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Research Article

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In vitro antiviral activity of *Tunisian propolis*

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Abstract The *in vitro* effect of propolis on several RNA and DNA viruses including Coxsackie virus type B3 (CVB-3) and Herpes virus type 2 (HSV-2), was investigated. The overuse of some drug as a usual therapy led to the emergence of resistant strains. Therefore, the search for new alternative molecules to overcome this obstacle is needed. In this objective, Propolis is used for its CVB-3 and HSV-2 activity. Propolis samples from four different origins in Tunisia were evaluated for their cytotoxicity on Vero cells by the MTT test and anti CVB-3 and HSV-2 activity by plaque reduction assay. Only the extract of propolis collected from the region of Zelligua exhibited activity against CVB-3 with a 50% inhibitory concentration (IC_{50}) and a selectivity index (SI) of 30 and 9µg/mL, respectively. In addition, the study of the antiviral mode of action revealed that this extract exerts a virucidal action both during the entry of viruses and the release of the newly formed virions. The active extract went through a liquid-liquid fractionation and only the Ethyl Acetate fraction was found active against CVB-3.

Keywords Propolis; antiviral activity; CBV-3; HSV-2; Vero cells

1. Introduction

Propolis is a sticky natural substance produced by bees upon collecting resins and exudates from plants. Bees use it as antiseptic glue to seal gaps, embalm dead intruders and preserve the hive from external contamination [1].

Propolis has a long history of use in folk medicine and numerous scientific studies have been published on its biological properties and its constituents, including anti-inflammatory, anti-diabetic, anti-tumoral [2] and antiviral [3] activities.

Authors have demonstrated these properties using propolis from different geographic locations. Although it is known that in different geographic zones propolis chemical composition varies due to the different plant sources [4]. In this work we wish to report the results of our study on the antiviral activity of propolis samples from different locations in Tunisia.

2. Materials and Methods

2.1. Cell lines

African green monkey kidney (Cercopithecus aethiops) cell line (Vero) (clinical isolate) was kindly provided by the Laboratory of Clinical Virology, Pasteur Institute of Tunis, Tunisia. The title of the viral stock solution was 5.7×10^6 plaque-forming units/mL (PFU/mL).

2.2. Viral strains

The viruses tested in this work are of two types: Coxsackie virus type B3 (CVB-3) and Herpes virus type 2 (HSV-2). CVB-3 is a non-enveloped RNA virus of the enterovirus genus of the family Picornaviridae while HSV-2 is a DNA virus of the family Herpesviridae and the subfamily Alpha Herpesvirinae.

Strains of CVB-3 and HSV-2 are clinical isolates that have been adapted to Vero cells by successive passages. Their viral titer is 1.5 x 10^8 1.42 x 10^6 respectively for CVB-3 and HSV-2.

2.3. Cellular culture

Vero cells are maintained in Roswell Park Memorial Institute (RPMI) medium containing 1% of antifungal mixture (Amphetericin B) and broad-spectrum antibiotics (penicillin 10,000 IU/ml and streptomycin 10 mg/ml).

These cells are maintained in flasks of 25 or 75 cm^3 containing RPMI medium with 5% fetal calf serum. Once at confluence, a so-called contact inhibition phenomenon occurs which prevents the cells from multiplying. The cells then undergo a trypsin action which will dissociate them and detach them from the surface of the wall, then are distributed in new flanges or in flat-bottomed 96-well plates.

For the culture of the viruses or for the study of the antiviral activity, the RPMI medium was enriched with only 2% of FBS. The cells are incubated in an oven at 37 °C under a humid atmosphere enriched with 5% CO_2.

2.4. Cytotoxicity assay

The cytotoxicity of propolis extract was carried out using MTT assay [5] by calculating the concentration of the extract necessary for the growth of 50% of the cells in culture (CC_{50}). Once the cells are confluent in flat-bottomed 96-well plates, the culture medium is removed and then replaced with 100µl of a dilution series of ½ propolis extract. For the cell control, the propolis extract was replaced by RPMI medium at 2% FCS. After 72 h incubation, the medium was removed and 50µL MTT solution (5 mg/mL in PBS) was added to each well. After 4 h incubation at 37 °C, 100 µL DMSO were used to solubilize the formed formazan in each well.

The absorbance was read in a reader plate (ELx800, BioTeck, USA) at 540 nm. The percentage of cytotoxic effect was calculated using the following equation:

$$\% \text{ cell inhibition} = [(\text{Absorbance of control} - \text{Absorbance of sample}) / (\text{Absorbance of control})] \times 100$$

The 50% cytotoxic concentration (CC_{50}), defined as the extract concentration able to reduce the cell viability by 50% when compared to untreated control, was determined by linear regression analysis from dose-response curve.

