

MONITORING OF THE ANTIVIRAL POTENTIAL OF BEE VENOM AND WAX EXTRACTS AGAINST ADENO-7 (DNA) AND RIFT VALLEY FEVER VIRUS (RNA) VIRUSES MODELS

By

MOSTAFA I. HASSAN^{1*}, ALY F. Mohamed, MONER A. AMER¹,
KOTB M. HAMMAD¹ AND SABER A. RIAD¹

Department of Zoology, Faculty of Science, Al-Azhar University¹ and Virology Sector, VACSERA², Dokki, Egypt (*Correspondence: mostafa012@gmail.com)

Abstract

This study monitored the antiviral potential of bee venom and four wax extracts, ethanol white and black beeswax (EWW/EBW) and acetone white and black beeswax (AWW/ABW) extracts. Two different virus models namely Adeno-7 as DNA model and RVFV as RNA virus models. End point calculation assay was used to calculate virus depletion titer. The depletion of viral infectivity titer of ABW to Adeno-7 virus showed strong antiviral activity recorded a depletion of viral infectivity titer (1.66 log (10)/ ml) that gave equal action with bee venom and more than interferon IFN (1 log (10)/ ml). On the other hand, antiviral activity of EBW showed a moderate potential, while AWW showed no antiviral activity. Finally EWW showed synergetic activity against Adeno-7 virus activity. Thus, activity of wax extracts to RVFV was arranged in order of IFN > bee venom > AWW & EBW > EWW and ABW recorded 3.34, 0.65, 0.5, 0.34 respectively. It is the first time to study the beeswax effect against DNA and RNA virus' models; acetone black beeswax recorded a depletion titer 1.66 log (10)/ ml.

Keywords: Bee venom, Bee's wax, Antiviral, Cytopathology, DNA & RNA virus models.

Introduction

Most people think in insects as enemies but some insects are friends not enemies. More than 900 therapeutics products were isolated from insects. Bee venom has antibacterial, antiparasitic, and antiviral properties (Guillaume *et al*, 2006). Many pharmacologically active components in honeybee, *Apis mellifera* venom, include peptides, enzymes, and biogenic amines that cause toxic reactions. The most important component is the peptide melittin, which comprises approximately 50% of bee venom (McKenna, 1993), the major components of honey bee venom-melittin, phospholipase A2 and hyaluronidase (Schumacher, 1993). Melittin has been used for cancer gene therapy and human immunodeficiency virus (HIV-1) inhibition (Liu *et al*, 2001). Secreted phospholipaseA2 (sPLA2) from honey bee venom have potent anti-human immunodeficiency virus (HIV) activities. The secretary PLA2s block HIV-1 entry into host cells via a mechanism linked to

PLA2 binding to cells (Fenard *et al*, 2001). Bee-wax is produced by worker bees for building the honeycomb structure used for brood rearing and storing of honey, and pollen. The honeybee's wax has an extremely wide spectrum of useful applications including candles, cosmetics, and furniture polishes, anti-inflammatory, antibacterial (Krell, 1996). As the antimicrobial venom peptides of honey bee are present both on adult bees' cuticle and on nest wax, it was suggested that these substances act as a social antiseptic device, venom is spread over body cuticle and on comb wax of *Apis* species (Baracchi *et al*, 2011).

The aim of this study was to monitor the antiviral potential of bee venom and four beeswax extracts as antiviral activity against DNA and RNA virus models.

Materials and Methods

Colonization of honey bees: Six honey bee hives were conducted at experimental

animal house, Al-Azhar Faculty of Science to produce wax and venom.

Bee venom collection (BV): Electric shock method was used to collect the bee venom from six bee hives during three weeks of 2014 (Bahreini *et al*, 2000). Two mg of dry bee venom were dissolved in 2ml media minimum essential medium Earl's Ramadan *et al*. (2009). Venom solution was filtrated by sterile 0.22µl syringe filter (Millipore, USA).

Collecting of bee's wax: Wax samples were collected from six bee reared in the animal house. White wax (WW) and black one (BW) were collected, then cut into small pieces, washed using tap water and left to dry at temperature (27-31°C) for 2 to 10 days WW, BW (10g) were extracted (Miroslava *et al*, 2012) in 80ml solvents 70% ethanol, and aqueous acetone designated (EWW, EBW, AWW & ABW), respectively. Wax extracts were acidified with hydrochloric acid to pH 1.5. Samples were extracted at 40°C in incubator for seven days, shaken daily and filtrated through Whatman filter paper No. 5. Supernatants were dried in a rotary evaporator at 40°C for 2-3 hrs. for ethanol and 40-60 minutes for acetone. Two mgs of dry bee's wax extracts were dissolved in 2ml of 0.8% sodium chloride solution 0.004ml Tween-80. Extracts solutions were 0.2 µm syringe filters filtrated.

