

Anti-HIV-1 activity of propolis in CD4⁺ lymphocyte and microglial cell cultures

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Abstract

An urgent need for additional agents to treat human immunodeficiency virus type 1 (HIV-1) infection led us to assess the anti-HIV-1 activity of the natural product propolis in CD4⁺ lymphocytes and microglial cell cultures. Propolis inhibited viral expression in a concentration-dependent manner (maximal suppression of 85 and 98% was observed at 66.6 µg/ml propolis in CD4⁺ and microglial cell cultures, respectively). Similar anti-HIV-1 activity was observed with propolis samples from several geographic regions. The mechanism of propolis antiviral property in CD4⁺ lymphocytes appeared to involve, in part, inhibition of viral entry. While propolis had an additive antiviral effect on the reverse transcriptase inhibitor zidovudine, it had no noticeable effect on the protease inhibitor indinavir. The results of this *in vitro* study support the need for clinical trials of propolis or one or more of its components in the treatment of HIV-1 infection.

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1. Introduction

A variety of natural products or their derivatives have been considered as potential candidates for the treatment of human immunodeficiency virus type 1 (HIV-1) infection (Cowan, 1999; De Clercq, 2000). Given the escalating incidence of HIV-1 resistance to standard antiretroviral drugs and the need for agents that are less toxic and expensive than the ones currently in use, the search for new treatments amongst these natural products is warranted. In the present study, one

such product, propolis, was evaluated for its effect on HIV-1 expression *in vitro*.

Propolis (also referred to as “bee glue”) is the generic name for a strongly adhesive resinous substance collected by honey bees from trees and leaf buds. While propolis is produced by a variety of plants, most plant species are not known because bee collection takes place high up in trees so, it is difficult to observe. Only one plant species, *Baccharis dracunculifolia*, is an established source of propolis (Santos et al., 2003), although several genera, i.e. *Populus*, *Clusia* and *Araucaria*, are regarded as additional sources of propolis (Bankova et al., 2000). Aptly named by the Greeks (according to some scholars by Aristotle), ‘pro’ (for or in defense) and ‘polis’ (the city), propolis is used to protect the entrance of the hive against intrusion of animals and within the hive against a wide spectrum of microorganisms (Banskota et al., 2001; Burdock, 1998).

Used for medicinal purposes since antiquity, propolis has been shown in more recent times to possess broad spec-

Abbreviations: Ag, antigen; AIDS, acquired immunodeficiency syndrome; HIV-1, human immunodeficiency virus type 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; CPRG, 5-bromo-4-chloro-3-indolyl-β-D-galactoside

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trum antimicrobial activity, including activity against many of the opportunistic pathogens associated with the acquired immunodeficiency syndrome (AIDS) (Banskota et al., 2001; Burdock, 1998). Studies of its antiviral properties have concentrated mainly on herpes simplex virus (Amoros et al., 1994; Vynograd et al., 2000) and influenza virus (Serkedjieva et al., 1992). Using a cell line (CEM cells), Harish et al. (1997) demonstrated that propolis potently inhibited HIV-1 expression. However, little or no data have been published regarding its antiretroviral activity in the primary cell targets of HIV-1, i.e. CD4⁺ lymphocytes and macrophages, which was the subject of this study.

2. Materials and methods

2.1. Propolis samples

Crude propolis purchased from Cannon Honey Bee Company (Minneapolis, MN) was used for most experiments. To determine whether the antiviral activity of the sample obtained from Cannon Honey Bee Company was representative of the activity of propolis collected from other geographic locations, samples of crude propolis were also obtained from colonies located in southeastern (provided by BeeHive Botanicals, Hayward, WI) and northern (provided by B&B Honey Farm, Houston, MN) Minnesota, three states in Brazil (Rio Grande do Sul, Rio de Janeiro and Minas Gerais; provided by Apis Flora in Ribeirão Preto, SP) and China (provided by BeeHive Botanicals). All of the above samples were collected by scraping the propolis from wooden beehive equipment and were conglomerates from an unknown number of bee colonies. To determine if there was inter-colony variation among colonies located in specific geographic locations and to obtain clean propolis samples (uncontaminated by beeswax and woodenware), we also trapped propolis using commercial traps (J&D Manufacturing, MI) from three colonies in each of three locations in Minnesota: University of Minnesota, St. Paul campus, Houston (southeastern MN) and Duluth (northern MN).

