Biomed & Pharmacother 1997;51:176-180

Original article

Anti-HIV activity of extracts from *Calendula officinalis* flowers

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Summary – Extracts of dried flowers from *Calendula officinalis* were examined for their ability to inhibit the human immunodeficiency virus type 1 (HIV-1) replication. Both organic and aqueous extracts were relatively nontoxic to human lymphocytic Molt-4 cells, but only the organic one exhibited potent anti-HIV activity in an in vitro MTT/tetrazolium-based assay. In addition, in the presence of the organic extract (500 µg/mL), the uninfected Molt-4 cells were completely protected for up to 24 h from fusion and subsequent death, caused by cocultivation with persistently infected U-937/HIV-1 cells. It was also found that the organic extract from *Calendula officinalis* flowers caused a significant dose- and time-dependent reduction of HIV-1 reverse transcription (RT) activity. An 85% RT inhibition was achieved after a 30 min treatment of partially purified enzyme in a cell-free system. These results suggested that organic extract of flowers from *Calendula officinalis* possesses anti-HIV properties of therapeutic interest.

HIV-1 / *Calendula officinalis*

INTRODUCTION

A number of laboratories are actively involved in the investigation of antiviral agents that interfere with human immunodeficiency virus type 1 (HIV-1) at different stages of viral replication. Unfortunately, the clinical trials for the administration of these compounds to acquired immunodeficiency syndrome (AIDS) patients revealed serious side effects [8]. Anti-HIV drugs with minimal toxic effects, which relate to different metablic pathways of HIV-1 infection, should prove important to prevent disease progression. It seems that a better strategy to find novel antivirals with less cytotoxicity is to look for natural substance(s).

Calendula officinalis (Asteraceae) is an annual herbaceous plant, used in Bulgarian folk medicine as an anti-inflammatory, antipyretic, antitumorogenic and cicatrizing remedy. Previous phytopharmacological studies on the extracts of *Calendula officinalis* flowers have confirmed the presence of bioactive secondary metabolites, flavonol glycosides [7, 9] and triterpenoids [3]. The role of triterpenoids in the anti-inflammatory activity of *Calendula officinalis* flowers was proven [3]. However, very little is known about the specific bioactivity of their flavonoids. Although the naturally occurring plant flavonoids are known as drugs with variable spectrum of antiviral activity against certain RNA and DNA human viruses [2], data on HIV-1 inhibition in vivo or in cell culture have not been reported [8]. However, inhibition of HIV-1 reverse transcriptase (HIV-1 RT) activity by a flavonoid compounds from *S baicalensis,* baicalein, was reported [5]. No antiviral activity had previously been associated with *Calendula officinalis.*

In our search for new sources of anti-HIV/AIDS agents, we have examined extracts of *Calendula officinalis* flowers and have found a cytoprotective effect by the organic extract in HIV-1 infected human lymphocytic Molt-4 cells. These results suggested that the organic extract of flowers from *Calendula officinalis* possesses anti-HIV properties of therapeutic interest.

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MATERIAL AND METHODS

Plant and plant extracts

Calendula officinalis was collected in the Rodopi Mountain of Bulgaria, at 1,700 m in altitude. Dried flowers of this plant (5 g) were cut into small pieces and extracted by a previously described method [10]. Stock solutions (100 mg/mL) of both vacuum-concentrated organic extract and freeze-dried aqueous extract were stored at – 30 °C until use.

Cells and viruses

Human CD4+ lymphocytic cell line Molt-4 clone 8 was used as the target cell in the anti-HIV and cytotoxicity assays. Persistently infected U-937/HIV-1 cells were used for virally-induced fusion [10]. Both cell lines were maintained in RPMI-1640 medium (Gibco BRL, Gaithsburg, MD, USA), supplemented with 10% heat-inactivated fetal calf serum and antibiotics.

The HIV-1 (IIIB) strain used throughout this study was propagated in the Jurkat cell line [10]. Virus stock contained 1.5×10^8 cpm/mL HIV-1 RT activity and an infectivity titer of $10^{-4.5}$ $TCID_{50\%}$/mL.

