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Anti-HIV-1 Activities of the Extracts from the Medicinal Plant *Linum grandiflorum* Desf.

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ABSTRACT

As part of our screening of anti-AIDS agents from natural sources e.g. *Ixora undulata*, *Paulownia tomentosa*, *Fortunella margarita*, *Aegle marmelos* and *Erythrina abyssinica*, the different organic and aqueous extracts of *Linum grandiflorum* leaves and seeds were evaluated *in vitro* by the microculture tetrazolium (MTT) assay. The activity of the tested extracts against multiplication of HIV-1 wild type III_B, N119, A17, and EFV^R in acutely infected cells was based on inhibition of virus-induced cytopathicity in MT-4 cells. Results revealed that both the MeOH and the $CHCl_3$ extracts of *L. grandiflorum* have significant inhibitory effects against HIV-1 induced infection with MT-4 cells. The MeOH extract of the leaves is more potent than other extracts against MT-4 cell cultures infected with the wild type HIV-1, strain III_B with an ED_{50} of 46 ± 6 µM, while the $CHCl_3$ extract of the seeds is more potent than other extracts against MT-4 cell cultures infected with the double mutation K103N+Y181C with an ED_{50} of 57 ± 4 µM.

Keywords: flavonoids, flowering flax, lignans, Linaceae, red flax, scarlet flax

Abbreviations: AIDS, acquired immunodeficiency syndrome; **AR**, aldose reductase; **ART**, antiretroviral therapy; **AZT**, zidovudine; $\mathbf{CC_{50}}$, 50% cytotoxic concentration; $\mathbf{CCID_{50}}$, the 50% HIV-1 tissue culture infectious dose; $\mathbf{EC_{50}}$, 50% effective concentration; **HIV**, human immunodeficiency virus; **MTT**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; **PTK**, protein tyrosine kinase; **QSAR**, quantitative structure activity relationship; **SAR**, structure activity relationship; **TI**, therapeutic index

INTRODUCTION

Since the first cases of AIDS were identified in 1981 in the United States (Cos *et al.* 2004), AIDS has become the largest and most devastating public health pandemic of our time, and has infected nearly 70 million people and left 25 million dead, and around the world, the number of people living with HIV is about 40.3 million (Veljkovic *et al.* 2007) (and may be more). Combination therapy of anti-HIV drugs now available has improved the quality of life and life-span of HIV/AIDS patients. Emergence of HIV drug resistance, side effects and the need for long-term antiretroviral treatment are the main causes for the failure of antiretroviral therapy (ART). Continuous development of new anti-HIV agents, targets and therapy appear to be inevitable. In some countries, traditional medicine is used to meet primary health care needs and to treat AIDS patients (Harnett *et al.* 2005; Zhang *et al.* 2005). Natural products are the most consistently successful source in drug discovery, and may offer more opportunities to find anti-HIV drugs or lead compounds. Many compounds with an anti-HIV-1 effect have been screened out from natural products and discovered to inhibit HIV at nearly all stages of the viral life cycle (Wang *et al.* 2004). They include alkaloids, sulphated polysaccharides, polyphenolics, flavonoids, coumarins, phenolics, tannins, triterpenes, lectins, phloroglucinols, lactones, iridoids, depsidones, *O*-caffeoyl derivatives, lignans, ribosome-inactivating proteins, saponins, xanthones, naphthadianthrones, photosensitisers, phosholipids, quinines and peptides (Ng *et al.* 1997; Vlietinck *et al.* 1998; Yang *et al.* 2001). Natural products provide a large reservoir for screening anti-HIV agents with novel structure and anti-viral mechanisms because of their structural diversity. A variety of natural products have been found to inhibit unique enzymes and proteins crucial to the life cycle of HIV, including efficient intervention with the reverse transcription process, virus entry, integrase and protease (De Clercq 2000; Cos *et al.* 2004). But the mechanism of anti-HIV activity of many more natural products is still unknown.

Linum grandiflorum, a medicinal plant, has many folkloric uses as food and medicine; it was used for the improvement of men and women's fertility and as a cyanogenetic agent (Duke *et al.* 2008). The seed oil of the genus *Linum* is used as a laxative and expectorant, to treat mental deficiencies in adults and relieves pain; the seeds are analgesic, emollient, pectoral and resolving (Gruenwald *et al.* 2000). The crushed seed makes a very useful poultice in the treatment of ulceration and inflammations (Bown 1995). The mucilage from the seed is valuable for remedy of coughs, cold and inflammation of urinary organs, the bark and the leaves are used in the treatment of gonorrhoea, and the flowers are cardiotonic and nervine (Duke *et al.* 2002, 2008). The genus *Linum* has a long folkloric history in the treatment of cancer (Phillips and Foy 1991; Duke *et al.* 2008).

