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The Anti-HIV Activity and Mechanisms of Action of Pure Compounds Isolated from *Rosa damascena*

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Water and methanol extracts of *Rosa damascena* exhibited moderate anti-HIV activity. The anti-viral activities of 9 compounds isolated from the methanol extract were compared. The tetrahydroxyflavanone (kaempferol, **1**), was effective in reducing the maturation of infectious progeny virus apparently due to selective inhibition of the viral protease. On the other hand the pentahydroxyflavone (quercetin, **2**) and two **3**-substituted derivatives of kaempferol appeared to inhibit HIV-infection by preventing binding of gp120 to CD4. 2-Phenylethanol-O-(6-O-galloyl)-β-D-glucopyranoside **8** interacted irreversibly with gp120 and neutralized virus infectivity. The differences in the modes of action of **1** and **8** can account for the apparent synergy of their anti-viral activities.

The water extract of the flower of *Rosa damascena* is an important constituent of the ancient herbal remedy called 'Safi', which means 'cleansing agent', and has been widely used in Pakistan, from ancient times without apparent harmful effects. The petals are a rich source of vitamin C and flavonoids. The water and methanol extracts of *Rosa damascena* were shown to inhibit HIV infection *in vitro* with a selectivity indices of >100 and 50 respectively. Using standard techniques for the separation of plant constituents, nine compounds were purified from the methanol extract including a new compound 2-phenylethanol-O-(6-O-galloyl)-β-D-glucopyranoside. Here we compare the anti-HIV activities of the nine compounds and show that the activity of the crude extract is due to the combined effects of different compounds acting additively against different stages of virus replication.

MATERIALS AND METHODS

Extraction and isolation. The air dried petals (48.1 g) were defatted with petroleum ether and $CHCl_3$ and extracted with $CHCl_3$-MeOH 9:1 and MeOH to give 1.3 and 2.8 g of residue respectively. The $CHCl_3$-MeOH extract was chromatographed on a Sephadex LH-20 column (100×5cm), with methanol as eluent. 8 ml fractions were collected and checked by TLC [Si gel plates, $CHCl_3$-MeOH-H_2O (80:18:2) and $CHCl_3$-MeOH-H_2O (70:30:3)]. Fractions 40-43 (A, 50.5 mg) contained pure **8;** fractions 28-32 (B, 221.7 mg) and 52-60 (C, 121.5 mg) were further fractionated by HPLC on a C18 μ-Bondapak column (30cm×7.8mm, flow rate 2 ml/min) using MeOH-H_2O (1:4 for B; 65:35 for C). Compound **7** (53.2 mg, RT = 30 min) was obtained from fraction B and **1** (20 mg, Rt = 12.5 min) and **2** (35.2 mg, Rt = 8 min) from fraction C.

Part of the MeOH extract (1.4 mg) was subjected to Sephadex LH-20 chromatography using the same procedure as for the $CHCl_3$-MeOH 9:1 extract. 8 ml fractions were analyzed by TLC [Si gel plates, $CHCl_3$-MeOH-H_2O (70:30:3) and nBuOH-AcOH-H_2O (60:15:25)]. Fractions 43-49 (D, 220 mg) and 51-62 (E, 80 mg) were fractionated by HPLC using MeOH:H_2O (2:3, flow rate 2 ml/min) as the eluent. From fraction D, pure compounds **3** (22 mg, Rt = 50 min), **4** (25 mg, Rt = 53 min) and **5** (32 mg, Rt = 37.5) were obtained; fraction E yielded **6** (14.2 mg, Rt = 12.5 min). Fractions 42-47 (F, 35 mg) contained pure **9.** Compounds 3,5,7,4′-tetrahydroxyflavone (kaempferol, **1**), 3,5,7,3′,4′-

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pentahydroxyflavone (quercetin, **2**), kaempferol-3-O-β-D-glucopyranoside **3,** kaempferol-7-O-β-D-glucopyranoside **4,** quercetin-7-O-β-D-glucopyranoside **5,** kaempferol-3-O-(6-O-trans-p-coumaroyl)-β-glucopyranoside **6,** 2-phenylethanol-O-β-D-glucopyranoside **7,** and quinic acid **9** were identified by FAB-Ms, ^{1}H and ^{13}C NMR analysis by comparison with literature values (1,2,3,4,5).

