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Inhibition of HIV replication by *Hyssop officinalis* extracts

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Summary

Crude extracts of dried leaves of *Hyssop officinalis* showed strong anti-HIV activity as measured by inhibition of syncytia formation, HIV reverse transcriptase (RT), and p17 and p24 antigen expression, but were non-toxic to the uninfected Molt-3 cells. Ether extracts from direct extraction (Procedure I), after removal of tannins (Procedure II), or from the residue after dialysis of the crude extract (Procedure III), showed good antiviral activity. Methanol extracts, subsequent to ether, chloroform and chloroform ethanol extractions, derived from procedure I or II, but not III, also showed very strong anti-HIV activity. In addition, the residual material after methanol extractions still showed strong activity. Caffeic acid was identified in the ether extract of procedure I by HPLC and UV spectroscopy. Commercial caffeic acid showed good antiviral activity in the RT assay and high to moderate activity in the syncytia assay and the p17 and p24 antigen expression. Tannic acid and gallic acid, common to other teas, could not be identified in our extracts. When commercial products of these two acids were tested in our assay systems, they showed high to moderate activity against HIV-1. *Hyssop officinalis* extracts contain caffeic acid, unidentified tannins, and possibly a third class of unidentified higher molecular weight compounds that exhibit strong anti-HIV activity, and may be useful in the treatment of patients with AIDS.

Hyssop officinalis; Caffeic acid; Anti-HIV activity

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Introduction

A number of laboratories are actively involved in the development of antiviral agents that interfere with human immunodeficiency virus (HIV-1) at different stages of viral replication (Sarin, 1988). These include: (a) nucleoside analogues that inhibit reverse transcriptase (RT), such as AZT, dideoxycytidine (ddC) and dideoxyinosine (ddI) (Sarin, 1988; Yarchoan et al., 1989a,b), other RT inhibitors such as phosphonoformic acid or foscarnet (Sarin et al., 1985a; Sandstrom et al., 1985), or combinations of these agents (Eriksson and Schinazi, 1989; Koshida et al., 1989) and alternating treatment with AZT and ddI (Spector et al., 1989); (b) binding or removal of cholesterol from viral membrane or virus infected cells by agents such as amphotericin methyl ester and AL-721 (Schafner et al., 1986; Sarin et al., 1985b); (c) inhibition of tat gene function by agents such as D-penicillamine (Chandra and Sarin, 1986; Chandra et al., 1987); (d) inhibition of virus packaging by agents such as avarol and avarone (Sarin et al., 1987); and (e) inhibition of regulatory gene functions or splicing by antisense oligonucleotides (Zamecnik et al., 1986; Goodchild et al., 1988; Agrawal et al., 1988, 1989; Sarin et al., 1988; Letsinger et al., 1989). Other inhibitors of HIV-1 replication include saponin (Nakashima et al., 1989), pentosan polysulfate (Baba et al., 1988), tetragalloylquinic acid (Nishizawa et al., 1989), GLQ 223, an inhibitor isolated from Chinese cucumber (McGrath et al., 1989), sulfolipids from cyanobacteria (Gustafson et al., 1989), cholic acid analogs (Baba et al., 1989), and more recently by a new class of compounds named TIBO (Pauwels et al., 1990).

In our search for new classes of antiviral agents, we have examined extracts of *Hyssop officinalis*. This herb was brought to our attention, when in September 1984, a 29-year-old black female heroin addict was brought to North Shore University Hospital for treatment of AIDS. The patient suffered from widely disseminated Kaposi's sarcoma (KS). She previously had been treated successfully for pneumocystis carinii pneumonia. She was partially blind from disseminated cytomegalovirus (CMV), and suffered from extensive oral and vaginal candidiasis, oral herpes simplex infection and chronic draining ulcers on her lower extremities. Blood cultures grew mycobacterium-avium intracellulare (MAI) organisms and urine cultures were positive for CMV. Arrangements were made for preterminal care at home. In March 1985, follow-up of the patient revealed that the skin lesions had improved significantly, and blood cultures were no longer positive for MAI. Overall, the patient felt much better and she was ambulatory. The mother of the patient stated that, for the previous month, the patient had been given an old Jamaican herbal remedy which was prepared in form of a tea by boiling a mixture of leaves from *Hyssop officinalis*, blessed thistle, and *Cassia angustifolia*. After this, she began to feel better. Because of this response, the patient continued to take this herbal extract intermittently, during which time her KS lesions continued to regress. In May of 1985, the patient succumbed to fulminant bacterial pneumonia.