In the present study the anti-HIV-1 activities of different extracts from the aerial parts (leaves (**Fig. 1A**) and seeds (**Fig. 1B**)) of *L. grandiflorum* were investigated. The results revealed that the MeOH and the $CHCl_3$ extracts of the leaves and seeds, respectively, have inhibitory effects against HIV-1-induced infections in MT-4 cells.

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Fig. 1 *Linum grandiflorum* Desf. leaves (**A**) and seeds (**B**).

MATERIALS AND METHODS

Plant materials

The aerial parts (leaves and seeds) of *Linum grandiflorum* Desf. were collected in March 2006, from El-Orman Garden, Giza Governorate, Egypt. The plant samples were kindly identified by The Head of Specialists of Plant Taxonomy at the garden, Ms. Tressa Labib. A voucher specimen (No. 38) of the whole plant was kept at the Herbarium of National Research Center (HNRC).

Extract preparation

The air-dried aerial parts [leaves (2.4 Kg) and seeds (184.46 g)] of one-month-old *L. grandiflorum* were extracted by percolation of the plant materials followed by fractionation using an improved fractionation method (Mohammed 2008) according to **Scheme 1**.

Antiviral assay procedures

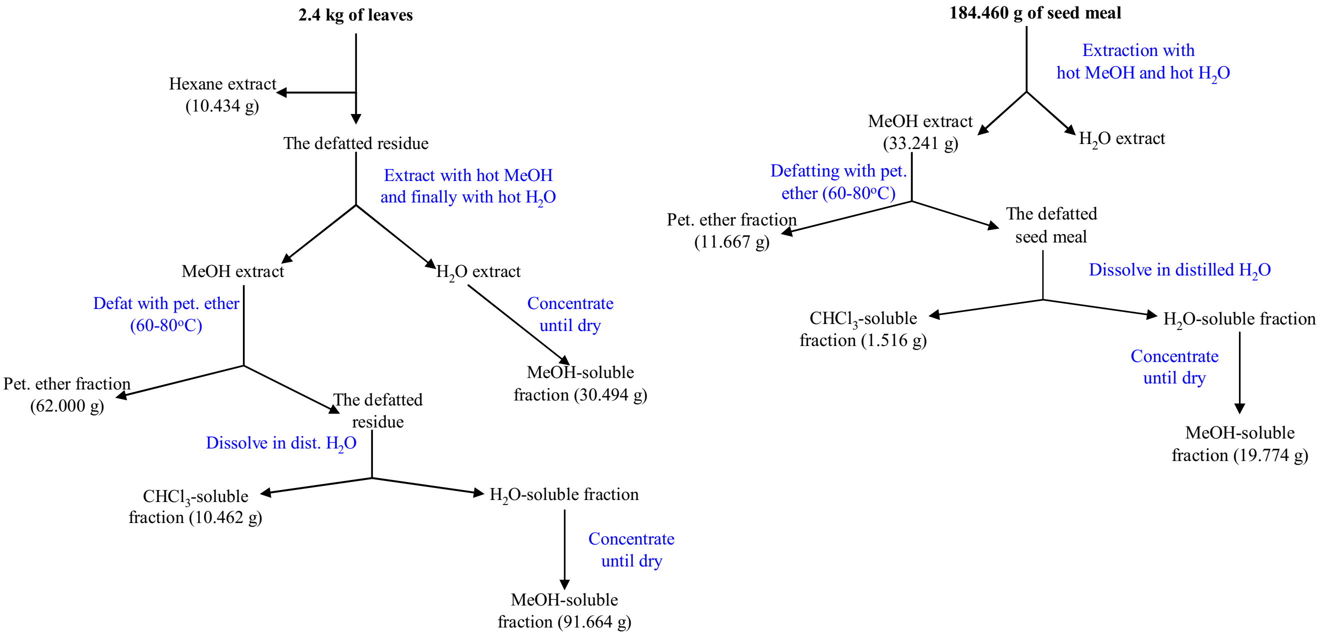
These were performed at the Department of Science and Biomedical Technology, Cittadella University, Monserrato, Italy. Samples were solubilized in DMSO at 100.000 γ and then diluted in RPMI 1640 culture medium.

