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Antiviral Activities of Extracts of *Euphorbia hirta* L. against HIV-1, HIV-2 and SIV_{mac251}

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Abstract. *The antiretroviral activities of extracts of Euphorbia hirta were investigated in vitro on the MT4 human T lymphocyte cell line. The cytotoxicities of the extracts were tested by means of the MTT cell proliferation assay, and then the direct effects of the aqueous extract on HIV-1, HIV-2 and SIV_{mac251} reverse transcriptase (RT) activity were determined. A dose-dependent inhibition of RT activity was observed for all three viruses. The HIV-1 inhibitory potency of E. hirta was studied further, and the activities of the aqueous and 50% methanolic extracts were compared. The 50% methanolic extract was found to exert a higher antiretroviral effect than that of the aqueous extract. The 50% MeOH extract was subjected to liquid-liquid partition with dichloromethane, ethyl acetate and water. Only the remaining aqueous phase exhibited significant antiviral activity; all the lipophilic extracts appeared to be inactive. After removal of the tannins from the aqueous extract, the viral replication inhibitory effect was markedly decreased, and it was therefore concluded that tannins are most probably responsible for the high antiretroviral activity.*

The currently used antiretroviral combination therapies have certainly improved the quality of life for HIV-infected people, but their high cost and limited availability do not allow the vast majority of patients in developing countries to benefit from these combination therapies. Moreover, these drugs are not always efficacious or well-tolerated, and drug resistance is rapidly emerging. For poor developing countries, therefore, it is very important to search for anti-HIV agents from local natural products, especially those of botanical origin, which can play a role in the management of HIV-1 infection and AIDS.

Recent antiviral screenings have demonstrated that some *Euphorbiaceae* species, *e.g. E. pekinensis*, *E. peplus*, *Phyllanthus nanus* and *P. amarus*, are effective against virus infections (1-4). With the aim of finding plants containing promising antiretroviral compounds, we have studied the activities of extracts from *E. hirta* L. (*Euphorbiaceae*) against three types of immunodeficiency viruses: HIV-1, HIV-2 and SIV_{mac251}.

This plant has been used in traditional medicine in many countries in Africa and Asia for the treatment of various illnesses, such as bowel complaints, coughs, dysentery, colic pains, bronchial affections and asthma (5). Besides these principal indications, other properties have also been recorded for *E. hirta*, *e.g.* hypotensive, tonic, antipyretic, anti-inflammatory and sedative effects (6). The latex of the plant is used in the treatment of conjunctivitis, fresh wounds and burns. Previous phytochemical studies on this plant have revealed the presence of diterpenoids, triterpenoids and flavonoids (7, 8). Moreover, Yoshida *et al.* reported the isolation of hydrolysable dimeric ellagitannins (euphorbin A, B, C and E) from the leaves of the plant (9-11).

In the present study, the antiretroviral effects of extracts of *E. hirta* against SIV_{mac251} (simian immunodeficiency virus strain mac251), HIV-1 and HIV-2 viruses (human immunodeficiency virus types 1 and 2) were evaluated.

Materials and Methods

Plant material. A commercial sample of the aerial parts of *E. hirta* L. (*Euphorbiaceae*) (Afedim tea OGYI-580/1995), purchased from Tamag Bt., Harkány, Hungary (GYNKI-B269/98 962552428), was used for the preparation of the extracts. A voucher specimen (No. 764) has been deposited at the Department of Pharmacognosy, University of Szeged.

Preparation of the extracts. In the first experiment, dried, ground plant material was extracted with physiological buffered saline (PBS) solution in an orbital shaker at room temperature for 24 hours (Figure

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Key Words: Euphorbia hirta, Euphorbiaceae, antiviral activity, HIV-1, HIV-2, SIV_{mac251}.

