ANTIVIRAL AND ANTI-QUORUM SENSING ACTIVITIES OF LYOPHILIZED AQUEOUS EXTRACT OF PROPOLIS FROM SÉTIF

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ABSTRACT

Aqueous extracts are preferentially used in traditional medicine in Algeria. Among these extracts, propolis is used for the treatment of wounds, boils, canker sores, burns. The purpose of this study was to evaluate the antiviral/antiquorum sensing (anti-QS) characteristics of Propolis aqueous extract collected from bee hive near Sétif (east of Algeria) against human pathogenic viruses and gram negative bacteria. First the Maximal Allowable Concentration (MAC) of the lyophilized aqueous extract of propolis was determined to avoid the toxicity of the extract. A human lymphoid cell line that contains Epstein Barr Virus (EBV) genome as an episome (P3HR-1) and HEp-2 infected with Coxsackie virus (CVB4), were selected according to their ability to deliver endemic infectious viruses at high titers. Anti-bacterial activity was screened by evaluating anti-QS capacity of the extract. Pre-treatment revealed that the MAC of the extract reduces the viral titer of Coxsackie virus by half a log from TCID<sub>50</sub> = 10<sup>7</sup> to TCID<sub>50</sub> = 10<sup>4</sup>, and protected HEp-2 against CVB4 infection. While no antiviral effect on the EBV replication was obtained. The anti-QS capability of the extract was showed against Chromobacterium violaceum strain 026, and by protecting Artemia from Vibrio harveyi BB120 infection. The aqueous extract pre-treatment has not antiviral protection for DNA viruses. It protected HEp-2 cells against CVB4 infection, degraded Acril Homoserine lactones (AHL), and protected animal Artemia.

Keywords: lyophilized aqueous extract; propolis; anti-QS; anti-viral; cytotoxicity

INTRODUCTION

In big cities like Algiers, there are herbalists essentially at the level of the markets, and their stalls are frequented by a broad public which goes from the adept diligent, convinced of the benefits of the alternative medicines, to the indigent patient in search of an accessible treatment (Hammiche et al., 2013). Hive products have been used by humans for millennia. Beehive products such as honey, propolis, and royal jelly were extensively used to treat several diseases (Paspuleti et al., 2017). Propolis is still the most used one in folk medicine worldwide. It is collected from sap flows, bark, and leaf buds of a considerable variety of plants, ranging from annual plants to perennial ones. However, the most plants visited for such collection are poplar and conifer trees (Nadjafti et al., 2007). Recent studies revealed a new type that was named Mediterranean Propolis, which contains high levels of diterpenoids. This propolis was collected from Greece (Popova et al., 2010; Celenli et al., 2013), Malta (Popova et al., 2011), Turkey (Silici et al., 2007; Duran et al., 2011), Algeria (Pincicelli et al., 2013; Soltani et al., 2017; Chaa et al., 2021). Bees use propolis as an immunity component that protects their community from micro and macroorganisms’ invasion. The propolis is gifted by propolis was harvested by scraping the hive honeycombs on a site (El Quarsia) adjacent to the central campus of the Ferhat Abbas University Sétif 1 between August and September 2012. 

Monte et al., 2014; Soltani et al., 2017, 2021). Plant extracts may have activities that inhibit bacterial virulence and pathogenicity. These effects are neither bactericidal nor bacteriostatic and do not put pressure to develop resistance (Chenia, 2013). In an editorial Williams (2006) reported that virulence factors constitute one of a variety of bacterial physiological processes regulated via “Quorum Sensing”. Antimicrobial chemotheraphy aimed at inhibiting the regulation of virulence factor expression could have a more global effect on the ability of an organism to establish infection (Alksne and Projan, 2000). Powerful antipathogenic compounds able to target bacterial signaling systems are present in nature. These compounds (Hentzer and Givskov, 2003) such as Cinnamaldehyde (Brackman et al., 2011) interfere directly with Quorum sensing (QS) signaling systems that control biofilm formation, pathogenicity, and virulence, which brings an attractive target for developing drugs that control microbial activity (Hentzer and Givskov, 2003). In the present study two goals were targeted: (i) The first goal evaluated the cytotoxic/protective power of the lyophilized aqueous extract of Propolis in the presence and absence of virus or bacteria; and (ii) the second goal focused on the investigation of an eventual mode of activity of the extract: its anti quorum sensing (anti-QS) power.