In view of this anecdotal observation, it was decided to investigate this herbal remedy for its antiviral activity against HIV-1. Early studies showed that the crude extracts of *Hyssop officinalis* produced the dominant antiviral activity against

HIV-1. Hence, we decided to evaluate Hyssop extract further in order to identify the compounds active against HIV-1.

Materials and Methods

All leaves for this study were purchased from the San Francisco Herb & Natural Food Co., Emeryville, CA 94608. *Cassia angustifolia* was harvested in southern India, whereas blessed thistle and *Hyssop officinalis* were grown in eastern Europe. A tea extract of each of these leaf varieties was prepared by boiling 25 g of dried leaves in 100 ml of distilled water for 30 min. Following filtration through a Whatman #4 (Whatman, Hillsboro, OR), the tea extract was then filter sterilized through a Millipore 25 μ filter (Millipore, Bedford, MA). For further extraction, the sterilized extracts were lyophilized to dryness, producing a brownish-black material of rubbery consistency. The hyssop-derived material was redissolved in distilled water and extracted following the schematic outline in Fig. 1, either directly (Procedure I) or following precipitation of tannins with gelatin (Difco, Detroit, MI) or lead acetate (Procedure II), or dialyzed against water through a dialysis membrane with an exclusion limit of a molecular weight of 3500, (Spectrum Medical Industries, Inc., Los Angeles, CA) (Procedure III). The extraction procedure is outlined in Fig. 1. In this figure, the roman numerals refer to the procedure used, and the capital letters to the individual fractions (code); both numerals and letters are used throughout text and tables.

For identification of small molecular weight substances with biologic activity, the ether extract (B/G) was submitted to HPLC separation on a μ Bondapak ODS column (Waters Assoc., Milford, MA). The HPLC apparatus consisted of a SEC-4 Solvent Environmental Control, a Series 410 LC Pump, a LC-235 Diode Array Detector, an ISS-100 Automatic Injector (Perkin-Elmer, Norwalk, CT) and an Epson Equity 1+ Computer (Epson America Inc., Torrance, CA). Two different elution systems were used. They consisted of either isocratic elution with 5% acetic acid in water, pH 4.6, or with a linear gradient consisting of 20% methanol/80% 0.033 M phosphate buffer, pH 3.0, to 40% methanol/60% 0.033 M phosphate buffer pH 3.0. Flow rate was 1.5 ml/min, and the setting for detection in the UV was 254 or 280 nm.

For the precipitation of tannins, the procedure of Kucera and Herrmann (Kucera and Herrmann, 1967) was used. In short, gelatin was dissolved in water to a concentration of 2% (W/V) and added dropwise to crude tea extract, and the resulting precipitate removed by centrifugation at 430 $\times$ g for 15 min in a Sorvall RC-5B (DuPont, Chadds Ford, PA) refrigerated superspeed centrifuge. The precipitate was washed three times with cold distilled water, and the tannin-gelatin complex resolubilized by incubation for 2 h at 37°C with a mixture of 0.1% trypsin (Difco) plus 0.1% EDTA (Fluka, Buchs, Switzerland), in magnesium and calcium free Eagle's balanced salt solution.

For the precipitation of tannins with lead acetate, saturated lead-II acetate solution was added dropwise to crude tea extract, the precipitate centrifuged at 2000 rpm for 15 min. The precipitate was washed three times with cold water, resuspended