2-Phenylethanol-O-(6-O-galloyl)-β-D-glucopyranoside **8** is a new natural product is a galloyl derivative of **7.** NMR data of **8** ($C_{21}H_{24}O_{10}$) clearly indicated the presence of 2-phenylethanol-O-β-D-glucopyranoside as the basic skeleton. The chemical shifts of the remaining six sp^2 carbon resonances and the carbonyl signal at δ 168.3 together with a two-proton singlet in the, ^{1}H NMR spectrum at δ 7.13, suggested the occurrence of also a galloyl residue. This was established to be linked to the glucose unit via a 6-O-ester linkage from the ~0.8-0.9 ppm downfield shifted H_2-6glu resonances and on the basis of the upfield shift (~2.5 ppm, γ-effect) and the downfield shift (~2.0 ppm, β-effect) exhibited respectively by C-5glu and C-6glu.

Antiviral assays. The anti-HIV activities and toxicities of compounds were assessed in C8166 human T lymphoblastoid cells infected with HIV-1_{MN} and H9 human T-cell lymphoma cells chronically infected with HIV-1_{IIIB} (6).

Microtiter plate wells were used to mix 4×10^4 C8166 cells per well with five-fold dilutions of the compounds prior to addition of 2 $CCID_{50}$ (50% cell culture infectious dose) units of virus and incubated at 37°C for 5 days. The inhibition of infection was monitored by examining syncytia, by measuring cell viability using the XTT-Formazan method (7) and by estimating viral antigen (gp120) by ELISA (8), which is a more sensitive assay and was used for calculating EC_{50} values. The cytotoxicity in drug treated uninfected cells was measured by the XTT-Formazan assay.

The inhibition of virus production from H9 cells was measured by mixing dilutions of compounds with chronically infected cells. After 5 days of incubation at 37°C, the infectivity of progeny virus released in the cell supernatant was titrated on C8166 cells and antigen gp120 was estimated by ELISA.

Infectivity assay. To measure the effects of compound on virus infectivity, HIV-1_{IIIB} (10^5-10^6 $TCID_{50}$) was incubated with compound at 37°C for 2 h, the mixture was serially diluted, mixed with C8166 cells and incubated at 37°C for 5 days. The infectivity end-point was determined by examining syncytium formation and by the XTT-Formazan assay. In all cases compound was diluted to well below the EC_{50} such that residual compound did not interfere with the virus titration.

Gp 120/sCD4 binding assay. Recombinant sCD4 (0.5 μg/well, ABT, ADP608) was bound to microtitre plate wells and after incubating with 25 μl of dilutions of compounds for 1 hr at 37°C, 25 μl of recombinant gp120 (0.04 μg/ml, CHO-expressed HIV-1_{IIIB} protein, Celltech, ADP604) was added and incubation at 37°C continued for another 3-4 hr. The binding of gp120 was detected using the human anti-HIV antibodies and anti-human Ig conjugated to horseradish peroxidase. Using WIACALC (Pharmacia Biotech) the amount of gp120 bound was calculated from linear logarithmic plots using three concentrations (0.04, 0.02 and 0.01 μg/ml) of gp120 alone as standards.

Enzyme assays. In vitro tests for the inhibitory effects of compounds on the HIV-1 enzyme reverse transcriptase (RT) were carried out using concentrated virus and RT-Detect kit (DuPont Medical Products) and the procedure supplied. The assay for proteinase activity was carried out according to the method described (9) using reagents supplied by the MRC AIDS Reagent Project.

Inhibition of viral core protein processing. Chronically infected H9 cells were washed and re-suspended in 1 ml RPMI containing different concentrations of compounds in 24 well plates. After 3 days at 37°C, the cells were harvested, centrifuged and the pellets solubilized in 100 μl of SDS buffer containing 0.1% Triton X-100 for analysis by SDS-PAGE and immunoblotting. The separated proteins were visualized using a mixture of 4 anti-p24 antibodies EH12E1, 1E8G2, 3D3 and 4H2B1 (10). The relative areas of protein bands p24 and p55 on the autoradiograms were scanned.

Evaluation of compounds in combination. Combination experiments were carried out using a range of 6 or 7 2-fold-diluted concentrations of each of two compounds both alone and in combination. C8166 cells and HIV-1_{IIIB} were used and effects were determined by XTT assay. For each combination isobolograms were plotted for concentrations of compounds exerting the same effect (50% or 25%). The straight line shown joins the single drug values and represents the line of zero-interaction or additivity. Points above and to the right of the line indicate antagonism, points below and to the left of the line indicate synergy (11,12).