Virus and cells

Cell lines and viruses were purchased from the NIH ADIS Research and Reference Reagent Program. MT-4, C8166, and H9/HIV-1$_{\text{IIIB}}$ cells were grown at 37°C in a 5% CO_2 atmosphere in RPMI 1640 medium (Reed and Muench 1938) supplemented with 10% fetal calf serum (FCS), 100 IU/mL penicillin G, and 100 µg/mL streptomycin. Cell cultures were checked periodically for the absence of mycoplasma contamination with a MycoTect Kit (Gibco). HIV-1$_{\text{IIIB}}$ was obtained from supernatants of persistently infected H9/HIV-1$_{\text{IIIB}}$ cells. The HIV-1 stock solutions had titers of 4.5×10^6 50% cell culture infectious dose (CCID$_{50}$)/mL. The Y181C mutant (NIH N119) was derived from an AZT-sensitive clinical isolate passaged initially in CEM and then in MT-4 cells in the presence of nevirapine (10 µM). The double mutant K103N +Y181C (NIH A17) was derived from the III$_{\text{B}}$ strain passaged in H9 cells in the presence of BI-RG 587 (1 µM). The triple mutant K103R+V179D+P225H (EFV$^{\text{R}}$ = resistant to Efavirenz$^{®}$ (Sustiva) was derived from an III$_{\text{B}}$ strain passaged in MT-4 cells in the presence of EFV$^{\text{R}}$ (up to 2 µM). N119, A17 and EFV$^{\text{R}}$ stock solutions had titers of 1.2×10^8, 2.1×10^7 and 4.0×10^7 CCID$_{50}$/mL, respectively.

HIV titration

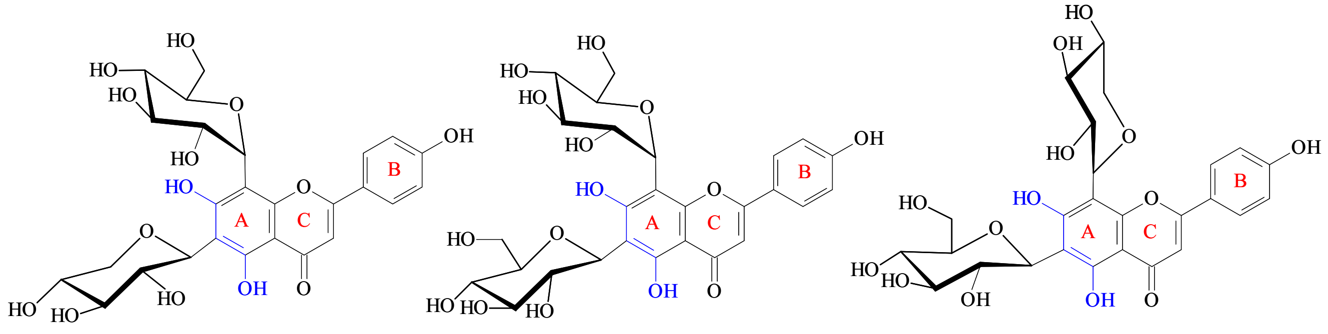
Titration of HIV was performed in C8166 cells by the standard limiting dilution method (dilution 1: 2, four replicates wells per dilution) in 96-well plates. The infectious virus titer was determined by light microscope (Olympus CK2) by trypan blue exclusion (Sigma-Aldrich) at 200X magnification, scoring of syncytia after 4 days of incubation. Virus titers were expressed as 50% cell culture infection doses per millilitre (Reed and Muench 1938).



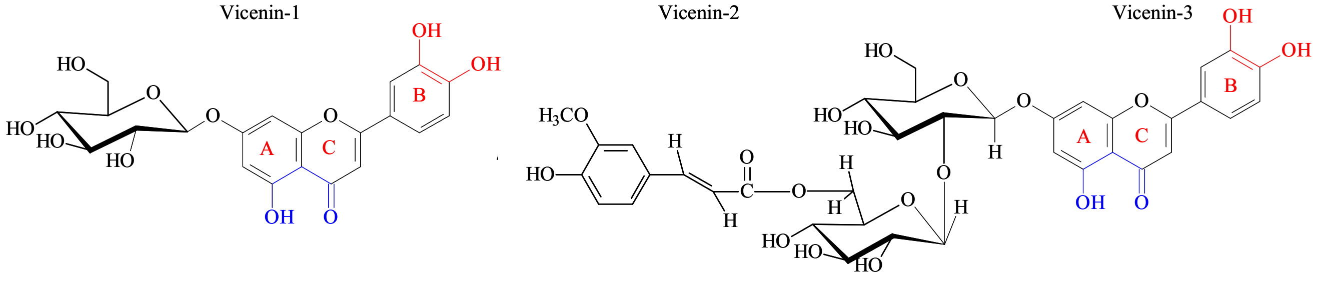
Scheme 1 The extraction procedure of *Linum grandiflorum* leaves and seeds.