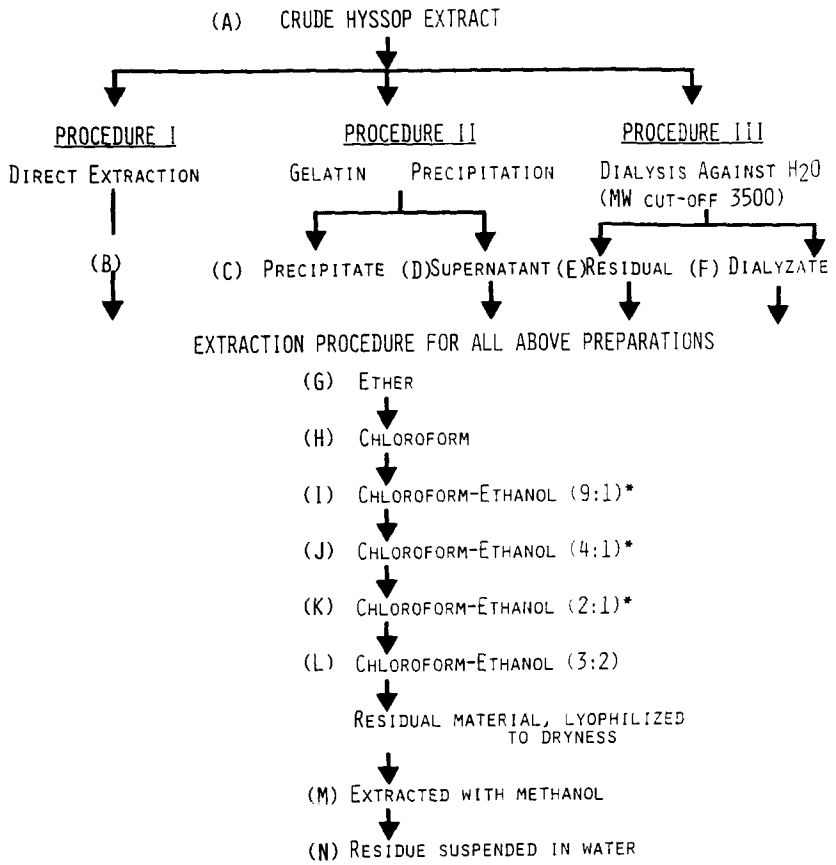

Fig. 1. Schematic outline of the extraction procedures used for the work-up of crude hyssop extracts. *In most instances, due to negligible biological activity of the chloroform (H), chloroform-ethanol (9:1) (I), (4:1) (J), (2:1) (K), and (3:2) (L) extracts, only a chloroform-ethanol (3:2) (L) extract was performed after the ether extract.

and lead removed by the batch procedure with Dowex Ag-50 W-X.

The isolated tannins were evaluated in the biological assay, but were not further analyzed chemically. Ten mg of commercial caffeic acid was dissolved in 0.5 ml 0.1 N NaOH (pH 6.0); gallic acid and tannic acid were dissolved in 1 ml of distilled water for evaluation of biological activity. The above solutions of caffeic and gallic acids were also used for identification by HPLC of these two compounds in ether extracts. Commercial caffeic acid was purchased from Fluka, Buchs, Switzerland; tannic acid (MW 1701.23) and gallic acid were purchased from the Aldrich Chemical Company, Inc., Milwaukee, WI 53233.

Anti-HIV-1 activity

Anti-HIV activity of the crude extract and the extracts outlined in Fig. 1, were determined by procedures outlined earlier (Sarin et al., 1985a, 1988). The inhibition of HIV-1 expression in Molt-3 cells in the presence of compounds was carried out by infecting 5×10^5 cells per ml with $2.5–5 \times 10^8$ virus particles of HIV-1 (IIIB). The infection of cells with 500–1000 virus particles per cell represents a multiplicity of infection of 0.5–1. Assays for inhibiting HIV-1 infection of cells by various agents was carried out by simultaneous addition of virus and drugs to the cells in culture. The cultures were carried out in 96-well plates and incubated in culture medium containing RPMI 1640, 10% fetal calf serum, 2 mM glutamine, and gentamicin (250 μg/ml) in a humidified atmosphere containing 5% CO_2/95% air at 37°C. After 4 days the cells and supernatant were examined for the level of HIV-1 expression by measuring number of syncytia, viral antigen expression as well as cell viability. The variation in the syncytia assay was less than 5%. When these assays were carried out for longer periods of time, the results were similar to the 4 day assay.

Syncytia measurement

The number of syncytia formed in Molt-3 cells was counted after triturating the cells to obtain an even distribution of syncytia in the culture. The average number of syncytia was determined by counting several fields in duplicate cultures.

Cell viability

Cell viability was measured in the presence of trypan blue, and the cells that excluded the dye were counted as viable cells.