Cell culture: Vero cells clone CCL-81, was grown in 199-Earls-medium supplemented with 10% fetal calf serum (FCS), 100µg/ml Penicillin and 10µg/ml Streptomycin. Fully grown monolayer was selected and the cells were trypsinized using trypsin solution (0.25%). Cell suspension was dispensed as 100µl/well using automatic multichannel pipettes in 96 well flat bottom tissue culture plates.

Cytotoxicity: Cell viability of Vero cells under the effect of test wax & venom samples was evaluated using MTT assay (Mosmann, 1983; Gerlier and Thomasset, 1986). Bee venom and wax extracts were added to cells at concentration

of 1000µg/ml, control was included. Cell viability percentage was calculated as the following equation $Viability\% = \frac{\text{Mean OD of sample}}{\text{mean OD control cell}} \times 100$.

Antiviral activity: Antiviral activity of bee venom and wax extracts against human adenovirus serotype 7 (Adeno-7) as DNA virus model and Rift Valley fever virus (RVFV) as RNA virus model were determined to evaluate the infectivity titer in Vero cells. According to Palamara *et al*. (1995) where confluent monolayer of Vero cells in 96-well tissue culture plates (TPP-Swiss), were divided into two sets, the first was treated with safe concentrations of bee venom and wax extracts (100µl/well) and incubated at 37°C as the following intervals; 3, 6 & 24 hrs.; other was treated with venom and wax extracts free media and incubated for same intervals as a negative cell control. CCID₅₀ was determined (Reed and Muench, 1938).

Comparison of antiviral activity of bee venom and wax with interferon: Vero cells at a concentration of 2x10⁵cells/ml were maintained to confluence in 96 well cell culture plates. Four rows of cultured cells were treated with 100 IU/well of interferon (Pegintron 80mg/0.5 (800. 000 IU/mg), Shering-Plough, USA) and the rest of the plate was supplemented with interferon (IFN) free media. Plates were incubated for 24 hrs. at 37°C. Medium was discarded and wells were inoculated with 10 fold serially diluted virus models Adeno-7 & RVFV and incubated at 37°C for 7 days. A 96 well cell culture plate for each virus was used. CCID₅₀ was determined (Reed and Muench, 1938). Antiviral activity of bee wax and venom was compared (Denizot and Lang 1986) as the difference between mean virus infectivity titer inoculated on INF treated & cells negative control was compared to difference between mean virus infectivity titer post-treatment.

Results

Bee venom and wax extracts cytotoxicity to Vero cells showed a relative relation

between concentration and viability%. IC50 was arranged in the order of EWW [1.6], AWW [1.6], EBW [12.5] ABW [0.5] and bee venom [0.4] µg/ml (Fig.1). The data showed that bee venom and wax extracts showed variable antiviral potentials on Adeno-7 virus (DNA) and Rift Valley fever virus (RNA) virus models.

Antiviral activity (Tab. 2) of EWW, ABW, EBW, AWW & BV against human adenovirus serotype 7 (Adeno-7) as DNA virus model showed an elevated virus infectivity titer post treatment recording 0.34 log (10) log (10) /ml, while rest of extracts showed a variable depletion rate of Adeno-7 virus infectivity titer in the order 1.66 log (10)/1 ml, 0.66 log (10)/1 ml, 0 log (10)/1 ml, and 1.66 log (10)/1 ml. The use of INF as 5ngm/ml showed a depletion of virus infectivity titer in order of 1 log (10)/1 ml post INF treatment compared with the virus control infectivity titer; 5.66 log (10)/1.

The antiviral activity EWW, ABW, EBW, AWW & BV against Rift Valley fever virus (RVFV) as RNA virus model (Tab. 3). A depletion of virus infectivity titer was 0.34 log (10)/1 ml, 0.34 log (10)/1 ml, 0.50 log (10)/1 ml, 0.50 log (10)/1 ml, and 0.65 log (10)/1 ml. While, the INF showed a depletion of virus infectivity titer in order of 3.34 log (10) /ml post INF treatment compared with virus control infectivity titer, 8 log (10)/1 ml.

Discussion

Bee venom contains at least 18 active components, including enzymes, peptides, and biogenic amines, which have varieties of the pharmaceutical properties (Park *et al*, 2004). The normal eukaryotic cells with a low membrane potential, the cell membranes of prokaryotic cells showed a large membrane potential. So, many lytic peptides in honeybee products selectively disrupt the prokaryotic cell membrane rather than those of eukaryotic cell membrane (Moon *et al*, 2006).

