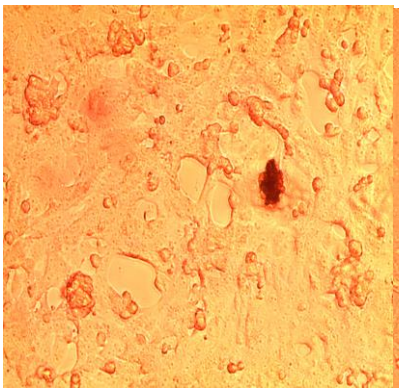

Fig-8

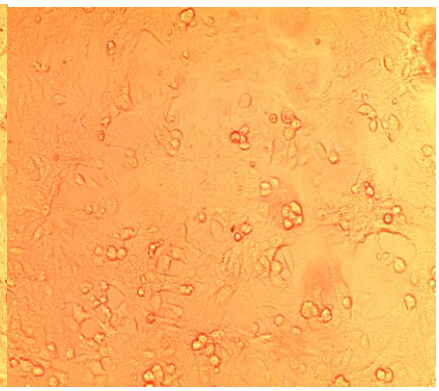

Fig-9

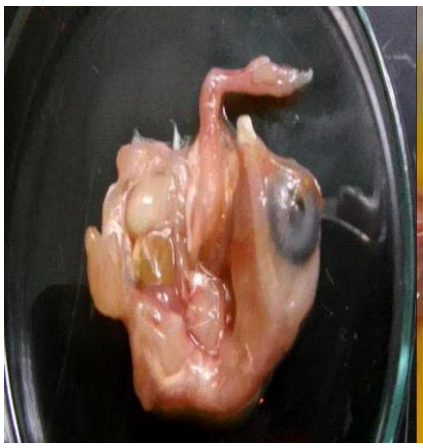

Fig-10

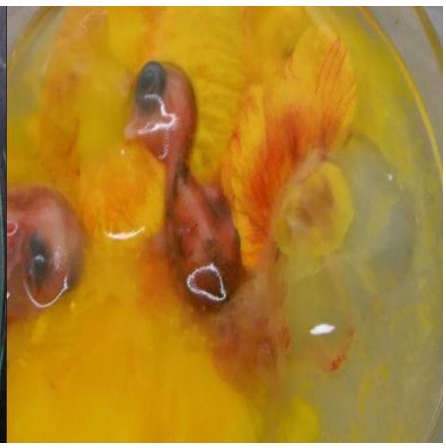

Fig-11

Figure-10: Test group showed chicken embryo treated with RVFV plus bee venom, showed normal body without congestion or hemorrhages.

Figure-11: Control positive group; chicken egg embryos treated with RVFV only, showed hemorrhagic patches on embryos and congested blood vessels

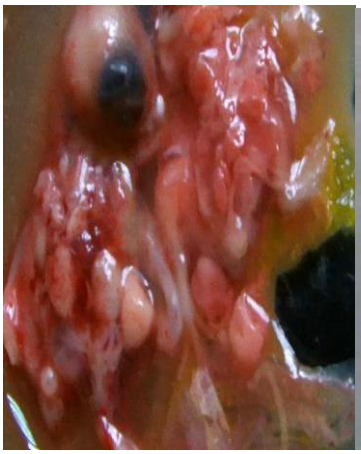
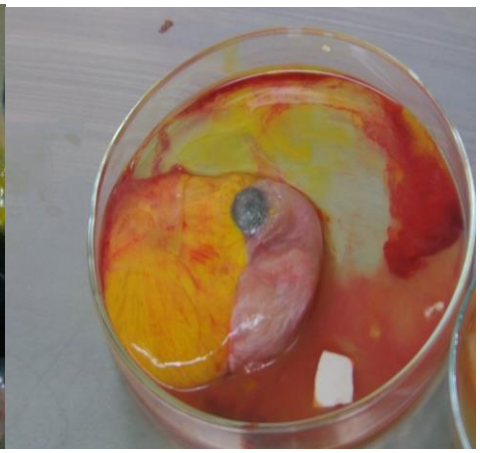

Fig-12 Fig-13

Figure-12: Control positive group which treated with RVFV only showed congestive hepatic tissue and hemorrhagic viscera, the liver showed necrotic foci.
Figure-13: RVFV mixed with bee venom, live egg embryo 13 days PI, showed normal growth and absence of congestion or hemorrhages.

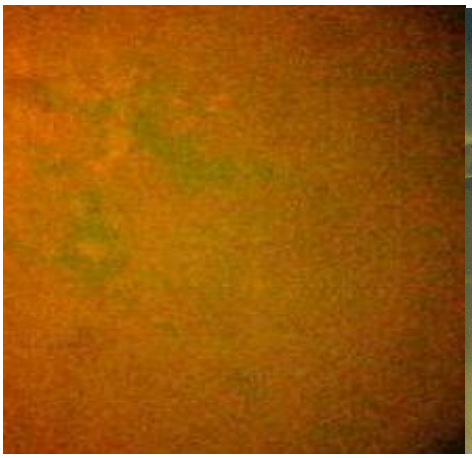


Fig-14 Fig-15

Fig-14: Liver homogenates of tested group, showed positive IF test
Fig-15: Showed positive AGPT.