HIV-1 antigen expression (p17/p24)

The immunofluorescence analysis for HIV-1 antigen expression was carried out as described (Sarin et al., 1985a, 1987). The cells were pelleted and resuspended in PBS at a concentration of 1×10^6 cells per ml. The cells were spotted on toxoplasmosis slides, air dried, and fixed in methanol/acetone (1:1) for 15 min at room temperature. The slides were next incubated with 10% normal goat serum at room temperature for 30 min and washed 4 times with PBS. Monoclonal antibody against HIV-1 core proteins p17 or p24 was added to each well, and the slides were incubated for 30 min in a humid chamber at 37°C. The slides were washed 4 times with PBS, incubated with FITC labeled goat anti-mouse IgG for 30 min at 37°C, and subsequently washed with PBS over night. The slides were counterstained with Evan's blue, washed with PBS, mounted with 50% (vol/vol) glycerol, and examined under a Zeiss fluorescence microscope. The percentages of cells exhibiting fluorescence in the drug-treated and untreated cultures were compared. The monoclonal antibodies used for immunofluorescence assays were

obtained from Biotech Research Labs, Rockville, Maryland.

Toxicity measurements were done on uninfected cells by measuring the number of the cells at various concentrations of the extracts and commercial compounds.

Reverse transcriptase assay

The reverse transcriptase assay is carried out as described by Sarin et al. (1985a, 1987). Briefly, the supernatants from virus infected cultures in the presence and absence of drug are collected and virus particles precipitated with a final concentration of 10% polyethylene glycol and 0.5 M NaCl. The virus pellet is suspended in 300 μl of buffer containing 50 mM Tris-HCl (pH 7.8), 5 mM dithiothreitol, 250 mM KCl and 0.25% Triton X-100. Reverse transcriptase activity in the solubilized pellet is assayed in a 50 μl reaction mixture containing 50 mM Tris-HCl (pH 7.8), 5 mM dithiothreitol, 100 mM KCl, 0.01% Triton X-100, 5μg dT_{15}-rA_n as template-primer, 10 mM $MgCl_2$, 15 μM (^{3}H-dTTP) (15 Ci/mmol) and 10 μl of the disrupted virus suspension. After incubation for 1 h at 37°C and subsequent addition of 50 μg of yeast tRNA, the incorporation into cold trichloroacetic acid insoluble DNA fraction is assayed by counting in a beta scintillation counter. Since all our assays are carried out in the presence of 10% serum, the observed inhibition of HIV is antiviral and not a nonspecific effect.

Results

Crude extract of *Cassia acutifolia* showed no biological activity against the HIV virus and blessed thistle crude extract had minimal activity at a dilution of 1:20 (not shown). Crude hyssop extracts (Table 1), showed high to good activity at dilutions from 1:50 to 1:300, especially in the p17 and p24 assay, ranging from 100 to 77% inhibition, whereas further dilutions from 1:1500 to 1:2500 revealed only moderate to low activity in both the syncytia and p24 assays (58 to 13 and 48 to 20% inhibition, respectively). At none of the dilutions evaluated was cellular toxicity observed with the crude extracts in all three experiments.

TABLE 1

Evaluation of hyssop tea extracts for anti-HIV activity

Number	Dilution	Crude extracts: inhibition of HIV expression (in %)				
		Syncytia	RT	p17	p24	Toxicity[b]
1	1:50	ND[a]	ND	95	97	
2	1:100	ND	ND	90	95	
3	1:200	ND	77	100	100	
4	1:300	ND	49	83	77	
5	1:1500	58	ND	ND	48	
6	1:2000	42	ND	ND	53	
7	1:2500	13	ND	ND	20	

[a]ND, not done.

[b]The extracts were not toxic to the cells at the tested dilutions of 1:50 to 1:2500.

TABLE 2

Evaluation of hyssop tea extracts for anti-HIV activity – Procedure I: direct extraction

Sample	Code	Dilution	Percent inhibition of HIV-1 expression			
			RT	p17	p24	Toxicity[a]
Ether extraction	B/G	1:50	ND	70	73	
		1:100	ND	60	55	
		1:200	59	44	34	
		1:300	31	39	19	
Residue (in MeOH) after $CHCl_3$-EtOH extractions	B/M	1:20	85	95	92	
		1:40	72	73	69	
Residue (in H_2O) after MeOH extractions	B/N	1:20	96	100	92	
		1:40	80	90	92	
		1:50	ND	91	91	
		1:100	ND	70	73	

ND, not done.