2.5. Antiviral activity evaluation

The antiviral activity is evaluated by calculating the concentration of the extract necessary for the inhibition of 50% of the cytopathic effect generated by the viral infection (IC_{50}). Once the cells are confluent in 96-well flat-bottomed plates, the culture medium is then replaced by 100µl of a dilution series of ½ propolis extract (starting from CC_{50} / 2) and 25µl of a viral suspension. The titer of the viral suspension used depends on the nature of the virus: it is 1.5 x 10^4 for HSV-2 and 3 x 10^3 for CVB-3. For the viral control, the propolis extract was replaced by the RPMI at 2%. After 48 hours of incubation at 37 °C, the contents of the wells were removed and then replaced with 50µl of a 5 mg/ml MTT solution. After 4 h incubation at 37°C, 100 µL DMSO were used to solubilize the formed formazan in each well.

The absorbance was read in a reader plate (ELx800, BioTeck, USA) at 540 nm. The percentage of cytotoxic effect was calculated using the following equation:

$$\% \text{ cell inhibition} = [(\text{Absorbance of control} - \text{Absorbance of sample}) / (\text{Absorbance of control})] \times 100$$

The 50% cytotoxic concentration (CC_{50}), defined as the extract concentration able to reduce the cell viability by 50% when compared to untreated control, was determined by linear regression analysis from dose-response curve.

2.6. Study of the antiviral mode of action

The purpose of the study of the antiviral mode of action is to determine at what level of the viral cycle the active extract acts.

2.6.1. Cell protection assay

The purpose of this technique is to determine if the active extract is able to block the cellular receptors recognized by the virus, thus preventing it from attaching to the host cell.

In this technique, 100 µl of propolis active extract at a concentration equal to CC_{50} / 2 were deposited on a sheet of Vero cells in confluence in 96-well flat-bottomed plates. For the viral control, the propolis extract is replaced by the RPMI at 2%. After 2 hours of contact, the extract is removed and replaced by 125 µl of RPMI medium at 2% and 25 µl of virus with the same title used for the evaluation of antiviral activity.

After 48 hours of incubation at 37 °C., the wells tested were observed by inverted microscopy in comparison with the viral controls. If the cellular aspect of the wells treated with the propolis active extract is identical to that of the viral control, this extract has no protective action against the virus. If, on the other hand, there is a difference in cell appearance between treated and untreated wells, then the extract appears to have a protective action on Vero cells. In this case, the contents of the wells have been eliminated and the protective power of the active extract is evaluated by the MTT method as previously described.

2.6.2. Virucidal action

The purpose of this technique is to determine if the active extract is able to block attachment proteins to cellular receptors, thus preventing it from attaching to the host cell.

In this technique, 10µl of propolis active extract at a concentration equal to CC_{50} / 2 and 10µl of virus at a titer equal to 100 times that used for the evaluation of the antiviral activity were brought into contact with 37 ° C. For the viral control, the propolis extract is replaced by the RPMI at 2%. After 2h, 980µl of RPMI 2% medium were added to the mixture "virus-active extract" (1/100 dilution). Then, 100µl of RPMI medium at 2% and 25µl this dilution were deposited on a sheet of Vero cells confluent in 96-well flat-bottomed plates. After 48 hours of incubation at 37 °C., the wells tested were observed by inverted microscopy in comparison with the viral controls. The same approach as that undertaken for the cell protection test is then performed.

2.6.3. Inhibition of viral infection

The purpose of this technique is to determine if the active extract acts after viral penetration and if so, at what stage of viral multiplication (replication, transcription, genome translation, protein processing and assembly or release of neo-formed virions) [6].

In this technique, 100µl of RPMI medium at 2% and 25µl of virus in the same way as that used for the evaluation of the antiviral activity were deposited on a sheet of Vero cells in confluence in 96-well plates-flat-bottomed. After 1 hour of incubation corresponding to the period of penetration of the viral genome, the mixture "virus-RPMI medium" is eliminated. The active extract of propolis is added at different post-infection times (1; 2; 3; 4; 5 and 6 hours). For the viral control, the propolis extract is replaced by the RPMI at 2%. After 48 hours of incubation at 37 °C., the wells tested were observed by inverted microscopy in comparison with the viral controls. The same approach as that undertaken for the cell protection test is then performed.

2.7. Fractionation of active extract

Crude propolis sample (10 g) was extracted with absolute ethanol (1:7, w/v), at 70°C, for 30 min and then filtered to obtain its ethanolic extract (EEP). The EEP was fractionated by liquid–liquid extraction, based on a polarity gradient, and hexane (HF), hot water (WF), and ethyl acetate (EAF) fractions were obtained, as previously detailed by Franchin et al. [7].

2.8. Statistical analysis

Cytotoxicity assays were performed in triplicate. CC_{50} and IC_{50} values are presented as the mean ± standard deviation (SD). All the other experiments were carried out in duplicate and the results are expressed as the mean of the obtained data.