References

.**Daubney R, Hudson JR, Garnham PC. Enzootic hepatitis or Rift Valley fever: an undescribed virus of sheep, cattle and man from East Africa(1931).** J Pathol Bacteriol; 34:545-579. doi: 10.1002/path.1700340418. PMID: 2133278

Murphy FA, Fauquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, Martelli GP, Mayo MA, Summers MD. Family Bunyaviridae(1995). In Virus Taxonomy. Classification and Nomenclature of Viruses. Sixth report of the international committee on taxonomy of viruses. Springer-Verlag, Wien.; p. 300-315., New York.

.**Linthicum KJ, Davies FG, Kairo A. Rift Valley Fever virus (family Bunyaviridae, genus Phlebovirus)(1985).** Isolation from Diptera collected during an inter-epizootic period in Kenya. Journal of Hygiene; 95(1):197-209. PMID: 2862206

.**Yadani FZ, Kohl A, Prehaud C, Billecocq A, Bouloy M, (1999):** The carboxyterminal acidic domain of Rift Valley Fever virus NSs protein is essential for the formation of filamentous structures but not for the nuclear localization of the protein. Journal of Virology; 73:5018-5025. PMID: 10233964

Billecocq A, Spiegel M, Vialat P, Kohl A, Weber F, Bouloy M, Haller O, (2004): NSs protein of Rift Valley fever virus blocks interferon production by inhibiting host gene transcription. *Journal of Virology*, 78:9798-9806. doi: 10.1128/JVI.78.18.9798-9806.2004. PMID: 15331713

Bouloy M, Janzen C, Vialat P, Khun H, Pavlovic J, Huerre M, Haller O, (2001): Genetic evidence for an interferon-antagonistic function of rift valley fever virus nonstructural protein NSs. *Journal of Virology*; 75:1371-1377. doi: 10.1128/JVI.75.3.1371-1377.2001. PMID: 11152510

Vialat P, Billecocq A, Kohl A, Bouloy M, (2000): The S segment of rift valley fever phlebovirus (Bunyaviridae) carries determinants for attenuation and virulence in mice. Journal of Virology; 74:1538-1543. PMID: 10627566

McElroy AK, Albariño CG and Nichol ST, (2009) :Development of a RVFV ELISA that can distinguish infected from vaccinated animals. Virology Journal; 6:125. doi:10.1186/1743-422X-6-125. PMID: 19678951.

Sumikura H, Anderson OK, Drewes AW, Arendt- Nelsen LA, (2003). A comparision of hyperalgesia and neurogenic inflammation induced by melittin and capsaicin in humans. Neurosci Lett.,; 337: 147-150.

Nam KW, Je KH, Lee JH, Kang SK, Mar W, (2003). Inhibition of COX-2 activity and pro-inflammatory cytokines (TNF-alpha and IL-1beta) production by water soluble sub-fractionated parts from honeybee (Apis melifera) venom. Archiv Pharmacol Res.,; 26: 383-388.

Park HJ, Lee SH, Son DJ, Oh KW, Kim KH, Song S, Kim GJ, Oh GT, Yoon DY, Tae Hong JT, (2004). Antiarthritic effect of bee venom: inhibition of inflammation mediator generation by suppression of NF-kB through interaction with the p50 subunit. Arthritis Rheumatism,; 50: 3504-3515

Jang HS, Kim SK, Han JB, Ahn HJ, Bae H, Min BI,(2005): Effects of bee venom on the pro-inflammatory responses in RAW264.7 macrophage cell line. J Ethnopharmacol,; 99: 157-160.

Amin MA, Abdel-Raheem IT, Madkor HR, (2008). Wound healing and anti-inflammatory activities of bee venom-chitosan blend films. J Drug Delivery Sci Technol.,; 18:424-430.

Yoon SY, Kwon YB, Kim HW, Roh DH, Seo HS, Han HJ, Lee HJ, Lee JH, (2008). Bee venom injection produces a peripheral anti-inflammatory effect by activation of a nitric oxide-dependent spinocoeruleus pathway. Neurosci Lett.,; 430: 163-168.

Habermann E, (1971). Chemistry, pharmacology and toxicology of bee, wasp and hornet venoms. In: Bucherl W, Buckley EE Eds., Venomous Animals and Their Toxins: Venomous Invertebrates, Vol. III, New York, Academic Press,; pp 61–93.

Banks BEC, Shipolini RA, (1986). Chemistry and pharmacology of honeybee venom. In: Piek T Ed., Venoms of the Hymenoptera. Academic Press, London,; pp 329–416.

Lee JH, Kwon YB, Han HJ, Mar WC, Lee H., Yang IS, Beitz AJ, Kang SK, (2001). Bee venom Pre-treatment has both an anti-nociceptive and anti-inflammatory effect on carrageenan induced inflammation. J Vet Med Sci,; 63: 251–259.

Pham DL and Prabhat KM, Seung YH, Seong-Gu H, (2010): Study of the Molecular Mechanism of Anti-inflammatory Activity of Bee venom in Lipopolysaccharide Stimulated RAW 264.7 Macrophages. Trop J Pharm Res, February; 9 (1): 19.