2.2. Ethanolic extracts of propolis

Ethanolic extracts of propolis were prepared as previously described (Kress, 1996). Briefly, propolis was ground and 20% ethanolic extracts of propolis were prepared (20 g of propolis completing the volume to 100 ml of 95% ethyl alcohol), protected from light, with moderate shaking at room temperature. After 1 week, extracts were filtered and diluted in culture medium to carry out experiments.

2.3. HIV-1 isolates

HIV-1_{AT}, a clinical isolate with characteristics of a T-tropic (X4) strain, and HIV-1_{SF162}, a monocytotropic (R5)

variant obtained from the NIH AIDS Research and Reference Reagent Program (National Institute of Allergy and Infectious Diseases, Bethesda, MD), were used in this study.

2.4. Cell cultures and cytotoxicity assays

Peripheral blood mononuclear cells were obtained from venous blood of healthy donors and previously described methods (Gekker et al., 2001) were used to prepare and isolate activated CD4⁺ lymphocytes ($\geq 98\%$ of cells stained positively with anti-CD4 antibodies). Microglial cells (the resident macrophages of the brain) were isolated from human fetal brain tissue, under a protocol approved by our Institutional Review Board and homogenous cell cultures ($\geq 99\%$ stained positively with anti-CD68 antibodies) were prepared as previously described (Peterson et al., 1999).

To assess the cytotoxic effect of propolis, cell viability was quantified microscopically using a trypan blue exclusion assay and also was assessed using an MTT assay 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide mitochondrial dehydrogenase as previously described (Jiang et al., 2001).

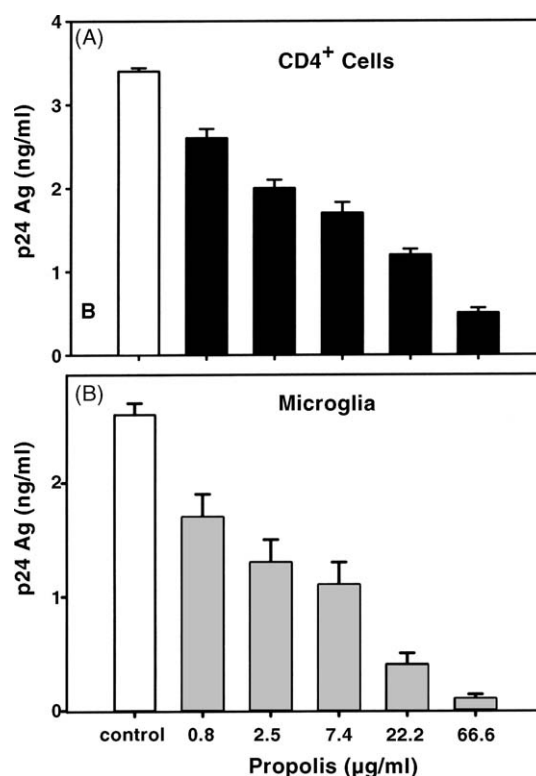


Fig. 1. Effect of propolis on HIV-1 expression in CD4⁺ lymphocyte and microglial cell cultures. Activated CD4⁺ lymphocytes were infected with HIV-1_{AT} (A) and microglial cells were infected with HIV-1_{SF162} (B) in the absence (control) or presence of propolis (Cannon Honey Bee Company preparation) at indicated concentrations. Data are mean \pm S.D. of triplicate values and are representative of three independent experiments using CD4⁺ lymphocytes from different donors and microglia isolated from different brain tissue specimens.