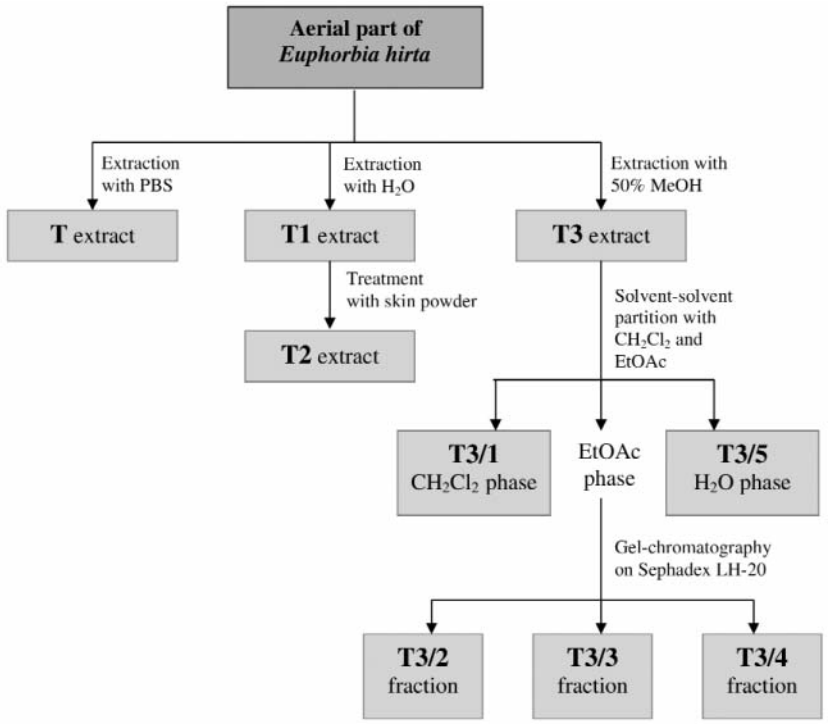

Figure 1. *Preparation of the extracts from the aerial parts of Euphorbia hirta.*

1). The suspension was then boiled for 30 min and centrifuged (5000 rpm, 20 min). The aliquots (T) of the supernatant were kept at –20˚C until use. The overall solute concentration of the aqueous extract was 20 mg/ml. In the second experiment, the same plant material (5 g) was extracted with water (3×50 ml) at room temperature and then filtered. One part of the extract was lyophilized (T1), while the other part was treated with skin powder, filtered, evaporated and lyophilized (T2). Dried and powdered plant material (50 g) was extracted with 50% MeOH (3×500 ml), and a subsample of the extract was filtered and lyophilized (T3). After removal of the MeOH, the remaining part of this extract was subjected to liquid-liquid partition with CH_2Cl_2 (3×100 ml) (T3/1) and then with EtOAc (3×100 ml). The EtOAc phase was fractionated on Sephadex LH-20 gel (Pharmacia Fine Chemicals), with elution with MeOH. The collected fractions were combined into three main fractions (T3/2, T3/3 and T3/4) on the basis of thin layer chromatography (TLC) monitoring according to their composition. The aqueous phase remaining after the organic solvent extraction was lyophilized, yielding fraction T3/5.

Total phenolic concentration. Extracts T1, T2 and T3 were characterized by their total phenolic content, measured by the Folin-Ciocalteu method as prescribed in European Pharmacopoeia 5 (12). Gallic acid (Fluka) was used as a standard, and total polyphenol concentrations were calculated in m/m% of the dried extract materials. The total phenol contents, expressed in terms of gallic acid, were as follows: 3.427±0.078% (T1), 0.496±0.013% (T2) and 2.188±0.143% (T3).

Cells. MT4 human T lymphocytes (13) were maintained in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (Sigma), 100 IU/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma). Experiments were performed in 96-well plates. Virus and appropriate dilutions of the drugs were added to the cells at the same time. Experiments were completed on days 7 or 8.

Virus. Clarified cell-free supernatants of HIV-1 (HTLV- IIIB), HIV-2 and SIV_{mac251}-infected MT4 cells were used in direct reverse transcriptase (RT) inhibition assays. The amounts of HIV-1 ($TCID_{50}$=50% tissue culture infectious dose) in the supernatants were determined by virus yield assay (14). 100-500 $TCID_{50}$/ml HIV-1/well was used to determine the anti-HIV-1 effects of suitable dilutions of *E. hirta* extracts. After incubation for 7-8 days, the yield of HIV-1 was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (15) or RT microassay (16).

Bioassays. MTT assay: The cytotoxic effect of *E. hirta* was measured by means of the colorimetric assay described by Mosmann (15). Briefly, cells were grown in 96-well flat-form plates with different concentrations of the plant material. At the end of the incubation period (usually 7 days), 20 μl of MTT (5 mg/ml dissolved in PBS) was added to each well. After incubation for 4 hours, the cell supernatant was removed, (according to our modification) 100 μl of acidic isopropanol (0.04 N HCl in isopropanol) were added, and the contents were mixed thoroughly. The absorbance was measured at 540 nm on an ELISA microplate-reader. The TC_{50} (50% cytotoxic concentration) values were determined.