RESULTS AND DISCUSSION

Anti-HIV Activity

The anti-HIV activities of compounds **1-9** (figure 1) are indicated in table 1. Kaempferol **1** and its 3-O-β-D glucopyranosides **3** and **6** exhibited the greatest activity against HIV infection of C8166 cells, whereas kaempferol-7-O-β-D-glucopyranoside **4** showed no effect even at a concentration of 250 μg/ml. Similarly, quercetin-7-O-β-D-glucopyranoside **5** was inactive compared to quercetin **2** which had an EC_{50} of 20 μg/ml.

Compound **8,** a new natural product exhibited some anti-HIV activity, presumably due to

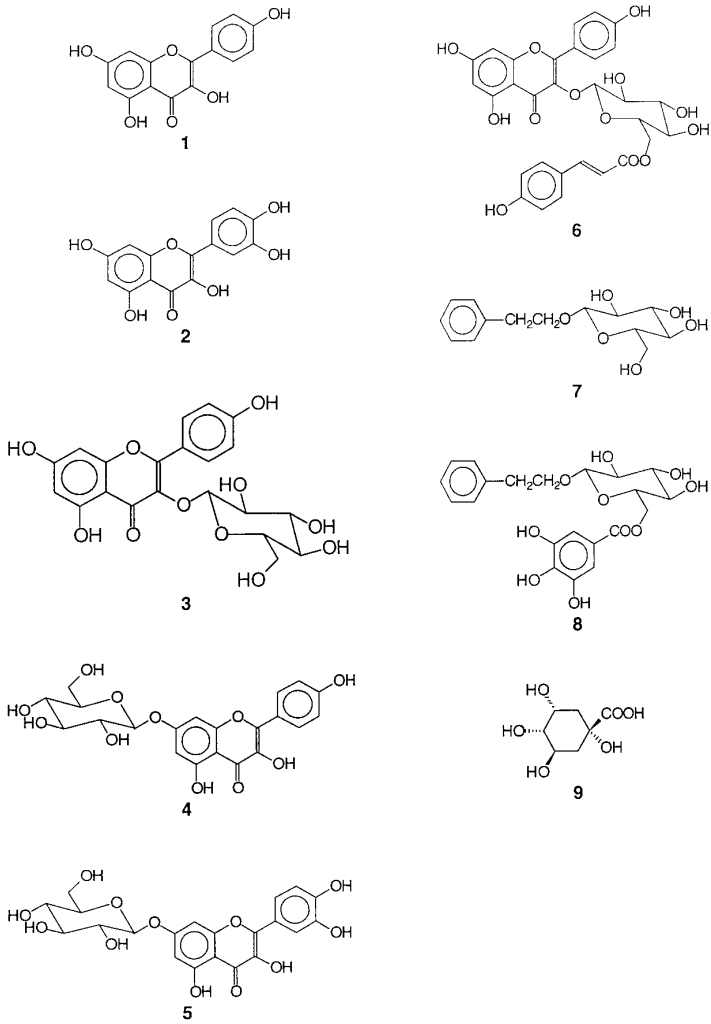

FIG. 1. Structures of compounds **1–9.**

the presence of the galloyl moiety since 2-phenylethanol-O-β-D-glucopyranoside **7** was inactive. The galloyl derivatives of epicatechin and quinic acids were previously shown to be more active than the parent compounds (13,20). Quinic acid **9** was inactive.

Compounds showing activity against acute infection of C8166 cells were also tested in chronically infected H9 cells which provides a more sensitive test of their effect on maturation of infectious virions. Compounds **1** and **2** reduced infectivity of HIV-1_{IIIB} by greater than 99% with EC_{50}s of 0.8 and 10, respectively (table 1). There was no difference in the level of gp120 released in the culture supernatant suggesting that there was no inhibition of viral-protein synthesis but that the processing of the precursor proteins was affected, resulting in the release of non-infectious progeny virus.

Mode of Action

Flavonoids have been shown to inhibit various HIV-coded and cellular enzymes (14,15,16,17,18,19) and to bind specifically to gp120 and to irreversibly inactivate virus infec-

TABLE 1
Effect of Compounds on HIV-1_{MN} Infection in C8166 and HIV-1_{IIIB} Infectivity in Chronically Infected H9 Cells

	C8166			H9		
Compound	EC_{50}	TC_{50}	SI	EC_{50}	TC_{50}	SI
Water extract	10	>1000	>100	ND	ND	—
Methanol extract	10	500	50	ND	ND	—
1	4	70	17.5	0.8	50	62.5
2	20	100	5	10	100	10
3	8	>200	>25	>200	>200	NA
4	>250	>250	NA	ND	ND	—
5	>250	>250	NA	ND	ND	—
6	8	>200	>25	80	80	1
7	>250	>250	NA	ND	ND	—
8	40	200	5	ND	ND	—
9	>200	>200	NA	ND	ND	—
AZT[a]	0.016	>1000	$>10^5$	>1000	>1000	NA
RO31-8959	<0.001	20	2×10^4	<0.001	20	2×10^4

Note. EC_{50} is the concentration of compound in μg/ml which inhibited by 50% the production of gp120 (C8166) or virus yield (H9). TC_{50} is the concentration that reduces uninfected cell growth by 50% as determined by XTT formazan method.

