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COMPARISON OF THE ANTI-HERPES SIMPLEX VIRUS ACTIVITIES OF PROPOLIS AND 3-METHYL-BUT-2-ENYL CAFFEATE

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ABSTRACT.—The in vitro activity against herpes simplex virus type 1 of 3-methyl-but-2-enyl caffeate isolated from poplar buds or prepared by synthesis was investigated. Under conditions of one or multiple multiplication cycles, this compound, which is a minor constituent of propolis, was found to reduce the viral titer by 3 $\log_{10}$, and viral DNA synthesis by 32-fold.

Propolis, a beehive product, is used in external applications to treat zona and herpetic cutaneo-mucosal infections (1,2). The activity of propolis in vitro against influenza virus (3,5), vaccinia virus, Newcastle disease virus (4), hepatitis B virus (6), herpes simplex virus type 1 (7), and avian herpes viruses (8), has been reported. Likewise, we have found evidence that propolis is very active in vitro against poliovirus and herpes viruses (HSV-1, a TK^- HSV-1 mutant, and HSV-2), whereas vesicular stomatis virus and adenovirus were less susceptible (9). The aromatic fraction of propolis is a complex mixture of more than 100 compounds including flavonoid aglycones, terpenoids, benzoic acids and esters, and substituted phenolic acids and esters (10–12). As shown by Greenaway *et al.* (10), propolis composition is directly related to that of bud exudates collected by bees from various trees (*Alnus* spp., *Betula* spp., or *Populus* spp.). We have previously investigated the in vitro activity of the major flavonoids identified in a propolis sample against herpes simplex virus type 1 (HSV-1), and a synergistic effect between flavones and flavonols was shown (13). However, even if flavonoids are the main components of propolis, minor compounds may also be involved in the biological activity (12). For this reason, we studied the anti-herpes activity of 3-methyl-but-2-enyl caffeate identified in propolis by Bankova *et al.* (12). This compound was isolated from a *Populus nigra* L. bud extract, the trees surrounding the beehive, and the same compound was synthesized as described by Hashimoto *et al.* (14).

3-Methyl-but-2-enyl caffeate isolated from poplar buds was designated **N** (natural) and the same compound prepared by synthesis was designated **S** (synthetic). The effect on HSV-1 replication was quantified by infectious titer reduction after single and several rounds of multiplication, the cultures being inoculated at a multiplicity of infection (MOI) of 1 and of 0.001, respectively. The results are shown in Table 1. Under both experimental conditions and with a concentration of 25 μg/ml, the decrease in infectious titer induced by the synthetic compound was slightly higher than that induced by the natural compound, and as high or equal to that induced by propolis. Relative to acyclovir, the reference antiviral for HSV-1, the activity of compound **S** was significant. Under the condition of multiple rounds where each step of the reproduction process might be affected by the antiviral, the **S** compound and acyclovir at concentrations of 25 and 50 μg/ml induced infectious titer decreases equal to or greater than 3 $\log_{10}$, acyclovir remaining active at the low concentrations of 6.25 and 12.5 μg/ml. Under the condition of a single cycle of replication, in which the MOI was 1 and

TABLE 1. Activities of Compounds **N**, **S**, Propolis, and Acyclovir on Production of Infectious Virus.[a,b]

Sample	1 Cycle (MOI=1)				Multiple Cycles (MOI=0.001)			
	Concentration (μg/ml)				Concentration (μg/ml)			
	6.25	12.5	25	50	6.25	12.5	25	50
N Compound	1	1.5	2.5	2.5	0.6	1.1	2	3.1
S Compound	1.75	2.5	2.8	2.8	0.6	1.25	3.25	3.6
Propolis extract ..	2.5	3	3	3.2	0.75	1	2	3
Acyclovir	—	—	—	—	2	3	3	3.25

[a] **N** and **S** refer to the natural and synthetic forms of 3-methyl-but-2-enyl caffeate, respectively.
[b] Activities were expressed as the reduction of virus titer ($\log_{10}$) in comparison of virus controls after one cycle of multiplication (MOI=1) or multiple cycles of multiplication (MOI=0.001).

drug was added 1 h after infection, the effects on adsorption and penetration can be excluded because the compounds were added after these events occurred. HSV-1 growth was again strongly inhibited (minus 3 $\log_{10}$ at a concentration of 25 μg/ml) which suggests that compound **S** and propolis exert an effect at a stage of the virus replication cycle after binding to and penetration into the host cell. They may therefore be considered as true antiviral agents.