Using the MTT assay, the selectivity index was determined as previously described (Amres et al., 2001), i.e. the ratio of the 50% cytotoxic concentration (CC₅₀) to the 50% effective concentration (EC₅₀).

2.5. Assessment of anti-HIV-1 activity

To evaluate the effect of propolis on HIV-1 expression, propolis was added at indicated concentrations to activated

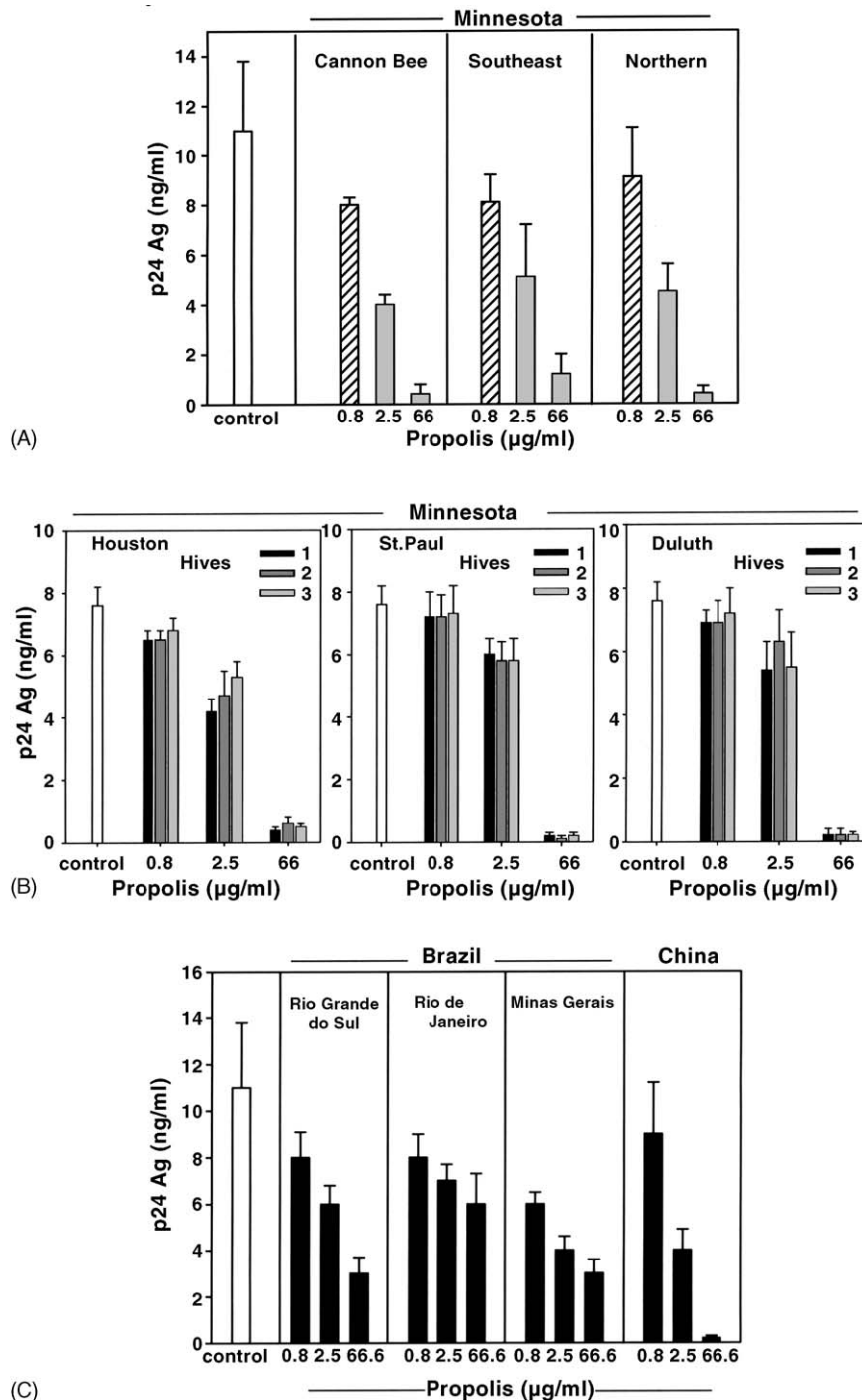


Fig. 2. Anti-HIV-1 activity of propolis from different geographic regions: (A) commercial sources in Minnesota: Cannon Bee Honey Company, southeast or northern Minnesota; (B) three colonies in each of three specific locations in Minnesota: Houston (southeastern), University of Minnesota, St. Paul campus and Duluth (northern); (C) three states in Brazil and China. Activated CD4⁺ lymphocytes were infected with HIV-1_{AT} in the absence (control) or presence of propolis preparations at the indicated concentrations. Data are mean ± S.D. of triplicate samples and are representative of two independent experiments using CD4⁺ cells from different donors.

CD4⁺ lymphocyte cultures at the time of infection with HIV-1_{AT} or to microglial cell cultures at the time of infection with HIV-1_{SF162}. After 2 h (CD4⁺ cells) or 18 h (microglia) of incubation, cells were washed and resuspended in culture medium alone (control) or in medium containing propolis. Cells were then incubated for 3 days (CD4⁺ cells) or 7 days (microglia) and supernatants were collected for measurement of p24 Ag levels. In one experiment, propolis was added to cell cultures that were simultaneously treated with zidovudine (AZT) or indinavir at drug concentrations that approximated their EC₅₀ values and after washing, cells were resuspended in culture medium alone (control) or medium containing propolis plus AZT or indinavir. HIV-1 expression in CD4⁺ lymphocytes and microglial cell cultures were assessed by measuring p24 antigen (Ag) levels in cell culture supernatants by ELISA, as previously described (Gekker et al., 2001; Peterson et al., 1999).