[a]None of the extracts listed were toxic to the cells.

In procedure I (direct extraction) (Table 2), ether extracts, at dilutions of 1:50 to 1:300 showed good to low activity, ranging from 73 to 19% inhibition for the p24 test system and 70 to 39% inhibition for the p17 test system. In the RT test, inhibition of 59% was achieved at 1:200 dilution in experiment 2. The chloroform and chloroform-ethanol extraction showed, in general, only moderate activity throughout all the experiments performed and are not listed. When the residual material (after the last or only chloroform-ethanol extraction) was lyophilized and subsequently extracted with methanol, high biological activity persisted. In this extract there was 85 to 95% inhibition and no toxicity was noted at 1:20 dilution in all three test systems and good activity, ranging from 69 to 73% inhibition, without cellular toxicity in the 1:40 dilutions. Subsequent to the methanol extraction, the residual material, after exhaustive evaporation of remaining methanol, was dissolved in distilled water and found to have high to good antiviral activity at dilutions of 1:20 to 1:100 (100 to 73% inhibition), again without cellular toxicity.

Crude material was treated with gelatin to remove tannins (Procedure II) (Table 3). The resulting precipitate was trypsinized to destroy the gelatin. The residual gelatin-free precipitate (predominantly tannins) redissolved material, exhibited moderate to good antiviral activity at dilutions of 1:20 and 1:40 in the p17 and p24 assays (51 to 70% inhibition), whereas in the RT test system, the activity was low to moderate (28 to 40% inhibition). No cellular toxicity was observed in this predominant tannin extract. The supernatant derived from the removal of tannins by gelatin precipitation was toxic to the Molt-3 cells. Ether extraction of this supernatant at dilutions of 1:200 and 1:300 showed only moderate antiviral activity. In contrast, the methanol extract of the lyophilized, presumably tannin-free supernatant (after ether and chloroform-ethanol extraction) exhibited high activity in all three test systems (99 to 84% inhibition) at dilutions of 1:20 and 1:40, without being toxic to the uninfected cells. The residual material (after methanol extraction), again showed good to high activity at dilutions 1:20 (74 to 85% inhibition)

TABLE 3

Evaluation of hyssop tea extracts for anti-HIV activity – Procedure II: gelatin or lead precipitation*

Sample	Code	Dilution		Percent inhibition of HIV-1 expression							
				RT		p17		p24		Toxicity	
		Gelatin	Lead	Gelatin	Lead	Gelatin	Lead	Gelatin	Lead	Gelatin	Lead
Tannins redissolved	C	1:20	1:200	40	42	66	65	70	51	NT[b]	NT
		1:40	1:300	28	44	63	51	51	53	NT	NT
Supernatant	D	1:20	ND[a]	99	ND	100	ND	100	ND	T[c]	ND
Ether extract of supernatant	D/G	1:200	1:200	36	51	20	57	0	34	NT	NT
		1:300	1:300	46	43	36	48	25	36	NT	NT
MeOH extract (after ether and $CHCl_3$-EtOH extract)	D/M	1:20	1:20	89	99	99	100	97	100	NT	T
		1:40	1:40	84	99	99	100	97	100	NT	T
Residue (in H_2O) after MeOH extract	D/N	1:20	1:20	74	36	83	54	85	46	NT	NT
		1:40	1:40	54	46	63	53	54	45	NT	NT

[a]ND, not done; [b]NT, not toxic; [c]T, toxic.

and good activity at 1:40 dilutions (54 to 63% inhibition) without being toxic to the cells at both dilutions.

When the tannins were precipitated by lead acetate (Table 3), the redissolved material showed moderate to good antiviral activity at concentrations 7–10 times lower than the tannins obtained with the gelatin procedure. These preparations were non toxic to the cells. The ether extract of the supernatant provided good to moderate activity at 1:200 and 1:300 dilutions (34 to 57% inhibition) without cellular toxicity. The methanol extract (after ether and chloroform-ethanol extractions), was, unlike the corresponding extracts after gelatin series, toxic to the cells. The residual material (in water) showed predominantly moderate activity (36–54% inhibition) but was not toxic to the cells.