The effect of **S** compound and propolis on viral DNA synthesis was measured by nucleic acid hybridization with a digoxigenin-labeled HSV-1 DNA probe (data not shown). DNA of untreated virus-infected control, serially diluted twofold, was detectable up to a 1:320 dilution whereas DNA of uninfected untreated cell control was not detected even at a 1:10 dilution, which proved the probe specificity. In HSV-1-infected cultures treated with 30 μg/ml propolis, DNA was only detectable at a 1/10 dilution; propolis therefore would have induced a 32-fold decrease in viral DNA production. At a concentration of 15 μg/ml, viral DNA synthesis was reduced eightfold. In cultures treated with synthetic 3-methyl-but-2-enyl caffeate, DNA synthesis was reduced eightfold and 32-fold with caffeate concentrations of 30 and 60 μg/ml, respectively. This experiment, performed with a HSV-1 viral DNA specific probe, proves that the **S** compound and propolis reduce viral DNA synthesis in a dose-dependent manner. However, this test does not allow us to conclude that replication is actually disrupted, or if inhibition results from another phenomenon occurring upstream, such as DNA polymerase inhibition for example. These results could account for the marked decreases in infectious titer observed after one multiplication cycle in **S** compound-treated and propolis-treated cultures.

It can thus be concluded, as suggested by Bankova *et al.* (12), that the antiviral activity of propolis is not only due to the main compounds (flavonoids), but also to the significant role played by minor compounds like 3-methyl-but-2-enyl caffeate. Our findings may be related to those of Serkedjieva *et al.* (5) who studied the anti-influenza action of propolis and of several esters of substituted cinnamic acids prepared by synthesis. At a concentration of 50 μg/ml, isopentyl ferulate, the most active compound, reduced the reproduction of influenza strain A/Hong Kong with a selectivity index equal to 8, and at a dose of 100 μg/ml, viral reproduction was inhibited by 2.5 $\log_{10}$. The good results obtained in cell culture against HSV-1 should nevertheless be tempered by the fact that 3-methyl-but-2-enyl caffeate is not an innocuous compound. Propolis and poplar buds are known to induce allergic dermatitis, in particular in professionals

(17), and according to Hausen *et al.* (18), the primary allergen could be precisely the 3-methyl-but-2-enyl caffeate. Patch tests performed by these authors on guinea pigs showed that the irritation threshold in these animals was reached with cutaneous applications of solution concentrations ranging from 3 to 10%. In our antiviral tests, the inhibiting doses were thus 100,000 times lower than the abovementioned irritant concentrations.

EXPERIMENTAL

PLANT MATERIAL.—Propolis was collected from a private apiary near Rennes, France, and buds from *Populus nigra* L. (Salicaceae) were harvested in March 1991 from trees near the apiary. Voucher specimens were deposited in the Department of Pharmacognosy, Faculty of Pharmacy, University of Rennes I, France.

EXTRACTION AND ISOLATION.—Dried and powdered propolis (11 g) or poplar buds (114 g) were successively treated with petroleum ether and Et_2O using a Soxhlet extractor. The Et_2O extracts were vacuum-dried to yield 6.7 g and 4.9 g of residue, respectively. Poplar bud crude extract was first chromatographed over Si gel tlc plates (60 F_{254} Merck) using C_6H_6-EtOAc-HO_2CH_3 (40:10:5) as solvent. The natural compound (**N**) was recognized by its R_f value (0.54) and its light-blue fluorescence. Si gel was scraped out and eluted using MeOH, then further purification was carried out by hplc. This yielded 21 mg of the natural compound (**N**).