MATERIAL AND METHODS

Material

Propolis

The raw propolis was harvested by scraping the hive honeycombs on a site (El Quarsia) adjacent to the central campus of the Ferhat Abbas University Sétif 1 between August and September 2012.
Bacterial Strains

Chromobacterium violaceum strain 026, Vibrio harveyi BB120, and Artemia cysts were from the laboratory of Dr. Natrah FMI Faculty of Agriculture, University Putra Malaysia.

Cell lines and viruses

Human lymphoid cell line (P3HR-1 cells) that contains Epstein Barr Virus (EBV) genome as an episome, HEP-2 cells derived from human squamous cell carcinoma of the larynx and Coxsackievirus (CVB4); have been provided respectively by Viral Oncogenesis Laboratory and the referenced Enterovirus Laboratory for the Coxsackievirus (Pasteur Institute of Algiers, Algeria). The CVB4 virus was propagated in HEP-2 cells in Eagles Minimum Medium (EMEM) enriched with 2% fetal calf serum (FCS) and stored at -80°C.

Methods

Lyophilized Aqueous Extract

The lyophilized aqueous extract of propolis was obtained as previously described by Haichour et al. (2021). Extracts solutions were prepared by dissolving 100 mg/mL of the lyophilized powder obtained in sterile water, filtered through Millipore millipore syringe driven filter unit (0.22µm). Then a serial dilution (1/2 and 1/4) of each solution is made for the anti-QS, and for antiviral activity, 25mg/mL were used in a serial dilution by half till 7.81µg/mL.

Cell culture

Cells of P3HR-1, were cultivated at a rate of 5.10^5 cells/mL in Roswell Park Memorial Institute (RPMI1640) medium supplemented with 10% FCS from Welgene (Hinuma et al., 1967), 1% of antibiotic (Penicillin-streptomycin; Sigma-Aldrich®), and 1% of the L-Glutamine (Sigma-Aldrich®); incubated at 37°C with 95% of humidity and 5% CO₂ (Zur Hausen et al., 1979).

Cells of HEP-2 were cultivated in Hank Minimum Essential Medium (HMEM) supplemented with 10% FCS, 1% L-Glutamine, and 100 µg/mL of Penicillin and 100 µg/mL of Gentamicin (Sigma-Aldrich®) (Gorpe, 2019).

Cytotoxicity test of the extract

Cytotoxicity of the extracts was performed according to Abid et al. (2012) with some modifications. instead of MTT (3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) crystal purple for HEP-2 and trypan blue for P3HR-1 cells which were reduced by viable cells (Strober, 2015) and. The viability of the cells was evaluated spectrophotometrically at 492 nm by the Crystal Purple (0.5%) staining method for HEP2 (Ait-Mbarek et al., 2007), and Trypan blue method for P3HR-1 (Strober, 2015). Cell suspensions (5 × 10^5 cells/mL) were distributed (100 µL/well) and cultivated as described in cell culture section using 96-well plates, and after incubation at 37°C for 24h they were treated with 50 µL of the extract at different concentrations, the cells were incubated for an additional time of 48 h (Abid et al., 2012). The blank consist of wells receiving cells without treatment. The cytotoxicity percentage in HEP-2 was calculated as [(U - T) / U] x 100, where U and T were respectively the OD492 of untreated and of treated cells. The 50% cytotoxic concentration (CC50) calculated by regression analysis was defined as the compound’s concentration (mg/mL) required for the reduction of cell viability by 50%. While for P3HR-1 cells the percentage of cell viability (Vi) was performed by inverse macroscopy enumeration using the following formula (Anonymous, 2006): Vi % = number of Clear cells/Total Cells number x 100.