Cytotoxicity and anti-HIV assays

The cytotoxicity of test extracts was evaluated in parallel with their antiviral activity in 96-well flat bottom microculture plates at an initial cell density of 6×10^4/well. Viability was estimated by the cellular metabolic reduction of the MTT-tetrazolium reagent (Sigma Chen Co, St Louis, MO, USA) to a colored formazan [6]. Uninfected and acutely HIV-infected Molt-4 cells (multiplicty of infection [moi] = 0.5), were maintained at 37 °C in the absence or presence of increasing concentrations, 1 to 10,000 µg/mL of test extracts and monitored up to 96 h post-infection. Each compound concentration was tested in triplicate. The cell viability was quantitated spectrophotometrically with a Multiskan MCC/340 DuPont Plate Reader at 540 and 690 nm. Data were plotted for a statistical analysis in a programs GraphPAD InStat & InPlot (GraphPAD Software, San Diego, CA, USA). The 50% cytotoxic dose ($CTD_{50\%}$) and 50% cytoprotective dose ($CPD_{50\%}$) were calculated by interpolation from the linear regression curve.

Syncytium formation inhibition assay

Uninfected Molt-4 cells and persistently U-937/HIV-1 infected cells were treated and cocultured (1:1) as previously described [10]. Cells were pretreated with 500 µg/mL of test extracts or 0.02 mM dextran sulfate, a compound of known fusion-blocking specificity [4], and incubated at 37 °C for 1 h, followed by washing before coculturing. After 8 and 24 h of cocultivation, the number of giant cells (syncytia) was recorded microscopically and expressed as a percentage of the number of syncytia in the positive controls (cocultures without pretreatment). Each experiment was performed in triplicate.

Reverse transcriptase enzyme inhibition assay

The HIV-1 RT inhibition assay was performed according to a previously described method [1]. The source of the partially purified enzyme was isolated from HIV-1 (IIIB) virions released by Jurkat/HIV-1 acutely infected cells in culture supernatants. Aliquots of HIV-1 RT with activity of 6×10^5 cpm/mL were preincubated with various concentrations of test extracts for 15 and 30 min at 37 °C and then added to a reaction cocktail (0.02 A_{260} units of poly [rA] · poly $[dT]_{12\text{-}18}$ template primer; 0.16 mM dATP; 52 µCi/mL of methyl-^{3}H TTP). Aliquots of plant extracts were added directly to the reaction cocktail for background activity. One µM of zidovudine (AZT), 50 µM of phosphonoformate (PFA, foscarnet), and 250 µM of nonsulfated dextran, MW 40000, were used as positive and negative controls. After 2 h incubation at 37 °C, the [^{3}H] TTP incorporated into DNA was quantified by counting in a Packard scintillation counter. The percentage of HIV-1 RT inhibition was determined by comparing the residual RT activity after treatment with test extracts and nonsulfated dextran, used as negative control. The extract concentrations inhibiting HIV-1 RT activity by 50% ($EID_{50\%}$) were calculated by interpolation from the linear regression curve.

RESULTS

Cellular viability and anti-HIV-1 cytoprotective activity

The viability of mock-infected Molt-4 cells was determined in order to evaluate the cytotoxicity of the test extracts. The results indicated that both aqueous and organic extracts from *Calendula officinalis* flowers were not cytotoxic at and below the concentrations of 1,000 µg/mL. The $CTD_{50\%}$ was calculated to be 3,600 and 5,300 µg/mL for aqueous and organic extracts, respectively.

When evaluated, the inhibitory effects of aqueous extract on HIV-1 replication in acutely infected lymphocytic Molt-4 cells showed no significant activity in protecting the HIV-infected cells. However, the organic extract proved remarkably effective and the maximal cytoprotection was observed at concentrations in the range of 10–30 μg/mL (fig 1). The $CPD_{50\%}$ was calculated to be 400 μg/mL by interpolation from the regression line.

Blocking effect on the HIV-1 induced fusion process

To evaluate the inhibitory potential of test extracts on HIV-1 mediated cell fusion, a syncytium formation inhibition assay was performed. In the absence of fusion-blocking agents, the interaction between uninfected Molt-4 cells and U-937/HIV-1 infected cells produced typical syncytium formation, easily distinguished from unfused cells. After 8 to 20 h of incubation, generally 100 to 300 syncytia per well were formed.

The two test extracts showed differential capacity in suppressing syncytium formation. The aqueous extract was less effective in suppressing cell fusion and only minimal inhibition on cell fusion was observed. However, treatment with the organic extract (500 μg/mL) strongly influenced the subsequent fusion process and completely protected for up to 24 h the uninfected Molt-4 cells from death, caused by infected U-937/HIV-1 cells. Pretreatment of both uninfected Molt-4 or persistently U-937/HIV-1 infected cells with the organic extract led to similar results (table I). As expected, dextran sulfate showed a good capacity for suppressing cell fusion.

