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ISOLATION OF A PREVIOUSLY UNIDENTIFIED POLYSACCHARIDE (MAR-10) FROM *HYSSOP OFFICINALIS* THAT EXHIBITS STRONG ACTIVITY AGAINST HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

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A polysaccharide (MAR-10) was isolated from the aqueous extract of the plant *Hyssop officinalis* and examined for its activity against HIV-1 (SF strain) in HUT78 T cell line and primary cultures of peripheral blood mononuclear cells. MAR-10, in a concentration-dependent manner, inhibited HIV-1 replication as demonstrated by the inhibition of HIV-1 p24 antigen and syncytia formation. Furthermore, MAR-10 had no significant direct toxicity or effect on lymphocyte functions or CD4+ and CD8+ T cell counts. In addition, MAR-10 has broad spectrum anti-glycosidase activity. Our study demonstrates that MAR-10 contains strong anti-HIV-1 activity that may be useful in the treatment of patients with HIV-1 infection.

Terrestrial plants have long been considered to be a rich source of biologically active secondary metabolites. Previously many plants belonging to the mint family (Labiatae) showed antiviral activity against a number of viruses, including HIV-1 (1-4). Tannins found in mint plant extracts and as secondary metabolites in food, including tea have been shown to have moderate anti-HIV activity (5). Earlier reports on *Hyssop officinalis* revealed that the crude methanolic and aqueous extracts contained strong anti-HIV 1 activity and the anti-HIV-1 activity was due to substances other than tannins (6). These reports prompted us to isolate and investigate the active components of the extract of *Hyssop officinalis*. Our *in vitro* study demonstrates strong anti-HIV-1 activity of a polysaccharide (MAR-10) isolated from the aqueous extract of *Hyssop officinalis* without any untoward side effects on lymphocyte subsets and their functions.

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MATERIALS AND METHODS

Isolation of a polysaccharide from aqueous extract of Hyssop officinalis: The dried leaves of *Hyssop officinalis* were purchased from China. The leaves (750g dry wt) were extracted with methanol for 48 hours using a soxhlet extractor. The methanolic extract was filtered and evaporated in vacuo. The resulting residue which was diluted with 1L of water and sequentially extracted with 2 X 1L of chloroform, ethyl acetate, and finally n-butanol. The residual aqueous layer was lyophilized and the butanol soluble portion was concentrated to a thick brown oil. TLC comparison of the material in the lyophilized aqueous layer and the butanol extract contained an almost identical distribution of compounds. The water soluble and butanol soluble fractions were combined and concentrated to afford 2.19g (2.9%) of a thick brown oil. Chromatography on silica gel ($CHCl_3$:MeOH:H_2O 26:15:3) gain a complex mixture of compounds that categorized according to their polarity. The intermediate polarity fraction was rechromatographed using the same solvent system and the intermediate polarity fraction was again collected and concentrated. When this fraction (602mg, 1%) was allowed to stand on room temperature for several days fine colorless crystals appeared (325 mg, 0.4%) which were identified by ^1H and ^{13}C-NMR as inositol. The mother liquor was chromatographed several times (SiO_2-$CHCl_3$:MeOH:H_2O 5:4:1 and Sephadex LH-20-MeOH:H_2O 1:1) to afford 84.2 mg (0.01%) of MAR-10. ^{13}C-and ^1H-NMR data indicate that MAR 10 is a substituted polysaccharide.

Measurement of glycohydrolase inhibition: The glycosidases (Sigma Chemicals, St. Louis, MO) were assayed under first order conditions ($\{S\] << K_m$, pH=6.4, 0.01M morpholinoethanesulfonate buffer, T=27^{0}C) with the corresponding p-nitrophenyl glycoside substrate (Sigma Chemicals, St. Louis, MO). The Ki values were obtained by comparing the first-order rate constants obtained in the absence and presence of various concentrations of MAR-10 (7).

Infection of HUT 78 cells: HUT 78 cells (a T cell line obtained from AIDS Reference Research Program, Rockville, MD) were incubated with SF strain of HIV-1 (AIDS Reference and Research Program, Rockville, MD) for 1 hour at 37^{0}C, unbound virus was washed x 3 with phosphate buffer saline (PBS), and then cells were incubated in the presence or absence of various concentrations of MAR 10 at 37^{0}C for 7 days. HIV-1 p24 antigen was measured by ELISA assay (DuPont, Delaware).