In the dialysis experiment (Procedure III) (Table 4), the residual material (in the dialysis bag), was subjected to ether extraction. This extract showed good antiviral activity (57–59% inhibition) at dilutions of 1:100, with no cellular toxicity, whereas the dilutions 1:200 and 1:400 showed predominantly moderate activity (29 to 52% inhibition) but again, without exhibiting toxicity to the cells. The methanol extract of this non-dialyzable portion (after chloroform-ethanol extraction) showed moderate to low activity at all dilutions (1:100 to 1:400) and no toxicity. The residual material (after methanol extraction) redissolved in water was slightly higher in its antiviral activity than the methanol extracts at comparable dilutions (1:100 to 1:400), inhibitory activity ranging from 17 to 78%. The dialyzate (outside bag) as well as their extracts with ether, chloroform-ethanol and methanol and residual water extracts were all toxic at dilutions 1:100, 1:200 and 1:400 and biologically inactive at higher dilutions.

HPLC separation was used for the detection of biologically active material. Two major peaks were identified in the ether extract derived from Procedure I. One of these two peaks checked by two HPLC systems and continuous UV spectrum was identified to be caffeic acid, whereas the other peak remains unidentified at this

TABLE 4

Evaluation of hyssop tea extracts for anti-HIV activity – Procedure III: dialysis (Residue after dialysis[a])

Sample	Code	Dilution	Percent inhibition of HIV-1 expression			
			Syncytia	p17	p24	Toxicity[b]
Ether extraction	E/G	1:100	57	58	59	
		1:200	52	33	45	
		1:400	48	29	37	
Residue in MeOH (after ether and $CHCl_3$ extraction)	E/M	1:100	50	33	34	
		1:200	38	17	26	
		1:400	7	17	23	
Residue in H_2O (after ether, $CHCl_3$-EtOH, MeOH extraction)	E/N	1:100	78	50	52	
		1:200	62	50	41	
		1:400	39	17	37	

[a]Residual material in the dialysis bag (E). The lyophilized and redissolved dialyzates (F), as well as their extracts with ether, chloroform-ethanol (3:2), methanol extracts and residual water extracts, were all toxic at dilutions of 1:100, 1:200, and 1:400, and inactive at higher dilutions.

[b]None of the extracts tested were toxic to the cells.

time. Thus, under the conditions outlined in Materials and Methods, caffeic acid elutes at 21.0 min in system 1, and at 13.9 min in system 2. When caffeic acid was added to the ether extract, a single peak with identical elution time was observed in both HPLC systems. Identity of caffeic acid derived from the ether extract (B/G) was further established by comparison of the UV spectra with commercial caffeic acid (Fig. 2). The same HPLC methods were used for a possible identification of gallic acid. However, it was not identified in the ether extract.

When pure commercial preparations of caffeic acid, gallic acid, and tannic acid were prepared at concentrations of 9.1, 10 and 10 mg/ml, respectively and subjected to the bioassay test for anti HIV-activity, the following results were obtained (Table 5): caffeic acid at a dilution of 1:10 and 1:20 was toxic to the uninfected cells, but at concentrations of 1:50 and 1:100 (containing 182 and 91 μg/ml, respectively) showed high activity in the syncytia, p17 and p24 assays (100 to 93% inhibition),

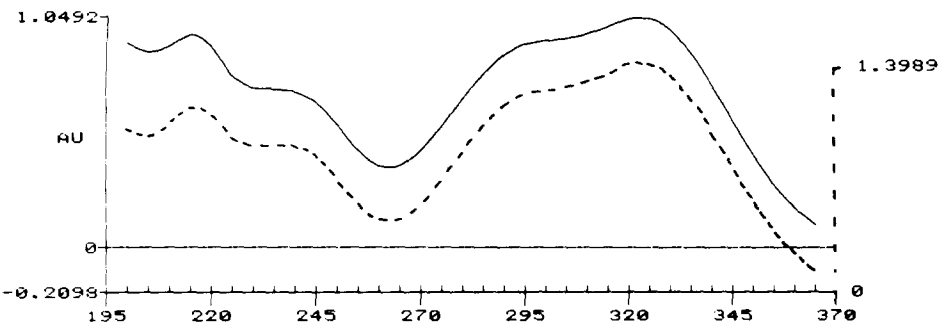

Fig. 2. Continuous UV spectra derived from injection of pure commercial caffeic acid (– – –) or ether extract (B/G) (——) derived from Procedure I. For HPLC method, see text.