Viruses’ inhibition assays

Confluent HEP-2 cells in microplates, were treated with decreasing concentrations of the extract from the MAC (Maximal Allowable Concentration: nontoxic) between 0.78 mg/mL and 0.09 mg/mL. After 2 hours of incubation; 100 µL TCID₅₀ (Tissue Culture Infectious Dose at 50%) of the viral suspension were added and incubated for 48 hours at 37°C under 5% CO₂. Viral inhibition effect (cell viability) and cytopathological change were assessed using values of Crystal violet (0.5%) for protecting cells of HEp2 (fig. 1 and P3HR1, fig. 2) with a CC₅₀ at 7 mg/mL for HEp-2 cells with an IC₅₀ of 0.53 mg/mL and a selective (therapeutic) index (SI) of 13.20. A

were incubated for 72h as previous conditions. Antiviral activity was performed by Trypan blue method or by Indirect Immune Fluorescence (IFI) (Strober, 2015).

Screening the anti-QS

Chromobacterium violaceum strain 026 (CV026) was grown in Luria Bertani broth (LB) with 20 µg/mL. Kanamycin (SIGMA) and 5 µg/mL of N-Hexanoyl-DL-Homoserine Lactone (SIGMA) for 48 h at a temperature of 32°C with 140 rpm aerobically to an estimated concentration of 10⁵ cell/mL. 20 mL of CV026 were mixed with 80 mL, soft LB Agar (0.8%, SIGMA) and homogenized. The mixture was poured onto hard Agar (1.5%) to have a double layer, after its solidification, wells were made in the upper layer using a sterile Pasteur pipette. 20 µL of the prepared extract of each dilution was filled in the prepared well. Negative controls consisted of distilled water; the positive control was Trans-cinnamaldehyde (SIGMA Aldrich) at 0.1M. Plates were incubated overnight at 32°C; CV026 was used to detect and respond to the presence of Acyl Homoserines lactones (AHL) molecules through the synthesis of purple pigmentation (violacen), absence of the purple color indicated inhibition of violacin and degradation of AHL molecules (degradation of QS activity). The diameters of the non pigmented observed zones were measured (Noorashikin et al., 2016). To confirm the degradation of AHL another assay was performed as follows.

AHL degradation assay

For this purpose, 10 µg/mL of N-Hexanoyl-DL-Homoserine Lactone (HHL, 8µL/2ml) were added to the positive control as well as the extract solution. This later was filtered through a 22µm filter and then 10 µL were spotted on the LB agar plate already inoculated with 100µL of CV026. The last step was repeated after 4 hours (Noorashikin et al., 2016). The QS degradation activity was observed through violacian inhibition, and the diameters of the non pigmented observed zones were measured.

Artemia Challenge

Artemia was used for testing the toxicity of the extract on living organisms, according to Soto–Rodriguez et al. (2003) with some modifications. First cysts of Artemia were decapsulated aseptically in sterile seawater to facilitate their hatching. Decapsulated cysts in falcon tubes were placed on aerator with constant light at 28°C, and incubated for 24h at least. Secondly, two batches of Artemia with 12.5µg/mL of the extract were prepared, one for testing its toxicity and the second for protecting Artemia against V. harveyi BB120 (10cell/mL); in addition to the control batches consisting of Artemia (i) without extract and bacteria (+ve one) (ii) and without extract but with bacteria (+ve one). The batches consist of 20mL of sterile seawater to which 100µL of yeast extract (10g/L) and twenty hatching Artemia were added. The experiment was repeated two times with two repetitions.