Inhibition of HIV-1 RT activity

The organic extract from *Calendula officinalis* flowers caused a significant reduction of HIV-1 RT activity in vitro. The inhibition of the specific enzyme activity (67–85%) was manifested at concentrations of 50, 100 and 200 μg/mL after a 30 min treatment of partially purified enzyme in a cell-free system. A time- and dose-dependent type of HIV-1 RT inhibition was observed (fig 2). The $EID_{50\%}$ inhibiting HIV-1 RT activity after 30 min of incubation was calculated to be 51 μg/mL, by interpolation from the regression line. In contrast, aqueous extract had a negligible effect on HIV-1 RT at nontoxic concentrations (up to 1,000 μg/mL). As expected, nonsulfated dextran showed no inhibition on HIV-1 RT, while 1 μM of AZT and 50 μM of PFA completely inhibited RT activity (data not shown). Background counts were between 1,800 and 2,400 cpm.

DISCUSSION

In the present study we have demonstrated that an organic extract, derived from *Calendula officinalis* flowers, possesses significant in vitro anti-HIV-1 activity and is an effective inhibitor of HIV-1 RT in a cell-free system.

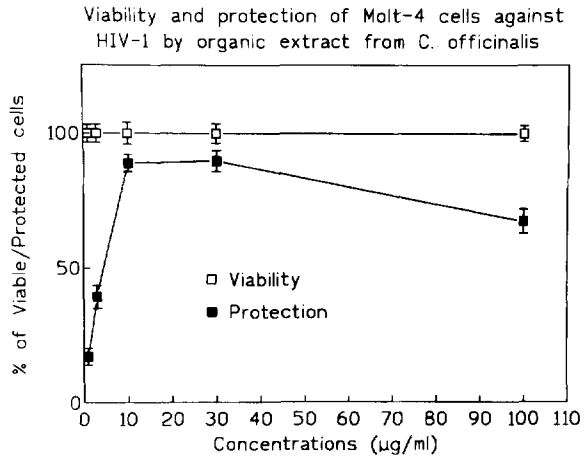

Fig 1. Viability and protection of Molt-4 cells against HIV-1 by organic extract from *Calendula officinalis* flowers.

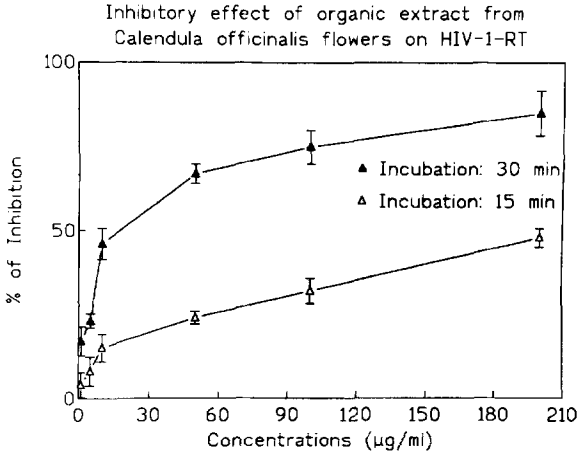

Fig 2. Inhibitory effect of organic extract from *Calendula officinalis* flowers on HIV-1 reverse transcriptase (RT).

Table I. Effect of extracts from *Calendula officinalis* flowers on the HIV-1 envelope-mediated cell fusion process.

Test extract/compounds	*Concentrations*	*% Inhibition*			
		*8 h pc****		*20 h pc****	
		*Assay A**	*Assay B***	*Assay A**	*Assay B***
Aqueous	500 µg/mL	10	6	40	13
Organic	500 µg/mL	100	100	100	100
Dextran sulfate	0.02 mM	94	100	51	87
RPMI-1640 medium	–	0	0	0	0

Assay A*: Uninfected Molt-4 cells were pretreated with the test compounds and then cocultured with infected U-937/HIV-1 cells; Assay B**: Infected U-937/HIV-1 cells were pretreated with the test compounds and then cocultured with uninfected Molt-4 cells; ***: Post co-cultivation.

The anti-HIV activity of test extracts was based on the inhibition of HIV-1 (IIIB) induced cytopathogenicity in CD4+ lymphocytic Molt-4, clone 8 cells. Viability of uninfected cells was not affected at the inhibitory concentrations. In the presence of 10–30 µg/mL of organic extract from *Calendula officinalis* flowers, the target cells were protected against HIV cytopathogenicity up to 90% (fig 1). Although maximal cytoprotection of the extract is in the range of 10–30 µg/mL, surprisingly, higher nontoxic concentrations were reproducibly less efficient in their protective effect. We suggest that this may be due to a nonspecific stearic hindrance and/or an antagonizing effect by some other concomitant extract's principle and thus of the progressive viral breakthrough.