In the present study, assessment of cell viability using methyl thiazolyltetrazolium assay (MTT) staining assay, as 100%

of cell viability was induced when the Vero cells were treated with 1.6µgm of bee venom and bee's wax, this concentration proved safe and agreed with Yin *et al*. (2005) who reported the safe concentration of honeybee venom on Vero cells could be less than 1 µg/ml. the cytotoxic effect may be attributed to the ATP depletion (Eduard *et al*, 2002).

In the present study, the antiviral activity of bee venom against human adenovirus (Adeno-7) as DNA virus model revealed a moderate depletion of infectivity titer in order of 1.66 log (10)/1 ml, compared to rh-IFN α-2a that showed a little pit depletion rate in the order of 1 log (10)/1 ml. ABW showed a better antiviral activity than AWW that might be due to the nature of each of them. On the contrary, antiviral activity of bee venom against RVFV as RNA virus model gave mild action compared to recombinant INF action that was superiorly affecting RVFV infectivity influence could be due to biological behavior of IFN, which enhances the induction of Mx protein and 2-5 Olygoadenylsynthetase 2,5- OAS (Guro, *et al*, 2006; Fahmy and Shoman, 2006) or interfering of IFN in viral replication cycle or inhibition of polymerase enzyme, (Mistchenko and Falcoff, 1987); Altinkilic and Brandner, 1988; Costa da Cunha *et al*, 1996; Samuel, 2001). The result also agreed with Ramadan *et al*. (2009) who recorded the virucidal activity of bee venom on *Herpes simplex* virus type-1(HSV-1) & Adenovirus type-7 (adeno-7) as a DNA virus models studies revealed no significant decrease in HSV-1 infectivity titer after 3, 6 & 24 hrs. (P>0.05) while 48 hrs. later a significant viral depletion rate was recorded in the order of 1 log (10)/0.1 ml was detected. The virucidal activity on Adeno-7 virus model showed no significance changes three hrs. post treatment. There was a significant depletion rate of virus infectivity titer 6 and 24 hrs. post treatment; 3.7 log (10)/0.1 ml and 2.75 log (10)/0.1 ml respectively.

Also, the evaluation of virucidal activity of bee venom against RNA model virus of RVV revealed that there was a significant decrease in WNV infectivity titer post thermal treatment incubation at 37°C for 3, 6, 24 & 48 hrs. post treatment gave 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 respectively. The antiviral activity of bee venom of HSV-1 as a DNA model virus showed a non-significant decrease in the infectivity titer post thermal treatment. In the meantime, the antiviral activity of bee venom against Adeno virus sero-type-7 revealed that there was a non-significant decrease in Adeno-7 titer 3, 6 & 24 hrs post incubation at 37°C. Evaluation of the antiviral activity of bee venom against RNA model virus RVFV showed a non-significant decrease in Rift Valley virus titer after incubation period of 3, 6 & 24 hrs post treatment at 37°C.

In the present study, antiviral activity of bee venom was also compared with that of the drug; interferon -2 a (IFN- α 2a). HSV-1 was insignificantly decreased recording 5.5 log (10)/0.1 ml in the bee venom treated cells compared to significant decrease when treated with (IFN -2a) recording 3.2 log (10)/0.1 ml. In the meantime antiviral activity against adenovirus-7 recorded a significant decrease in virus infectivity titer recording 3.4 log (10)/0.1 ml post bee venom compared with 3.25 log (10)/0.1 ml post IFN treatment which mean that the in vitro studies of the effect of standard IFN used was the same as bee venom on the Adeno-7 virus. RNA model virus; RVFV was well influenced by bee venom than IFN where the virus infectivity titer recorded 1 log (10) /0.1 ml post bee venom treatment compared with 2.5 log (10)/0.1 ml virus infectivity titer post IFN treatment. The present results agreed with Mistchenko and Falcoff (1987) and Samuel (2001). But, the bee venom (BV) action against RVF virus (RVFV) as RNA virus model, recorded weak depletion of infectivity titer in order of 0.65 log (10)/1 ml. While