Statistical analysis

Data were analyzed using Graph Pad Prism 8.4.2 Statistical software (Graph Pad Software, USA). Analysis of Variance (ANOVA), one way and Tukey’s (anti QS, Hep2 protection); two way and Tukey’s (Artemia viability, P3HR-1, and Hep-2 cell multiplication), two way and Sidak’s multiple comparisons test (AHL degradation). Data were presented as mean ± standard error (SEM) and differences were considered significant at P < 0.05.

RESULTS

Cytotoxicity and antiviral effect of lyophilized aqueous extracts

The lyophilized aqueous extract of propolis showed a cytotoxic effect on the viability of tested cells (HEP-2, fig 1 and P3HR1, fig. 2) with a CC₅₀ at 7 mg/mL for HEP-2 cells and 0.230 mg/mL for P3HR1 cells. While concentrations of 0.78, 0.39, and 0.19 mg/mL; showed no inhibition effect on HEP-2 cells (100% of the cells are viable); the same was observed with P3HR1 at concentrations bellow 125µg/mL. Concentrations of 25, 12.5, 6.25, 3.12, 1.56 mg/mL, and 500, 250 µg/mL acted respectively on the growth and appearance of HEP-2 and P3HR1 cells with different inhibition percentages compared to untreated control. A concentration of 25 mg/mL inhibited 60% of HEP-2 cells; at 12.5, 6.25, 3.12, and 1.56 mg/mL, the inhibition percentage was respectively about 54, 49, 40 and 28% as shown by the Purple Crystal method; and P3HR1 cells were inhibited at a rate of 67.96% when treated with 500µg/mL shown by Trypan blue method.

By evaluating its antiviral effect and compared to positive control, the MAC of the extract reduces the viral titer of Coxsackievirus by half a log from TCID₅₀ = 10⁰/T to TCID₅₀ = 10⁶/T. Concerning EBV and after induction of P3HR1cells by TPA, the results obtained showed no antiviral effect on the replication of the virus, the expression of the Viral Capsid Antigens (VCA) by IFI was very significant. Otherwise, the aqueous extract of propolis showed a protective effect against CVB4 infection (fig.3), it inhibited the multiplication of the virus in HEP-2 cells, with an IC₅₀ of 0.53 mg/mL and a selective (therapeutic) index (SI) of 13.20. A
total destruction of the cellular layer was obtained with concentrations of 0.19, 0.09, 0.04, 0.02, 0.01 and 0.006 mg/mL; while concentrations of 0.78 and 0.39 mg/mL showed a protective effect against CVB4 infection, with protection percentages of 76% and 37% respectively.

Figure 1 Cytotoxicity of the lyophilized aqueous extract of Propolis on Hep2 cells

****: Statistically very significant (P<0.0001)

Figure 2 Cytotoxicity of the lyophilized aqueous extract of Propolis on P3HR1 cells

***Statistically significant (P<0.0004)

Figure 3 Protection of Hep2 cells against infection by CVB4

Antibacterial activity versus Anti-QS activity

Artemia Challenge

In the in vivo virulence test, Artemia survived differently when it was infected with Vibrio harveyi BB120 in the presence and absence of the extract; amongst twenty Artemia used in the first batch 19 ±1 were still alive, they were grown and moved well after 24h of feeding. While in the second batch treated with propolis extract and inoculated with V. harveyi BB120, 77.6% of the organisms were still alive and being well after 24h of feeding too. On the other hand, the positive control with V. harveyi BB120 decreased to 12 ±1 organisms (60% alive) after 6 h of treatment only (fig. 4).

Figure 4 Protection of Artemia against infection by Vibrio harveyi BB120

Negative control: Neither treatment nor BB120; T: treatment with aqueous extract at 12.5µg/mL; T+BB120: treatment with aqueous extract at 12.5µg/mL+ Vibrio harveyi BB120 at 10^6 cell/mL; positive control: Vibrio harveyi BB120 at 10^6 cell/mL; ****: Statistically very significant (P<0.0001)

Anti-QS activity of the lyophilized aqueous extract

To understand partially how the extract protected Artemia against V. harveyi BB120, its anti-QS capacity was evaluated. As compared to a positive control consisting of Cinnamaldehyde, the inhibition, by the extract, of QS was demonstrated by the loss of the purple pigment in strain 026 of Chromobacterium violaceum, thus showing a clear zone around the wells and the diameter of which was concentration-dependent. The negative control was only water which remained purple (fig. 5, 6).