2.6. Assessment of viral entry

To determine whether propolis affects HIV-1 entry into CD4⁺ lymphocytes, we used a vaccinia virus-based assay which quantifies cell fusion-dependent reporter gene activation in response to HIV-1 IIIB-Env glycoprotein-mediated membrane fusion (Nussbaum et al., 1994; Stantchev and Broder, 2000) with minor modifications (Lokensgard et al., 2002). Vaccinia virus-infected activated CD4⁺ lymphocytes were treated with propolis prior to mixing with vaccinia virus-infected HeLa S3 cells and the amount of β-galactosidase in the cultures was quantified by using the CPRG substrate. β-Galactosidase concentrations in the lysates were determined from a standard curve.

2.7. Statistical analysis

For comparison of means of multiple groups, analysis of variance (ANOVA) was performed followed by Sheffe's *F*-test. For analysis of the effect of propolis on viral entry in which propolis values from treated samples were expressed as % inhibition relative to untreated (control) samples, a mixed effects repeated measures model was used that accounts for within-person correlations and intrinsic differences among individuals. The Tukey method was applied to adjust for multiple comparisons and the mixed procedure in SAS Version 8.2 was used to perform this analysis.

3. Results and discussion

Prior to investigating its effect on HIV-1 expression, an experiment was performed to determine whether propolis (Cannon Honey Bee Company) was toxic to CD4⁺ lymphocytes or microglia. After 4 days of incubation in the absence (control) or presence of propolis (at concentrations ranging between 0.82 and 200 μg/ml), cell viability was quantified by trypan blue dye exclusion and MTT assay. At propolis

concentrations of ≤66.6 μg/ml, the viability of treated cells by both assays did not differ from control cells. Thus, for all experiments, propolis extract was used at concentrations of 66.6 μg/ml or less.