the use of INF showed high depletion of infectivity titer in order of 3.34 log (10)/1 ml. This meant that *in-vitro* studies indicate weak action of honeybee venom (BV) on RVFV compared to the chemical drug INF action, which agreed with Altinkilic and Brandner (1988) and Costa da Cunha *et al.* (1996). In the same time, antiviral activity of ethanol white bee extract EWW against RVFV as RNA virus model, recorded mild depletion of infectivity titer in order of 0.34 log (10)/1 ml. While the use of INF showed high depletion of infectivity titer in order of 3.34 log (10)/1 ml. On the other hand, antiviral activity of ethanol white honeybee extract EWW against human adenovirus (Adeno-7) as DNA virus model, recorded depletion infectivity titer in order of 0.34 log (10)/1 ml. The use of INF showed high depletion of infectivity titer in order of 1 log (10)/1 ml. This indicated low action of ethanol white honeybee extract EWW on (RVFV), against human adenovirus (Adeno-7) compared to the chemical drug INF action. The recorded data revealed a resemblance to Mistchenko and Falcoff, (1987); Samuel (2001). Antiviral activity of acetone black honeybee wax extract ABW against Rift Valley fever virus as RNA virus model, recorded a depletion of infectivity titer in order of 0.34 log (10)/1 ml. While, the use of INF showed a depletion of infectivity titer in order of 3.34 log (10)/1 ml. But, the antiviral activity of acetone black honeybee extract ABW against human adenovirus (Adeno-7) as DNA virus model, recorded high depletion of infectivity titer in the order of 1.66 log (10)/1 ml. While the use of INF showed a depletion of infectivity titer in order of 1 log (10)/1 ml. So, *in-vitro* studies indicated low action of acetone black bee extract ABW on (RVFV), strong action against human adenovirus (Adeno-7) compared to INF action, data showed a resemblance to Mistchenko and Falcoff (1987); Samuel (2001); Moon *et al.* (2006) and Ghanem (2011).

Also, the results agreed with Filotti *et*

al. (1967) despite they regarded the venom virucidal activity 24 hrs. post treatment. De-Clercq *et al.* (2000) proved that bee venom melittin inactivates HIV and aborts cell-to-cell fusion and HIV transmission due to high-affinity interaction with gp120, Mitsuishi *et al.* (2006) indicated that the direct addition of bee venom phospholipase A2 to 293A cells suppressed adenovirus plaque formation in number and size. De-Clercq *et al.* (2000) and Mitsuishi *et al.* (2006) proved that bee venom melittin inactivates HIV interaction with gp120

The Egyptian bees' wax have biological role as antibacterial, antiparasitic and antiviral properties not reported as a case of bee venom antimicrobial properties. Da Cunha *et al.* (2013) found that geopropolis is a type of propolis containing resin, wax and soil, collected by threatened stingless bee species showed antimicrobial and antiproliferative potential against cancer cell lines. Majiene *et al.* (2004) reported that propolis, a natural product produced by bees and contains plants' exudates mixed with bee wax, its ethanolic extracts were used for diseases' treatment and prevention, as having antibacterial, antiviral, antiinflammatory, antifungal, anesthetic and immunomodulating properties with structural compounds types of terpenoids, aromatic and aliphatic acid esters. Most of terpenoids are mono- and sesquiterpens: azulene, alphasabolol, citral, valerenol, etc., which compositions depended on plants origin of propolis collected.

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Table 1: Viability % of Vero cell lines post treatment with serially diluted wax extracts & venom diluted using (MTT).

concentration µg/ml	Cell viability (%)			
	EWW	ABW	EBW	AWW
100	9.60	17.42	32.54	8.10
50	34.69	24.51	50.17	16.63
25	48.60	43.72	66.01	25.16
12.5	64.44	67.37	98.62 *	30.60
6.3	70.60	91.17	98.70	33.19
1.6	100.42*	105.65*	97.12	47.66
0.8	101.71	97.33	97.62	54.19
0.4	98.70	97.69	97.26	66.59
0.2	102.57	97.55	96.27	79.77
0.1	99.27	101.20	98.55	88.30
0.05	98.62	99.13	100.56	91.67 *
0.03	98.19	100.63	97.86	91.31

* = bee's wax safe concentrations.

Table 2: Antiviral activity of bee wax extracts venom on human adenovirus serotype 7 (Adeno-7) compared to INF.

Sample	V.T.Pre-TTT	V.T.Post-TTT	Log difference	(%)
EWW	5.66	6.00	↑ +0.34	0.00
ABW		4.00	1.66	55.33
EBW		5.00	0.66	16.50
AWW		5.66	0.00	0.00
BV		4.00	1.66	55.33
INF		4.66	1.00	27.32

V.T.Pre-TTT = virus titer pre-treatment, V.T.Post-TTT = virus titer post treatment.

Table 3: Antiviral activity of bee wax extracts, bee venom against Rift Valley fever virus as compared to INF.

Sample	V.T.Pre-TTT	V.T.Post-TTT	Log difference	%
EWW	8	7.66	0.34	5.16
ABW		7.66	0.34	5.16
EBW		7.50	0.50	7.69
AWW		7.50	0.50	7.69
BV		7.25	0.65	10.40
INF		4.66	3.34	91.25