Figure 5 AntiQuorum Sensing screening

P1: lyophilized aqueous extract of propolis at 100mg/mL, water: negative control, cinnam: positive control (cinnamaldehyde at 0.1M)

Figure 6 AntiQuorum Sensing activity of Propolis lyophilized aqueous extract

100, 50 and 25mg: aqueous extract of propolis diluted/ml, water: negative control, cinnam: positive control (cinamaldehyde at 0.1M); Anti-Qs diameter: diameter of the degradation zone of the quorum sensing compound produced by Chromobacterium violaceum strain 026. ****: Statistically very significant (P<0.0001), ***: Statistically significant (P<0.0004)
Degradation of QS molecules

The degradation power of the extract (fig.7) was confirmed using the agar diffusion method, by the absence of pigmentation (inhibition of the violacarin purple color regulated by QS) in the inoculated zone. This latter was increased after 72h (fig. 8) as compared to positive and negative controls. The extract inhibited the pigmentation with mean a diameter of 12.05±1.11 mm, while the positive control inhibited with a mean diameter of 14.20±.1.42 mm.

The statistical analysis revealed that no significant differences were obtained for all the antiviral tests, while a significant difference was obtained for AHR degradation one.

before the virus entry into cells or after their release to the supernatant (Bufalo et al., 2009).

In addition to the cytotoxic activity against the growth of lineage cells (HEp-2 and P3HR1), the expression of surface antigens (VCA) of the EBV rather than the product of infection in tumorderived cells would be attributable to an anti-proliferative effect. Pretreatment and continuous treatment of propolis extract in NIH/3T3 cell cultures showed an impressive inhibition of malignant cell transformation by Moloney murine Sarcoma Virus (MuSV) (Huleihel and Ishano, 2001).

The antiviral and the anti proliferative activities of the extract of propolis could be attributed to its contents in flavonoids (3,047 ± 0,004 mg/g), polyphenols (721 ± 0,001 mg/g), and its four identified derivatives of cinnamic acid (Soltani, 2017; Soltani et al., 2021).

Cinnamaldehyde a derivative of cinnamic acid has been reported as an inducer of apoptosis on various cancer cell lines in vitro, that transduced the apoptotic signal via reactive oxygen species (ROS) generation (Ka et al., 2003; Li et al., 2016). Investigating flavonoids from plant extracts against Coxsackievirus B3 (CVB3), Abid et al. (2012) demonstrated that flavonoids enhanced antiviral activity at noncytotoxic concentrations. While earlier, Bufalo et al. (2009) demonstrated that cinnamonic acid a compound from propolis has also an antiviral activity against replication of PV1. Hazan et al. (2021) demonstrated that direct contact with acetylclov and other pure molecules e.g. quercetin, caffeic acid and chlorogenic acid were very effective in inhibiting Herpes simplex 1 virus (HSV1) and varicella zoster virus (VZV) while in the case of adenovirus type 5 (AD5) most of the substances were not so efficient. The propolis aqueous extract was most effective in pre-treatment of AD5 – infected cells, more efficient than the propolis tincture, as well as more efficient than acetylclov; while post-treatment it did not protect HSV1 infected cells (Hazam et al., 2017). These findings may explain partially the non activity of the lyophilized aqueous extract on EBV (DNA virus) and vice versa.

The inhibition of cell growth, necrosis, apoptosis (Oršolić et al., 2001, 2003), and metastasis formation (Oršolić and Bašić, 2003, 2005a) in tumor cells are the main mechanisms by which propolis acts. Metastasis is mediated by immunomodulatory activity by increasing macrophages activity (Oršolić and Bašić, 2003).