TABLE 5

Evaluation of commercial tannic, caffeic and gallic acids for anti-HIV activity

Sample	Dilution (conc. μg/ml)	Percent inhibition of HIV-1 expression			
		Syncytia	p17	p24	RT
Tannic acid	1:100 (100)	T[a]	T	T	T
	1:200 (50)	99	93	93	70
	1:300 (33)	82	86	79	46
	1:400 (25)	35	60	36	40
	1:600 (17)	0	0	0	0
Caffeic acid	1:50 (182)	99	100	100	71
	1:100 (91)	97	93	93	60
	1:200 (46)	93	50	57	32
Gallic acid	1:500 (20)	T	T	T	T
	1:1000 (10)	75	29	36	23
	1:2000 (5)	67	7	0	0

All samples had a stock solution of 10 mg/ml in H_2O except caffeic acid which contained 9.1 mg in 1 ml of 0.1 N NaOH.
[a]Toxic to uninfected cells.

and good activity in the RT assay (71 to 60% inhibition), and further dilution (1:200, containing 46 μg/ml) showed high to moderate activities in the four assay systems (93–32% inhibition). Tannic acid, with a molecular weight of 1701.23, at a concentration of 100 μg/ml was toxic in all four assay systems, whereas further dilutions to concentrations of 50, 33 and 25 μg/ml provided high to moderate antiviral activity (82 to 36% inhibition). Gallic acid, at a concentration of 20 μg/ml, was toxic to the uninfected cells and at lower concentrations (10 and 5 μg/ml) its activity was good only in the syncytia assay and exhibited moderate to no activity in the other test systems.

Discussion

Hyssop officinalis is one of many plants reported to have antiviral activity (Herrmann and Kucera, 1967a). Mint plants, such as *Melissa officinalis* (lemon balm) mentioned by Theophrast, Plinius and Hippocrates to be a useful medicament (Schenck and Brieskorn, 1944), as well as peppermint and sage, have been reported to contain the same condensed tannin, consisting of trimers of caffeic acid (3,4-dihydroxycinnamic acid) (Herrmann, 1954). This tannin demonstrated substantial inhibition of Newcastle disease virus (NDV) (Kucera et al., 1965). A variety of the mint family (Labiatae) exhibited antiviral activity against NDV, herpes simplex, vaccinia, Semliki Forest and West Nile virus in egg and cell-culture systems (Herrmann and Kucera, 1967a). The anti NDV activity was also attributed to a tannin with affinity for NDV and mumps virus, whereas a non-tannin fraction was demonstrated to have antiviral effects against herpes simplex virus. Whereas aqueous extracts of sage (*Salvia cyprea*), majoram (*Origanium majorana*), wild thyme (*Thymus serpyllum*), American pennyroyal (*Hedeoma pulegioides*), Crea monda (*Satureia* sp.), and French and Spanish thymes, exhibited antiviral activity against NDV and herpes simplex, thyme (*Thymus* sp.) and hyssop (*Hyssop officinalis*) extracts showed antiviral activity only against herpes simplex virus, and none of these plant extracts showed antiviral activity superior to *Melissa officinalis* (Herrmann and Kucera, 1967a). In preliminary experiments, our crude extracts from hyssop demonstrated good antiherpes activity in vitro (unpublished results). All the plants of the mint family contain a common tannin (Herrmann and Kucera, 1967b), and these tannins show antiviral activity by interaction with the surface of host cell and neutralization of the virus (Kucera et al., 1965; Fukuchi et al., 1989). Such a mechanism might also apply to our extracts.

In *Melissa officinalis* extracts, a second, non-tannin component with antiviral activity against herpes simplex and vaccinia virus was shown to have properties of a polyphenol, comparable to caffeic acid or an analog of caffeic acid (Herrmann and Kucera, 1967a). Commercial caffeic acid has also been reported to have antiviral activity against herpes simplex, vaccinia, influenza A and B, and NDV virus in egg and cell culture test systems (Herrmann and Kucera, 1967a). None of the extracts of these plants, to our knowledge, have been reported by others to have an effect on HIV replication. However, extracts of plants such as licorice root (Ito

et al., 1987a), *Trichosanthes kirilowii*, a chinese herb, and the bitter lemon plant, *Momordica charantia* (Andrews, 1989) were reported to have activity against HIV.