RT assay: A microassay was used for the measurement of RT activity, as described elsewhere (16). Aliquots of supernatants of virus-infected MT4 cells were used in direct RT inhibition assays.

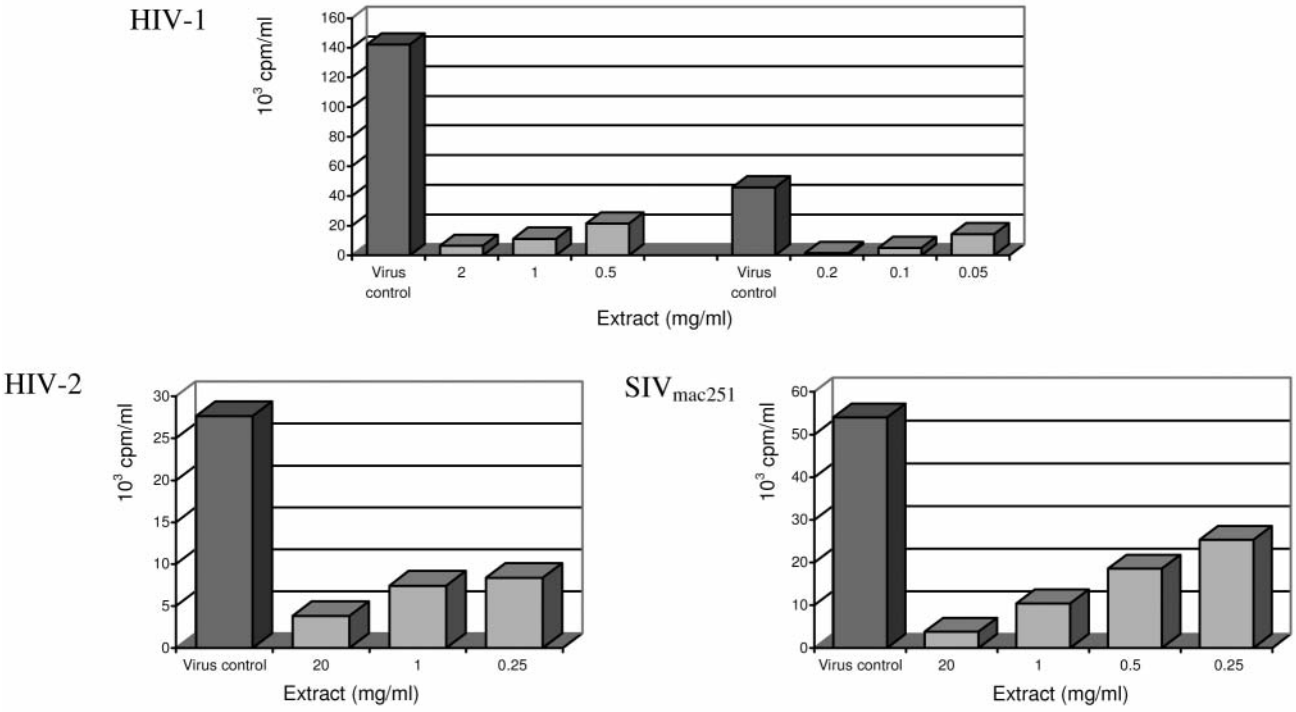

Figure 2. *Direct effects of PBS-extract (T) of Euphorbia hirta on HIV-1, HIV-2 and SIV_{mac251} reverse transcriptase.*

Four µl of poly(rA)-oligo$(dT)_{12-18}$ (Sigma) as template were immobilized on Whatman DE 81 filter paper squares. Three µl of [^{3}H]dTTP and virus pellet were resuspended in RT buffer, then mixed with different concentrations of the plant extracts (10:1), and these samples were dropped onto the squares. They were incubated for 2 hours, washed, dried and measured (cpm) in a scintillation cocktail, using a Packard 1600 CA liquid scintillation analyser.