Infection of peripheral blood mononuclear cells: Peripheral blood mononuclear cells (PBMNC) from healthy adult volunteers were incubated with PHA (5μg/ml) at 37^{0}C for 3 days. Cells were washed and incubated with HIV-1 SF strain (HIV-1 p24 of 3,000 pg/10^6 cells) for 1 hour at 37^{0}C. At the end of incubation, unbound virus was removed by washing the cells x 3 with PBS and resuspended in medium RPMI-1640 supplemented with antibiotics, 20% fetal bovine serum (Hyclone), and recombinant IL-2. Cells were incubated at 37^{0}C in the presence or absence of various concentrations of MAR-10 for 15 days (maximum HIV-1 activity was observed). One half of the culture medium was removed from cell cultures twice weekly and replenish with fresh medium containing original concentrations of MAR-10. HIV-1 p24 antigen was measured by ELISA technique.

Syncytia formation: The cell fusion assay procedure as described by Johnson and Walker (8) was used with minor modification. In brief, HIV-infected HUT78 cells (2 x10^4/well) in 96 well flat bottom plates were incubated at 37^{0}C in the presence or absence of various concentrations (1-50 μg/ml) of MAR-10. After 1 hour of incubation, 3 x 10^4 MT-2 cells (AIDS Reference and Research Program, Rockville, MD) were added to HUT78 cells. Cultures were examined at 4 and 24 hours intervals. Total number of syncytia were counted from each well and results are expressed as # of syncytia/well.

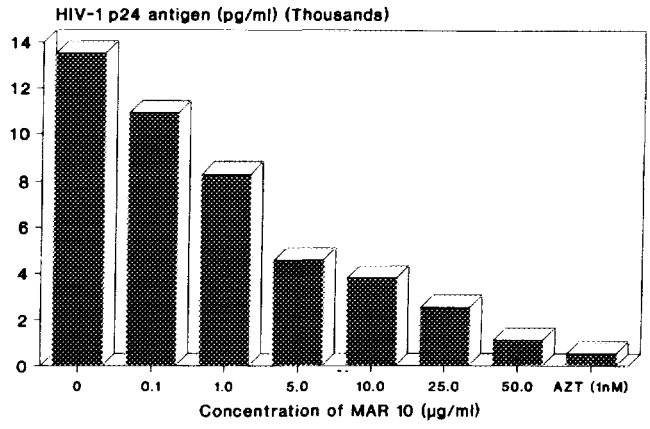

Figure 1. Inhibition of HIV-1 p24 antigen in *in vitro* infected peripheral blood mononuclear cells by MAR-10. Data represent mean of three separate experiments.

Measurement of cell viability: HIV-1 infected HUT 78 cells and PBMNC incubated in the presence or absence of various concentrations of MAR 10 were incubated with propidium iodide and cell viability was measured with FACScan (Becton-Dickinson, San Jose CA). Data are expressed as percent viable cells. All experiments were done in triplicate and PBMNC from three different donors.

Lymphocyte proliferation: Peripheral blood mononuclear cells from two healthy donors were activated with phytohemagglutinin (PHA 10μg/ml) and pokeweed mitogen (PWM) in the presence or absence of various concentrations of MAR 10 for 3 days at 37^{0}C. Cultures were pulsed with 1μCi/well of ^{3}H thymidine for final 18 hours of cultures and ^{3}H thymidine incorporation was measured by scintillation counter. All experiments were done in triplicate and data are expressed as counts per minute.

Lymphocyte subsets: Peripheral blood mononuclear cells from healthy volunteers were incubated at 37^{0}C for 3 days in the presence or absence of various concentrations of MAR

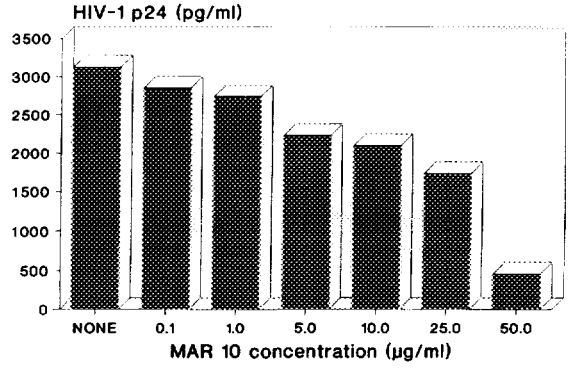

Figure 2. Inhibition of HIV-1 p24 antigen in *in vitro* infected HUT78 T cell line by MAR-10. Data represent mean of three separate experiments.