The present studies showing anti HIV activity of an aqueous extract of *Hyssop officinalis* and caffeic acid extend our knowledge of the previously reported antiviral activity of plant extracts (Herrmann and Kucera, 1967a; Schenck and Brieskorn, 1944; Kucera et al., 1965; Herrmann and Kucera, 1967b) to antiviral activity against HIV. The observed greater antiviral activity of the crude extracts prior to extraction is possibly due to inactivation of the active principles during extraction or due to synergism of the individual active agents in the crude material. The chemical identification of the tannins precipitated from the crude hyssop extract, as well as the elucidation of the structure of the other biologically active agents, was beyond the scope of our present study and will be the subject of another report. However, in analogy to the precipitation of tannins with the same methodology as used for extracts from related plants and also Hyssop (Kucera and Herrmann, 1967b; Herrmann and Kucera, 1967; Schenck and Brieskorn, 1944), we concluded that the residual material (Table 2) and the material obtained by gelatin precipitation and trypsinization, and lead acetate precipitation (Table 3), were tannins of unknown molecular weight.

Commercial tannic acid ($C_{76}H_{52}O_{46}$), with a molecular weight of 1701, showed high to moderate anti-HIV activity at low concentrations (50–25 μg/ml), but was toxic at concentrations of 100 μg/ml (Table 5). It is possible that further testing of the tannins described in the literature, may yield some tannins with anti-HIV activity. Our results confirm the potent HIV inhibitory activity reported by Nishizawa et al. (1989) for their unidentified commercial tannins, although already at a concentration of 50 μg/ml our commercial tannin showed a 70% inhibition. Steric differences (cis-trans isomerism) may explain the discrepancy.

According to Wheeler (Wheeler, 1979), tannins are widely distributed in food, including tea. In contrast, tannic acid, a well defined compound with the empirical formula of $C_{76}H_{53}O_{46}$ (a hydrolyzable tannin yielding gallic acid and glucose), was never reported as a constituent of tea. Therefore, it is likely that strong antiviral activity of the residual material in methanol and final water extracts (after ether, chloroform, chloroform-ethanol extractions) in Procedure I, after removal of the tannins by Procedure II, and of the residual material after dialysis in Procedure III, is due to substances other than tannins. Such biologically active substances may include proteins, glycoproteins, carbohydrates, and flavanols. Indeed, nondialyzable polysaccharides, polyphenols and proteins have been isolated in yields of 10–12% from black tea (Millin et al., 1969), and two out of eight polysaccharide fractions derived from pine cones (*Pinus parvifloria* Sieb et Zucc) were reported recently to have potent immunopotentiating effects and strong inhibition of HIV-1 replication (Lai et al., 1990). In view of the recently reported inhibition of the replication of HIV in vitro by dextran sulfate and heparin (Ito et al., 1987b), other low and high molecular weight anionic polymers may well be found with similar biological activity. While our extracts might well contain polysaccharides, polyphenols, etc., studies on reaction mechanisms will have to await further extensive studies with purified material.

The contribution of caffeic acid to the overall biological anti-HIV activity of the hyssop tea extract is, at present, undetermined. Further examination of biological effects of caffeic acid (both cis and transforms) and its relative instability in aqueous alkaline solutions are warranted. Although caffeic acid has been reported to possess antiviral activity (Herrmann and Kucera, 1967a), it is possible that caffeic acid derivatives or metabolites might also be worthwhile candidates for tests for anti HIV activity. Recently, a caffeic acid oxidation product showed evidence of therapeutic benefit in a cutaneous herpes simplex test system (Helbig et al., 1987).

Our studies indicate that the hyssop leaf extracts contain antiviral activity that may be useful in the treatment of patients with AIDS and Kaposi sarcoma. Further studies will determine the chemical identification of the active ingredients in *Hyssop officinalis* extracts and possibly will identify reaction mechanisms for identified substances.

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