Results

The antiretroviral effects of extracts of *E. hirta* against SIV_{mac251}, HIV-1 and HIV-2 viruses were evaluated with bioassay methods. First the cytotoxic effect of the extract prepared with PBS (T) was determined on MT4 cells by means of the MTT assay (50% cytotoxic concentration, TC_{50} 442 µg/ml), and then the direct effects on RT were investigated in non-cytotoxic concentrations. A dose-dependent inhibition of RT was observed on HIV-1, HIV-2 and SIV_{mac251} (Figure 2), with IC_{50} values (inhibitory concentration for 50% yield reduction) of 38, 22 and 177 µg/ml, respectively. In the next experiment, the antiviral activities of aqueous (T1) and 50% methanolic extracts (T3) were evaluated against the most sensitive of these viruses, HIV-1. High antiretroviral activity was recorded for both extracts, with IC_{50} values of 9 µg/ml (T1) and 5 µg/ml (T3). After removal of the tannins from the aqueous extract (T1) by treatment with skin powder (T2), the viral replication inhibitory effect markedly decreased (IC_{50} 81 µg/ml) (Table I). On detannination, the total phenolic content determined by the Folin Ciocalteu method fell from 3.427% (T1) to 0.496% (T2). The 50% MeOH extract (T3) was subjected to solvent-solvent partitioning, yielding a dichloromethane (T3/1), an ethyl acetate and an aqueous phase (T3/5). The EtOAc fraction was further separated by gel-chromatography, resulting in fractions T3/2, T3/3 and T3/4. The HIV-1 inhibitory effects of these fractions were investigated by the MTT assay. All the lipophilic extracts proved to be inactive, only the residual aqueous phase (T3/5) exhibited significant antiviral activity (Table II).

Discussion

Our results suggest that most probably the tannins are responsible for the high antiretroviral activity, similarly as in case of *E. pekinensis*, in which galloylated compounds have been identified as active principles against HIV-1 replication (1). In other *Euphorbia* species (*E. paralias*, *E. maschallian* and *E. myrsinites*) (17-19), different types of diterpenes have been identified as antiviral compounds, but in *E. hirta* the relevance of diterpenes as inhibitors of HIV-1 can be excluded, since the lipophilic CH_2Cl_2 and EtOAc extracts, in which the diterpenes most probably accumulate, were found to be inactive. As far as the mechanism of action is concerned, previous studies have demonstrated HIV protease (20) and HIV integrase inhibitory activities (1) of *Euphorbia* extracts, but our experiments demonstrated RT inhibitory activities

Table I. *Cytotoxicities and anti-HIV-1 activities of extracts from E. hirta.*

Extracts	TC_{50} (μg/ml)	IC_{50} (μg/ml)	TI_{50}
PBS extract (T)	442±88	38±6.0	12
Aqueous extract (T1)	174±21	9±4.6	19
Tannin-free extract (T2)	376±82	81±22	5
50% MeOH extract (T3)	193±56	5±0.5	39

TC_{50}: 50% cytotoxic concentration; IC_{50}: inhibitory concentration for 50% yield reduction; TI_{50}: therapeutic index. All values are averages of the results of three independent experiments. TC_{50} was determined by MTT assay. IC_{50} was determined by RT assay. $TI_{50}=TC_{50}/IC_{50}$.

Table II. *Cytotoxicities and anti-HIV-1 activities of extracts obtained from the 50% MeOH extract (T3) of E. hirta.*

Extracts	TC_{50} (μg/ml)	IC_{50} (μg/ml)	TI_{50}
CH_2Cl_2 extract (T3/1)	52±22	not effective	-
EtOAc extract Fr 1 (T3/2)	131±27	not effective	-
Fr 2 (T3/3)	59±20	not effective	-
Fr 3 (T3/4)	68±13	not effective	-
Residual aqueous phase (T3/5)	223±21	10±2	22

All values are averages of the results of three independent experiments. TC_{50} was determined by MTT assay. IC_{50} was determined by MTT assay. $TI_{50}=TC_{50}/IC_{50}$.

against SIV_{mac251}, HIV-1 and HIV-2. The noteworthy efficacy of these *E. hirta* extracts against three types of immunodeficiency viruses makes this plant a promising subject for further drug development research.

Conclusion

The aqueous and 50% MeOH extracts of *Euphorbia hirta* demonstrated a specific inhibition of SIV_{mac251}, HIV-1 and HIV-2 replication in MT4 cells *in vitro*. Solvent-solvent partition and gel-chromatographic separation of the highly active 50% MeOH extract, and RT assay of the obtained fractions, revealed that the tannin-containing aqueous extract possessed the highest potency with low cytotoxicity; it was therefore concluded that tannins are most probably responsible for the high antiretroviral activity.

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