To determine the effect of propolis on HIV-1 expression, cells were treated with various concentrations of propolis obtained from Cannon Honey Bee Company. As is shown in Fig. 1, propolis inhibited in a concentration-dependent manner the expression of HIV-1 in CD4⁺ lymphocyte and microglial cell cultures. In CD4⁺ cell cultures, 66.6 μg/ml propolis inhibited by >85% expression of the X4 HIV-1 variant (Fig. 1A) and in microglial cell cultures, 66.6 μg/ml propolis inhibited viral expression of the R5 HIV-1_{SF162} isolate by 98% (Fig. 1B). The selectivity index (CC₅₀/EC₅₀) was 6.7 for CD4⁺ lymphocytes and 16.3 for microglial cells.

To determine whether the antiviral potency of propolis varied in samples from different regions of Minnesota, preparations from southeast and northern Minnesota were compared to the Cannon Honey Bee Company in CD4⁺ lymphocyte cultures. As is shown in Fig. 2A, the anti-HIV-1 activity of these propolis preparations were similar. Also, propolis collected from three separate colonies in three areas of the state (Houston, St. Paul and Duluth, MN) were found to have comparable antiviral activity (Fig. 2B). When propolis samples from three states in Brazil and one sample from China were assessed, all demonstrated anti-HIV-1 activity, but the sample from Rio de Janeiro appeared least effective (<50% inhibition of viral expression at 66.6 μg/ml propolis) (Fig. 2C).

Due to mounting resistance of HIV-1 to reverse transcriptase inhibitor (RTIs) and protease inhibitor (PI)s, antiviral research has been directed in recent years at finding drugs that work via different mechanisms, such as interfering with HIV-1 entry into cells. Using a cell fusion assay that measures HIV-1 cell entry into CD4⁺ cells, propolis (Cannon Honey Bee Company) suppressed cell fusion at all concentrations tested with an EC₅₀ of approximately

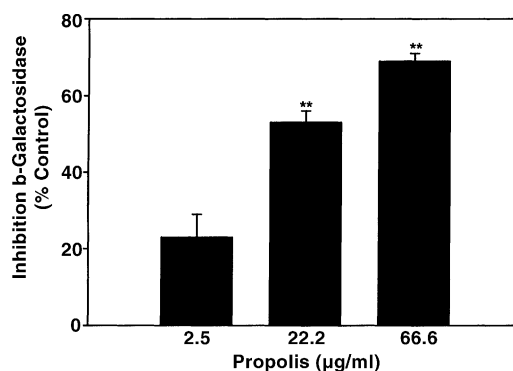


Fig. 3. Effect of propolis on viral entry. CD4⁺ cells were used as partners with IIIB-Env expressing HeLa S3 cells in the absence (control) or presence of propolis and fusion was measured using the reporter gene activation assay analyzed by the colorimetric CPRG method. Data (mean ± S.E.M. of values from three different donors) are expressed as percent inhibition of β-galactosidase relative to control values. ***P* < 0.01 vs. control (by mixed model analysis incorporating intra person correction).

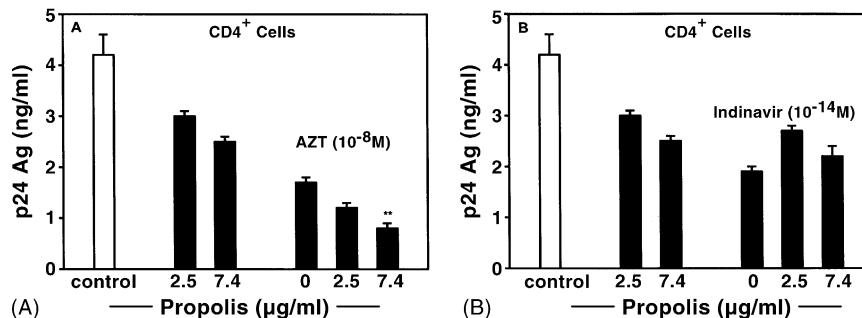


Fig. 4. Effect of propolis on antiretroviral drugs in CD4⁺ lymphocyte cultures. CD4⁺ cells were infected with HIV-1_{AT} in the absence (control) or presence of propolis alone or in combination with: (A) AZT or (B) indinavir. Data are mean \pm S.D. of triplicate values and are representative of three independent experiments using CD4⁺ lymphocytes from different donors. ** $P < 0.01$, compared to AZT in the absence of propolis by (ANOVA).