SYNTHESIS OF 3-METHYL-BUT-2-ENYL CAFFEATE.—A quantity (320 mg) of the synthetic compound (**S**) was synthesized as described by Hashimoto *et al.* (14). Spectral data of compounds **N** and **S** were in full agreement with those given by Bankova *et al.* (12), Hashimoto *et al.* (14), and Wollenweber *et al.* (15).

CELLS AND VIRUSES.—African green monkey kidney cells (Vero cell line: ATCC CCL81) were grown in Eagle's minimum essential medium (MEM) supplemented with 10% newborn calf serum, 160 units/ml penicillin and 80 μg/ml gentamicin. A stock of herpes simplex virus type 1, strain H 29 S, was prepared and titration was performed by the limiting dilution method, using six wells of a 96-well Nunc microplate per dilution. The virus titer was estimated from cytopathogenicity and expressed as 50% tissue culture infectious doses per ml ($TCID_{50}$/ml).

ANTIVIRAL ACTIVITY.—Antiviral activity was determined under two culture conditions. At a low multiplicity of infection, MOI=0.001, drug and virus were added simultaneously to Vero cells and the incubation time at 37° was 72 h. First, the cytopathic effect inhibition was assessed by microscopic observation of cell layers, relative to untreated virus controls; subsequently, after three freeze-thaw cycles and low-speed centrifugation, supernatant viral yields were determined. Propolis and the **N** and **S** compounds were added to the culture medium at the same concentrations, i.e., 6.25, 12.5, 25, and 50 μg/ml; each test was carried out in sextuplicate and each experiment was repeated three times. Toxicity controls, cell controls, and virus controls were run simultaneously. Antiviral activity was expressed as the reduction factor (log_{10}) of the viral titer relative to untreated controls. In the second experimental procedure, Vero cells were infected at a multiplicity of infection of about 1. After 60 min at 37°, the excess virus was discarded and the monolayers washed twice with MEM. Then, MEM containing 0–50 μg/ml of drug was added. A single-cycle condition was achieved by incubation for 18 h with virus. Yields were then determined as above.

STUDY OF VIRAL DNA SYNTHESIS INHIBITION BY NUCLEIC ACID HYBRIDIZATION.—*Purification of HSV-1 DNA.*—Vero cells grown in 25 cm^2 tissue culture flasks were infected at a high multiplicity (MOI=1) and HSV-1 adsorbed for 1 h at room temperature. The cultures were refed with maintenance medium (controls) or propolis-added medium (5, 10, 15, and 30 μg/ml) or **S** compound (15, 30, 60 μg/ml) (assays). Cultures were reincubated at 37° for 18 h. The medium was discarded and cells were disrupted with 100 μl of lysis buffer. Then DNA was purified as previously described by Boom *et al.* (19), using a diatom suspension. Samples were dissolved in 100 μl of sterile H_2O.

Hybridization probe.—The digoxigenin-labeled DNA probe was a non-radioactive probe prepared by the polymerase-chain reaction (PCR) according to the method described by Griffais *et al.* (20,21). The DNA template was the U_{57} gene of HSV-1 obtained by a first PCR (21). This template was amplified using two primers: 5′-CTCACAGCCCCGAT-3′ and 5′-GTCCCG-CGTTGC-3′ kindly provided by Dr. P. André. They were mixed with *Thermus aquaticus* polymerase (Perkin-Elmer Cetus, Norwalk, CT) and deoxynucleotide triphosphates: dATP, dCTP, dGTP, and dUTP linked to digoxigenin, dig-dUTP, (Boehringer Mannheim, France). The solution was subjected to 38 cycles of amplification in 3 steps at 92°, 55°, and 72°. Amplification was verified using agarose gel electrophoresis.

Hybridization procedure.—DNA extracts were serially diluted twofold from 1/10 to 1/320 and dilutions were heated up to 95° to denature the DNA. Samples were deposited on nylon mem-

brane Hybond N (Amersham International Plc, UK) fixed to a Hybri-slot manifold (BRL, MD). Then, the filters were prehybridized in thermally sealed plastic bags for 3 h at 42° in the prehybridization solution containing 1% blocking agent (Boehringer Mannheim, France). After denaturation, the non-radioactive probe was added into the bag and hybridization was carried out at 42° for 18 h with gentle shaking.