Furthermore, organic extract is capable of suppressing cell fusion, thus protecting uninfected Molt-4 cells against subsequent HIV-induced cytolysis caused by persistently infected U-937/HIV-1 cells (table I). Suppression of HIV-1 mediated fusion was observed at higher doses (500 µg/mL) as used in the assay; however, active compound(s) may be as efficient at lower concentrations. These results indicate that the organic extract from *Calendula officinalis* flowers contains an active constituent(s), which is capable of inhibiting some early event of the HIV-1 replicative cycle. Previous studies have demonstrated that the main bioactive principles of the extracts of *Calendula officinalis* flowers are flavonol glycosides in the lipophilic (organic) extract [3, 7, 9]. It is most likely that the compound(s) giving the reported effect is of the same nature. Moreover, the plant flavonoids are known antiviral agents [2]. Our results suggest that this extract is probably blocking virus adsorption and/or internalization at the early event of the HIV-1 replicative cycle. It is possible that the extract is obstructive for the CD4-gp120 binding by blocking or coating relevant binding sites on the viral or cell membrane.

In addition, the results demonstrate that organic extract of *Calendula officinalis* flowers is a potent inhibitor of HIV-1 RT at concentrations much lower than the maximal nontoxic concentration (1,000 µg/mL). The reduction of the specific HIV-1 RT enzyme activity was fully manifested after a 30 min treatment. A significant difference was observed between the values of the residual RT activity at 15 and 30 min of incubation with different concentrations of the organic extract, indicating a time- and a concentration-dependent reduction in the activity of HIV-1 RT (fig 2). A previous study demonstrated an inhibitory effect of a plant flavonoid compound, baicalein, on the RT activity in vitro [5]. It is therefore possible that a part of the anti-HIV activity we have observed results from a direct effect of such compound found in our extract, which inhibits RT activity.

We can conclude that the organic extract from *Calendula officinalis* flowers contain antiviral activity with possible therapeutic significance. Further work is required to isolate the active compounds from the promising plant extract and to identify reaction mechanisms for the active principles.

REFERENCES

1 Kalvatchev Z, Walder R, Garzaro D. Inhibition of HIV-1 reverse transcriptase activity by extracts from *Fomitela supina, Phellinus rhabarbarinus, Trichaptum perrottettii* and *Trametes cubensis. Fitoterapia* 1995;66:257

2 Kaul T, Middleton E, Ogra P. Antiviral effect of flavonoids on human viruses. *J Med Virol* 1985;15:71
3 Loggia R, Tubaro A, Sosa S, Becker H, Saar ST, Isaac O. The role of triterpenoids in the topical anti-inflammatory activity of *Calendula officinalis* flowers. *Planta Med* 1994;60:516
4 Mitsuya H, Looney D, Kuno S, Ueno R, Wong-Staal F, Broder S. Dextran sulfate suppression of viruses in the HIV family: inhibition of virion binding to CF+ cells. *Science* 1988;240:646
5 Ono K, Nakane H, Fukushima M, Chermann JC, Barre-Sinoussi F. Inhibition of reverse transcriptase activity by a flavonoid compound, 5, 6, 7-trihydroxyflavone. *Biochem Biophys Res Commun* 1989;160:982
6 Pauwels R, Balzarini J, Baba M, Snoeck R, Schols D, Herdewijn P, Desmyter J, De Clercq E. Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. *J Virol Methods* 1988;20:309
7 Pietta P, Bruno A, Mauri P, Rava A. Separation of flavonol-2-o-glycosides from *Calendula officinalis* and *Sambucus nigra* by high-performance liquid and micellar electrokinetic capillary chromatography. *J Chromatogr* 1992;593:165
8 Scinazi R, Mead J, Feorino P. Insights into HIV chemotherapy. *AIDS Res Hum Retroviruses* 1992;8:963
9 Vidal-Olliver E, Elias R, Faure F, Babadjamian A, Crespin F, Balansard G, Boudon G. Flavonol glycosides from *Calendula officinalis* flowers. *Planta Med* 1989;55:73
10 Walder R, Kalvatchev Z, Garzaro D, Barrios M. In vitro activity of extracts from Polyporaceae against human immunodeficiency virus type 1. *Phytotherapy Res* 1996;10:497