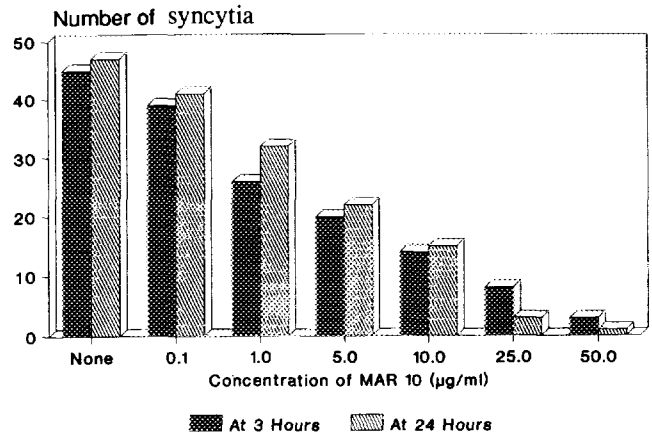

Figure 3. Inhibitory effect of MAR-10 on syncytia formation between MT-2 and HIV-1 infected HUT 78 cells. Data represent mean of triplicate experiments.

10. At the end of incubation, cells were washed x 3 with PBS and stained with FITC-conjugated antibodies against CD3 (pan T cell), CD4 (helper/inducer), CD8 (cytotoxic) and CD20 (Pan B cell) antigens. Ten thousand cells were counted with FACScan and data are expressed as percent positive cells for each phenotype.

RESULTS AND DISCUSSION

A large number of agents have been described that exhibit anti-HIV-1 activity (9). However, only nucleoside analogues that inhibit RT, such as azidothymidine, dideoxycytidine, dideoxyinosine and D4T have been approved for general use. Prolonged use of these agents is limited because of their toxicity and development of drug resistance (10, 11). A number of plants belonging to mint family have been shown to contain antiviral activity against various viruses, including HIV (1-4). Mint as well as peppermint and sage (*Salvia cyprea*)

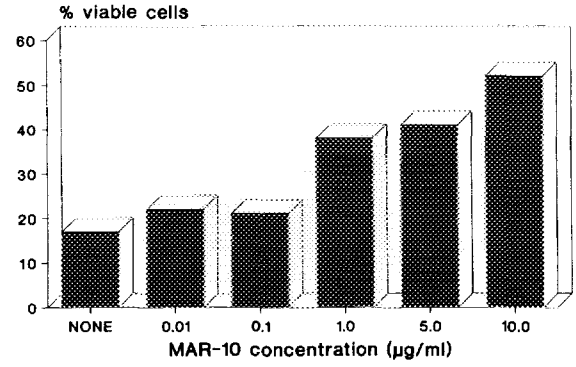

Figure 4. Effect of MAR-10 on the viability of peripheral blood mononuclear cells. Data represent mean of three separate experiments.

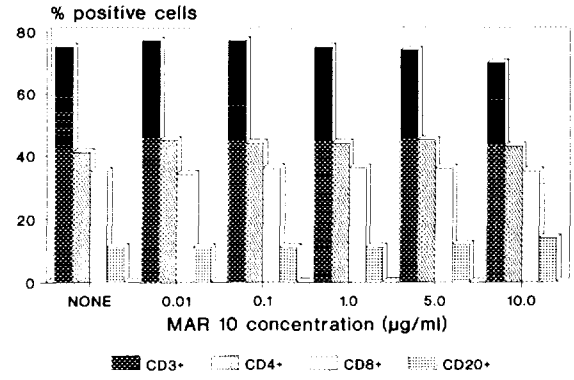

Figure 5. Effect of MAR-10 on proportions of T cells (CD3+), T cell subsets (CD4+ and CD8+) and B cells (CD20+). Data are mean of triplicate experiments.