[a] AZT μM. ND, not done; NA, not applicable.

tivity (20). It has however been difficult to differentiate between nonspecific binding of some of these compounds to proteins and specific interactions (21). For example quercetin **2** inhibited CD4/gp120 interaction (figure 2) and the *in vitro* activities of the viral enzymes reverse transcriptase (RT) and protease (table 2). Kaempferol on the other hand was more inhibitory to the viral protease (IC_{50} 2μg/ml) and had little effect on either RT activity (table 2) or gp120/

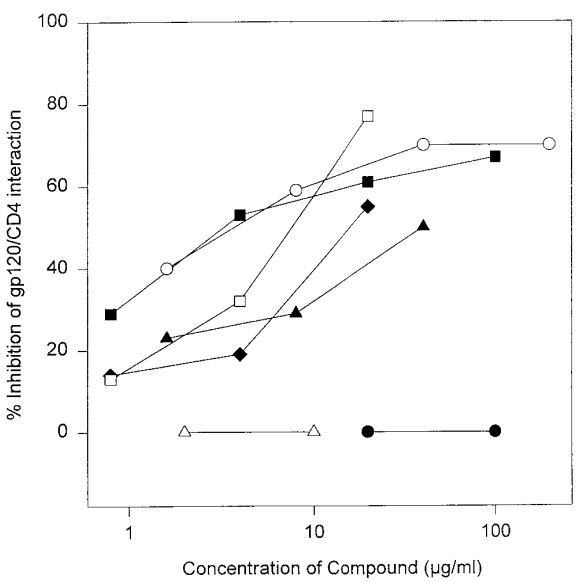

FIG. 2. Inhibition of gp120/CD4 interaction. (—●—) Compound **1;** (—■—) Compound **2;** (—▲—) Compound **3;** (—♦—) Compound **6;** (—○—) Compound **8;** (—□—) Dextran sulphate; (—△—) AZT.

TABLE 2
Inhibition of HIV-1 Enzymes

Compound	EC_{50} (RT) (μg/ml)	EC_{50} (protease) (μg/ml)
1	>100	2
2	100	20
3	ND	>200
6	>100	>200
8	50	>200
Protease inhibitor (RO31-8959)	ND	0.001

CD4 interaction (fig 2). In contrast, the 3 substituted derivatives of kaempferol **3** and **6** had little effect on protease activity but caused a significant reduction in gp120/CD4 interaction. Although these compounds had similar EC_{50}s against HIV-1 infection of C8166 cells, the ability of kaempferol to reduce virus infectivity in chronic H9 infection correlates with its inhibitory activity against the viral protease.

The lower multiplicities of virus infection of C8166 cells renders these assays more sensitive to the effects of kaempferol than the assays used in the previous report (20) of the inactivity of a more toxic commercial preparation. The ability of compound **1** to reduce cleavage of p55 was tested by SDS-PAGE and immunoblotting analysis. The ratio of mature capsid protein p24 to the precursor p55 decreased from 3.8 to 0.8 with increasing concentrations of compound (table 3).

Compound **8** differed from the flavones in that it inhibited RT, CD4/gp120 interaction and inactivated viral infectivity by binding irreversibly to gp120. The virus titer was reduced to 10% of control when treated with 100 μg/ml whereas compounds **1, 2, 3** and **6,** like dextran sulphate did not neutralize virus infectivity.

Inhibition of Infection by Combinations of Compounds

Since the mechanisms of action of compounds **1, 2, 3, 6** and **8** are different, the possibility that the activity in the extract is the result of synergistic action was studied. Compounds **1** and **8** which target different stages in the HIV replicative cycle were chosen. Isobolograms are shown in figure 3. The results indicate that the effects of **1** and **8** are additive to that of AZT. The combination of **1** and **8** appears to be synergistic.