Chen YN, Li KC, Li GW, Shang D, Liu NZ, Lu M, Zhang JW, JI YH, Gao GD, Chen J,(2005). Pain hypersensitivity in mammals, and among the five identified reverse-phase high pressure liquid chromatography. Neurosci.,; 138: 631–640.

Schumacher, M. J. (1993). Significance of Africanized bees for public health. Arch Med Intern; 155:2038-2043.

McKenna, W. (1993). killer bees: what the allergist should know. Pediatr. Asthma allergy and immunol; 6:275-285.

Guillaume C.; Calzada C.; Lagarde M.; Schrével J. and Deregnaucourt C. (2006). Interplay between lipoproteins and bee venom phospholipase A2 in relation to their anti-plasmodium toxicity. J. Lipid Res., 47: 1493 - 1506.

-Fenard, D.; Lambeau, G.; Maurin, T.; Lefebvre, J.C.; and Doglio, A. (2001). A peptide derived from bee venom-secreted phospholipase A2 inhibits replication of T-cell tropic HIV- 1 strains via interaction with the CXCR4 chemokine receptor. Mol. Pharmacol. 60:341-347

Mraz, C. (1983). Methods of collecting bee venom and its utilization. Apiacta, 18: 33-34, 54

Reed, L.J.; Muench, H. (1938). "A simple method of estimating fifty percent endpoints". The American Journal of Hygiene **27**: 493–497.

Aoki, F.Y. and Messiha, N. (1999). Assessing In Vitro Herpes Simplex Inhibitory Activity of Stannous Fluoride, Embro Research Corporation. W. J .Embro ،DDS, Manitoba University, Canada

Pelczar J. Michael, Chan E.C.S, Krieg R. Noel,(1998). Microbiology, fifth Edition, Tata McGraw-Hill Publishing Company Limited

European pharmacopoeia, biological tests (1980): 2nd edition, part1 P.V 2.13 Maisonneane Sainte- Ruffie.

Fennell JF, Shipman WH and Cole LJ (1968): Antibacterial action of mellitin, a polypeptide from bee venom, Proc,Soc, Exp, Biol. Med., 127:707-710.

Schmalijhon CS, Nichol ST (2007): Bunyaviridae. Fields Virology, Eds kaipe DH, Howley PM (Lippincott Williams & Wilkins, Philadelphia), 5th Ed, Vol 2,pp1741-1788.

Swanpoel R, Coetzer JAW (1994): Rift Valley fever. Infectious diseases of livestock: with Special Reference to Southern Africa. Eds Coatzer JWA, Thomson GK, Justin RC, Kriek NPJ (Oxford Univ Press, Cape Town), pp 688-717.

Gargon TP, Clark GG, Dohn DJ, Turell MJ, Bailey cl (1988): Vector Potential of selected North American mosguito species for RVFV. Am J Trop Med Hyg 38 (2)440-446.

Enserin K M (2008): Entomology. A mosguito goes global.Science 320 (5878) :864-866.

Stone R (2010) : Infectious diseases. Rival teams identify a virus behind deaths in Central China .Science 330 (6000) :20-21.

McMullan LK,et al. (2012) : A new phlebovirus associated with severe febrile illness in Missouri .N Engl J Med 367(9):834-841.

Fry BG,etal.(2009) :The toxigenomic multiverse: Convergent recruitment of proteins into animal venoms. Annu Rev Genomics Hum Genet 10:483-511.

Vonk FJ, et al . (2008): Evolutionary origin and development of snake fongs. Nature 454 (7204) 630-633.

Palm NW, Rosenstein RK, Medzhitov K (2012): Allergic host defences. Nature484(7395):465-633.

Fry BG ,Roelants K , NormanJA (2009) :tentacle of venom toxic protain Gonvergence in the Kingdom Animelia. J Mol EVOL 6 8 (4):311-321.

Raghuramon H ,Chattopadhyay A(2007): Mellittin:A membrane –active peptide with diverse functions. Biosci Rep 27(4-5) :189-223.

Van den Bogaart G, Guzman JV, Mika JT ,Poolman B (2008): On the mechanism of pore formation by mellitin.J Biol chem 283 (49):33854- 33857.

Kourie JI , Shorthouse AA(2000): properties of cytotoxic peptide –formed ion channels. Am J Physiol Cell Physiol 278 (6):c1063-c 1087.

Muller UR (2010): insect venoms. Chem Immunol Allergy 95:141-156.

Brown TC, Tankersley MS (2011): The sting of the honeybee:An allergic perspective.Ann Allergy Asthma Immunol 107(6):463-470, quiz 471.

Strowig T, Henao-Mejia J, Elinav E, Flavell R (2012): Inflammasomes in health and disease. Nature 481(7381):278-286.

Sambrook J and Russell DW, 2001: Commonly used techniques in molecular cloning Appendix 8 in "Molecular Cloning" Vol.33rd edition. Cold Spring Harbor Laboratory Press, Cold Spring, Harbor, NY, USA.