Detection of HSV-1 DNA probe binding.—The hybrids were detected by enzyme-linked immunoassay using an antibody conjugate: antidigoxigenin sheep antibody conjugated to alkaline phosphatase. The color reaction was initiated at alkaline pH by the addition of 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium (NBT). (The detection kit was purchased from Boehringer Mannheim, France). A blue precipitate indicated the presence of HSV-1 DNA.

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LITERATURE CITED

1. M. Popescu, C. Polinicencu, P. Atansiu, and E. Predescu, Romanian Patent 86,003, July 24, 1982; *Chem. Abstr.*, **103**, 220838q (1985).
2. F. Giurcoaneanu, I. Crisan, V. Esanu, V. Cioca, and N. Cajal, *Virologie*, **39**, 1, 21 (1988).
3. L.F. Shevchenko, O.A. Chasovodtseva, and A.N. Peschanskii, *Khimioprofil, Khimioter. Grippa, Mater. Vses. Simp. 1st*, 1971, p. 57; *Chem. Abstr.*, **82**, 68322e (1972).
4. V. Maximova-Todorova, N. Manolova, G. Gegova, J. Sekerjieva, S. Uzunov, S. Pancheva, N. Marekov, and V. Bankova, *Acta Microbiol. Bulg.*, **17**, 79 (1985).
5. J. Serkedjieva, N. Manolova, and V. Bankova, *J. Nat. Prod.*, **55**, 294 (1992).
6. A. Morfei, O. Burducea, R. Neuman, N. Cajal, Y. Copelovici, and I. Crisan, *Ag. Rev. Roum. Med.*, **31**, 273 (1980); *Chem. Abstr.*, **95**, 166958e (1980).
7. I. Crisan, A. Mutiu, N. Sahnazarov, V. Cioca, V. Esanu, and A. Popescu, *Apimondia*, **59** (1978).
8. B. König and J.H. Dustmann, *Naturwissenschaften*, **41**, 43 (1988).
9. M. Amoros, F. Sauvager, L. Girre, and M. Cormier, *Apidologie*, **23**, 231 (1992).
10. W. Greenaway, T. Scaysbrook, and F.R. Whatley, *Proc. R. Soc. London, Ser. B*, **232**, 249 (1987).
11. W. Greenaway, T. Scaysbrook, and F.R. Whatley, *Z. Naturforsch.*, **43C**, 301 (1988).
12. V. Bankova, A. Dyulgerov, S. Popov, and N. Marekov, *Z. Naturforsch.*, **42C**, 147 (1987).
13. M. Amoros, C.M.O. Simoes, L. Girre, F. Sauvager, and M. Cormier, *J. Nat. Prod.*, **55**, 1732 (1992).
14. T. Hashimoto, M. Tori, Y. Asakawa, and E. Wollenweber, *Z. Naturforsch.*, **43C**, 470 (1988).
15. E. Wollenweber, Y. Asakawa, D. Schillo, V. Lehmann, and H. Weigel, *Z. Naturforsch.*, **42C**, 1030 (1987).
16. V.S. Bankova, S.S. Popov, and N.L. Marekov, *Phytochemistry*, **28**, 871 (1989).
17. B.M. Hausen, E. Wollenweber, H. Senff, and B. Post, *Contact Dermatitis*, **17**, 163 (1987).
18. B.M. Hausen, E. Wollenweber, H. Senff, and B. Post, *Contact Dermatitis*, **17**, 171 (1987).
19. R. Boom, C.T.A. Sol, M.M.M. Salimans, C.L. Jansen, P.M.E. Wertheim-Van Dillen, and J. Van Der Noordaa, *J. Clin. Microb.*, **28**, 495 (1990).
20. R. Griffais, P.M. André, and M. Thibon, *Res. Virol.*, **141**, 331 (1990).
21. R. Griffais, P.M. André, and M. Thibon, *Nucleic Acids Res.*, **19**, 3887 (1991).

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