In conclusion, small differences in structure of closely related compounds isolated from *R. damascena* can influence their binding to various proteins and their specificity and mode of action in inhibiting HIV-infection *in vitro.* Thus an additional hydroxyl group at the 3′ position

TABLE 3
Inhibition of Viral Precursor Protein Processing

Compound	Concentration (μg/ml)	Ratio p24:p55
1	30	0.8
	6	2.1
	1.2	3.8
Control	0	3.5
RO 31-8959	10 μM	No p24 detected

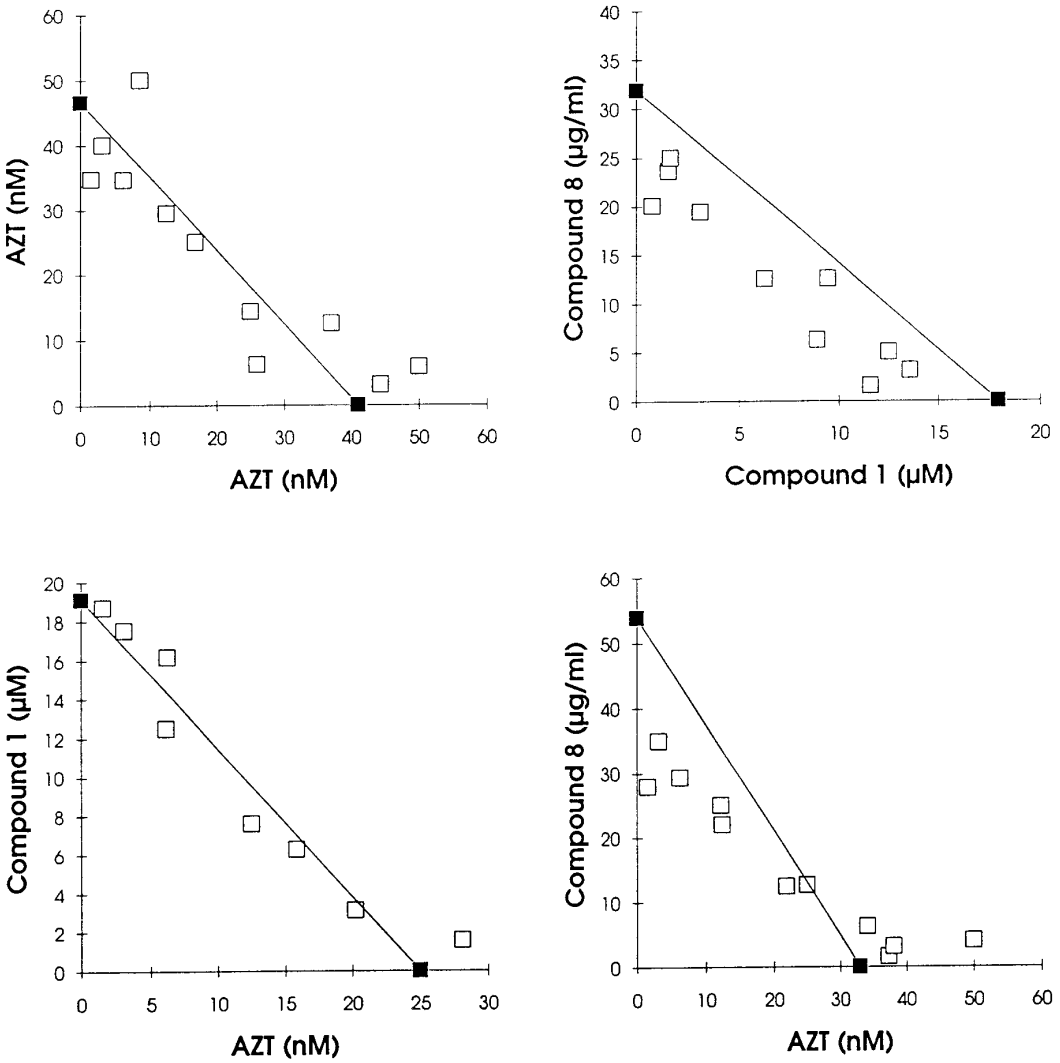

FIG. 3. Isobolograms for combinations of compounds **1, 8,** and AZT.

of ring B causes quercetin to be less specific than kaempferol in its interactions with HIV-1 proteins. A similar difference caused the flavanone 3,5,7,3′,4′-pentahydroxyflavanone to be less specific in its interaction with viral proteins than 3,5,7,4′-tetrahydroxyflavanone, isolated from *Cuscuta reflexa,* which selectively bound to gp120 (22). In another recent publication (23) certain flavonoids were reported to have another novel antiviral property. Of 33 flavonoids evaluated, only chrysin, acacetin and apigenin completely inhibited HIV expression in TNF-α-activated latently infected OM-10.1 cells.

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