3. Results and Discussion

3.1. Evaluation of the cytotoxicity of propolis extracts

The cytotoxicity of the propolis extracts was evaluated by the calculation of the cytotoxic concentration 50% (CC_{50}) (Table 1).

Table 1: Result of the evaluation of the cytotoxicity and antiviral activity of propolis extracts and the active fraction against CVB-3

Extract	CC_{50}	IC_{50}	IS = CC_{50}/CI_{50}
Zelligua	30 ± 2.66	3.33 ± 0.88	9 ± 0.45
Bizerte	30 ± 3.36	11.58 ± 2.49	2.59 ± 0.62
Zouarine	27 ± 4.95	13.10 ± 2.08	2.06 ± 1.12
BK	36 ± 6.62	24.13 ± 4.36	1.49 ± 0.82
EAF of propolis from Zelligua	157 ± 4.79	3.12 ± 1.02	50.32 ± 1.38

The cytotoxicity test was performed on Vero cells. The values of CC_{50} and IC_{50} are expressed in µg / ml. The tests were performed in triplicate independently and the values of CC_{50}, IC_{50} and IS are the average of the results of 3 tests +/- standard deviation.

Overall, the four extracts showed similar CC_{50} values, varying between 27 and 30µg/ml indicating that the toxicity of Tunisian does not depend on the bioclimatic stage in which it was harvested.

The CC_{50} values of these four extracts are considered low, indicating that this product is relatively toxic. Amoros et al 1992 found similar results with French propolis and concluded that the concentration of 30µg/ml appeared to be the highest dose without any discernible toxic effect on cell growth. [10]

3.2. Evaluation of the antiviral activity of propolis extracts

The four propolis extracts did not reveal any activity on HSV-2 but with the exception of the extract collected in the BK region, the other 3 extracts showed activity against CVB-3. Indeed, an extract is considered significantly active against CVB-3 if its SI ≥ 5, and relatively active against this virus if its SI ≥ 2.

In contrast to the cytotoxicity which did not reveal a significant difference between the four extracts, the antiviral activity showed significant differences between these extracts according to their geographical location since with the exception of the propolis sample collected in the Zelligua region which showed a significant antiviral, the samples collected in the regions of Bizerte and Zouarine showed a relative antiviral activity with a SI of about 2, while the one collected in the region of BK did not revealed antiviral activity (Table 1).

3.3. Study of the antiviral mode of action of the most active extract of propolis

The study of the antiviral mode of action was made on the most active extract of propolis, in this case that collected in the region of Zelligua.

To identify at which stage the active extract affected CVB-3 replication, three modes of action were investigated: cell protection, virucidal action and inhibition of viral infection. Our results showed that this extract has completely inhibited virus replication by direct contact (virucidal effect) and also during and after virus penetration (from 0 to 5 h). However, no activity was found when cells were pre-treated with this extract (cell protection).

Ioirich et al. (1965) showed that propolis had virulicidal action *in vitro* against influenza virus (type A) [9].

3.4. Fractionation of the most active extract of propolis

The most active extract of propolis collected in the Zelligua region underwent a fractionation step by liquid-liquid extraction.

Crude propolis from Zelligua region was macerated in Hexane over night and under stirring. After filtration, an hexane fraction (HF) is obtained. During this phase, a process of delipidation is going through and the fraction obtained is called FAT. The dry residue was macerated in hot water over night and after filtration, water fraction (WF) which is full of sugars is obtained. The dry residue was macerated in ethyl acetate over night and after filtration, ethyl acetate fraction (EAF), which is polyphenolic fraction.

3.5. Evaluation of the antiviral activity of propolis fractions

The three fractions obtained by liquid-liquid extraction, (HF, WF and EAF) were evaluated for their cytotoxicity and anti-CVB-3 activity. Only the ethyl acetate fraction (EAF) showed an activity. Better still, this fractionation significantly improved the antiviral activity since the selectivity index went from 9 for the most active starting extract (collected in the Zelligua region) to 50.32 for the acetate ethyl fraction. However, the IC_{50} did not change between the starting extract and the active fraction (Table 1). Variability was mainly observed at the level of CC_{50}, which increased significantly (from 30 to 157 µg / ml) and allowed significantly the reduction of cytotoxicity. As a result, if the antiviral power, materialized by the selectivity index, has improved significantly, it is partly because the fractionation has made it possible to rid the active ethyl acetate fraction of toxic compounds which decrease the antiviral yield of propolis.

4. Conclusion

Four different Ethanolic extracts of Tunisian propolis were investigated for its HSV-2 and CVB-3 activities. Only the extract of propolis collected from the region of Zelligua exhibited activity against CVB-3. This active extract showed a virucidal action both during the entry of viruses and the release of the newly formed virions. This active extract went through a liquid-liquid fractionation and only the ethyl acetate fraction was found active against CVB-3. This fraction is probably the polyphenolic fraction from propolis. Further studies are needed to isolate and identify the active compound in this fraction.

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