Using continuous and discontinuous exposure methods on P3HR1 cell lines in an unpublished data, Rihane and Ouanes (2017) showed a dose dependent apoptotic effect and DNA fragmentation of the lyophilized aqueous extract of propolis tested in the present study. The anti metastatic activity as well as its antitumor activity (Oršolić et al., 2005), were the result of synergistic activities of a water-soluble derivative of propolis (WSDP) components (Oršolić and Bašić, 2005b) as flavonoid e.g. caffeic acid a cinnamic acid derivate (Oršolić et al., 2001) and polyphenolic compounds (Oršolić et al., 2003).

The lyophilized aqueous extract of propolis protected Artemia against bacterial infection and inhibited the QS activity. This extract has proved bactericidal activities against several bacteria; Vibrio harveyi, Photobacterium damselae (Soltani et al., 2017), Staphylococcus aureus ATCC25923, Bacillus cereus ATCC10876, Pseudomonas aeruginosa ATCC27853, Klebsiella pneumoniae ATCC700603 and Enterobacter cloacae (Soltani et al., 2021), respectively two marine opportunist (Soltani et al., 2017) and human pathogenic bacteria (Soltani et al., 2021). The lowest bactericidal activity was found against P. damselae (Soltani et al., 2017), P. aeruginosa ATCC27853, Klebsiella pneumoniae ATCC700603 and Enterobacter cloacae (Soltani et al., 2021), while in Staphylococcus aureus it was in the case of V. harveyi (Soltani et al., 2017) Staphylococcus aureus ATCC25923 and Bacillus cereus ATCC10876 (Soltani et al., 2021). Monte et al. (2014) have already demonstrated that furfural acid (hydroxycinnamic acid) was more effective than gallic acid (hydroxybenzoic acid) against Escherichia coli and Staphylococcus aureus.

In the presence of Vibrio harveyi BB120, with and without the addition of the extract Artemia survives differently. The extract significantly increased the survival of Artemia after infection. Earlier, Brackman et al. (2011) reported the increased survival of Ctenoharabdis elegans nematodes infected with V. harveyi, V. anguillarum and V. vulnificus in the presence of cinnamamic acid and its structural analogs.

These activities can be assigned to its components as, benzoic acid, cinnamate, and its derivatites which were present in the extract (Soltani et al., 2017). Cinnamic acid derivative, cinnamaldehyde, and most analogs reduced the Vibrio species starvation response, biofilm formation. V. vulnificus, V. anguillarum, V. cholerae and V. cholerae, protease production, V. anguillarum and V. cholerae, and pigment production in V. anguillarum. They blocked at least 2× (autoducer-2) QS (Brackman et al., 2011).

Our data showed decreased violacein production when the extract was added exogenously, as observed through halo zone formation, as obtained by Kalia et al. (2018). As expected, zones of QSI inhibition were also observed with the control cinnamaldehyde. This latter has been reported as HSL degrader (Noorashikin et al., 2016). Finally, the aqueous propolis extract, had antiviral activity against CVB 4, do not provide antiviral protection in the case of EBV, and had an anti-QS activity which correlated with the QSI (quorum sensing inhibition) activity that disquered QS AHL bacterial communication mechanism. On the base of these results, more investigations are needed for the study of the Algerian propolis extract.
CONCLUSION

The use of natural compounds to attenuate viral and bacterial pathogenicity is an attractive approach, particularly if, at the dosages used these inhibitors are nontoxic for living organisms. In this study, the aqueous extract of propolis from Sefīl possess anti viral activity against B4 Coxackievirus and an anti-QS effect in C. violaceum. The use of the extract as quorum sensing-disrupting compound protects living organisms as Artemia larvae from V. harveyi BB120 without a negative effect on the growth of the larvae.

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