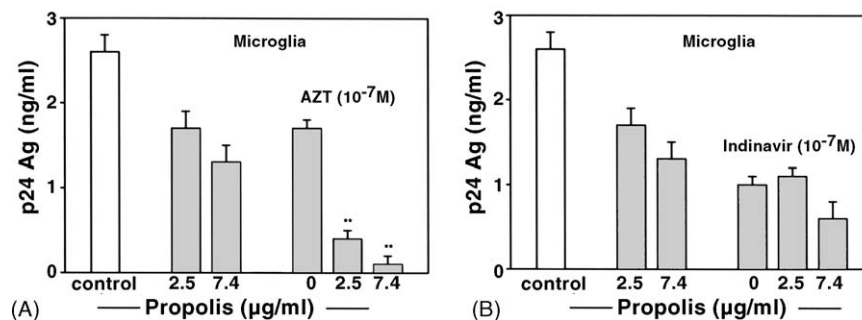


Fig. 5. Effect of propolis on antiretroviral drugs in microglial cell cultures. Microglial cells were infected with HIV-1_{SF162} in the absence (control) or presence of propolis alone or in combination with: (A) AZT or (B) indinavir. Data are mean \pm S.D. of triplicate values and are representative of three independent experiments using microglial cells from different brain tissue specimens. ** $P < 0.01$, compared to AZT in the absence of propolis by (ANOVA).

22.2 µg/ml (Fig. 3). Using CD4⁺ lymphocytes from three different donors, 22.2 µg/ml propolis suppressed cell fusion by $52.3 \pm 4.9\%$ (mean \pm S.E.). Thus, it appears that the antiviral effect of propolis in CD4⁺ lymphocytes is mediated, at least in part, by inhibiting viral entry into cells.

Currently, antiretroviral therapy involves the use of drug combinations with different mechanisms of action. To study potential interactions between propolis and standard antiretroviral agents, propolis (Cannon Honey Bee Company) was added to CD4⁺ lymphocyte and microglial cell cultures that were simultaneously treated with AZT or indinavir. As is shown in Fig. 4, at a concentration of 7.4 µg/ml, propolis appeared to have an additive effect on AZT-mediated viral suppression (Fig. 4), but it had no effect on indinavir (Fig. 4) in CD4⁺ lymphocyte cultures. When microglial cells were studied, propolis again had an additive effect on AZT (Fig. 5) and no significant effect on indinavir (Fig. 5).

4. Conclusions

In conclusion, the results of these in vitro studies suggest that propolis has potent antiviral activity against X4 and R5 HIV-1 variants in the major cell types that are infected by this virus in vivo. Our studies with CD4⁺ lymphocytes suggest that propolis from several geographic regions has similar activity and that propolis operates, at least in part, as a viral

entry inhibitor. Also, our results suggest it is unlikely that propolis will antagonize the anti-HIV-1 activity of RTIs such as AZT or PIs such as indinavir. The antiretroviral component(s) of propolis, a natural product which contains over 180 constituents (Burdock, 1998), is unknown. However, flavonoids (Wang et al., 1998; Critchfield et al., 1996; Xu et al., 2000), moronic acid derivatives (Ito et al., 2001) and caffeic acid (Burke et al., 1995), all of which are found in propolis, have been demonstrated by several research groups to possess anti-HIV-1 activity. The widespread use of propolis as a herbal remedy for almost three millennia and the results of toxicology studies in rodents (Burdock, 1998) suggest that propolis is relatively safe. Nonetheless, the absence of reports on either efficacy or safety of propolis in the treatment of HIV-1 infection coupled with recent evidence of adverse interactions of other natural products on the bioavailability of PIs (Piscitelli et al., 2000, 2002) mandate that before propolis is considered for clinical use that safety and pharmacokinetic studies be carried out in HIV-1-infected patients receiving standard antiretroviral agents.

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