plant extracts have been reported to contain tannins and trimers of caffeic acid (5). Tannins are the most frequently found secondary metabolites in food, including tea and have been show to have anti-viral, including anti-HIV activity (12). Herrmann and Kucera (1,2) also reported antiviral activity of *Hyssop officinalis*. Kreis et al (6) reported strong anti-HIV activity of methanolic and water extracts of *Hyssop officinalis* due to unknown substances other than tannin. Lai et al (13) reported strong anti-HIV activity of two polysaccharides derived from pine cones (*Pinus parvifloria* Sieb et Zucc). In the present study, we have isolated an unidentified polysaccharide (MAR-10) from *Hyssop officinalis* that exhibited anti-HIV-1 activity as demonstrated by strong inhibition of HIV-1 p24 antigen in primary

TABLE 1

EFFECT OF COMPOUND MAR-10 ON LYMPHOCYTE FUNCTION

Compound µg/ml	cpm		
	MAR-10	MAR-10 + PHA	MAR-10 + PWM
0	288 ± 75.6	97684 ± 3734	28417 ± 11844
0.01	565 ± 222	98998 ± 5750	15999 ± 3866
0.1	627 ± 140	106145 ± 20472	20988 ± 226
1.0	533 ± 300	92666 ± 24319	17351 ± 2725
5.0	492 ± 39	82498 ± 14814	19192 ± 444
10.0	470 ± 148	94228 ± 15719	20865 ± 1565

Peripheral blood mononuclear cells were incubated with various concentrations of MAR-10 in the presence or absence of PHA (activator of T cell function) and PWM (activator of CD4+ T cell-dependent B cells) for 3 days and ^{3}H thymidine incorporation was measured. Data are expressed as mean ± SD of triplicates in counts per minute (cpm).

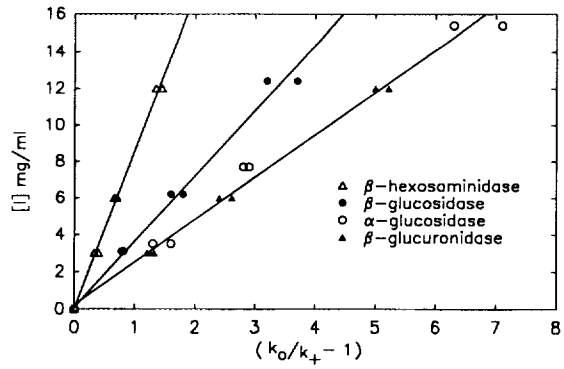

Figure 6. Inhibition of glycohydrolases by MAR-10. The ratio of first-order rate constant in the absence of MAR-10 (k_0) to that in the presence of MAR-10 (k_+) is a linear function of MAR-10. The slope of each of the lines in this plot is K_I.

peripheral blood mononuclear cells cultures (Figure 1) or in HUT78 T cell line (Figure 2) and of syncytia formation (Figure 3). Furthermore, no direct toxic effect and some protective effect was observed on infected cells (Figure 4) and no significant suppressive effect was observed on the proportions of T cells, T cell subsets and B cells (Figure 5) or on lymphocyte proliferation to PHA (T cell mitogen) and PWM (a T cell-dependent B cell mitogen) (Table 1). Our preliminary study shows that MAR-10 has a broad spectrum glycosidase activity, binding reasonably well to the four enzymes tested (Figure 6). A compound with such a "relaxed specificity" for glycohydrolase inhibition is unusual (14). MAR-10 inhibits sweet almond β-glucosidase with K_I=3.6mg/ml, bovine liver β-glucuronidase with K_I=2.4mg/ml, brewer's yeast α-glucosidase with K_I=2.4 mg/ml, and Jack bean hexosaminidase with K_I=8.6mg/ml. Mammalian glucosidases appear to play a role in infection with a variety of viruses, including HIV (15-17).

In summary, we have isolated a previously unidentified polysaccharide (MAR-10) from *Hyssop officinalis* that exhibits strong HIV-1 activity in several assay systems and has no toxic or inhibitory effect on lymphocyte proportions and functions. Further characterization of this polysaccharide is under investigation. Our data suggest that this polysaccharide may be a potential agent for the treatment of patients with HIV-1 infection.

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