Table 1 The cytotoxicities and anti-HIV-1 activities of the extracts from *Linum grandiflorum*.

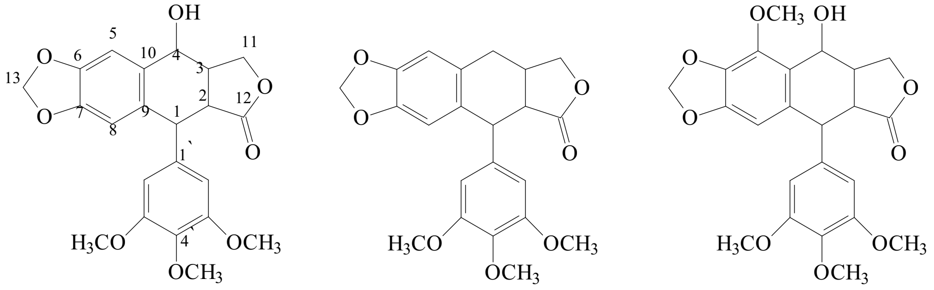
Samples tested	**MT-4/CC$_{50}$**	**WTIII$_B$**	**Y181C**	**K103N-Y181C**	**EFV^R**
Hexane extract of leaves	≥100	>100	>100	>100	>100
Pet. ether fraction from MeOH extract of leaves	>100	>100	>100	>100	>100
$CHCl_3$ fraction from MeOH extract of leaves	>100	>100	>100	100	>100
MeOH fraction from MeOH extract of leaves	>100	>100	>100	>100	>100
MeOH fraction from H_2O extract of leaves	>100	46 ± 6	>100	100	>100
Pet. ether fraction (oily) from MeOH extract of seeds	>100	>100	>100	>100	>100
$CHCl_3$ fraction from MeOH extract of seeds	>100	>100	>100	57 ± 4	>100
MeOH fraction from MeOH extract of seeds	>100	>100	>100	>100	>100



Vicenin-1 Vicenin-2 Vicenin-3



Glucoluteolin Luteolin 7-*O*-D-(6```-*E*-feruloyl)glucopyranosyl(1-2)-D-glucopyranoside



Podophyllotoxin Deoxypodophyllotoxin 5-Methoxypodophyllotoxin

Fig. 2 Previously (Mohammed *et al.* 2009) isolated phenolics from *Linum grandiflorum* Desf.

Anti-HIV assays

The activity of test compounds against multiplication of HIV-1 wild type III$_B$, N119, A17, and EFV^R in acutely infected cells was based on inhibition of virus-induced cytopathicity in MT-4 cells. Briefly, 50 µL of culture medium containing 1 × 10^4 cells was added to each well of flat-bottom microtiter trays containing 50 µL of culture medium with or without various concentrations of test samples. Then 20 µL of HIV-1 suspensions, containing the appropriate amount of CCID$_{50}$ to cause complete cytopathicity at day 4, was added. After incubation at 37°C, cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Pauwels *et al.* 1988). The cytotoxicity of the tested compounds was evaluated in parallel with their antiviral activity and was based on the viability of mock-infected cells, as monitored by the MTT method.

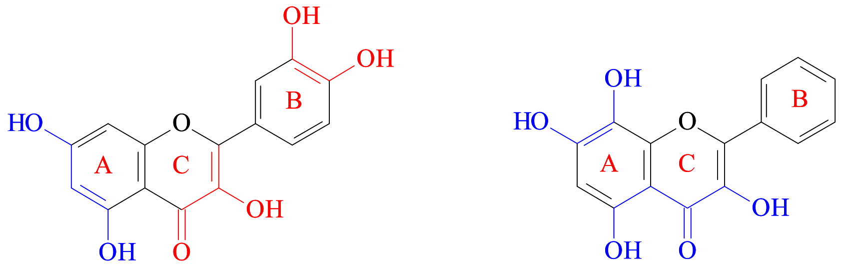
Experimental design and statistical analyses

The results were expressed as means ± S.E.M. and all statistical comparisons were made by means of the Student's *t*-test. $P < 0.05$ was regarded as significant, using SPSS (Version 11).

RESULTS AND DISCUSSION

The cytotoxicity and anti-HIV-1 activity of the extracts from *L. grandiflorum* are summarized in **Table 1**, which clarify that the extracts were non-cytotoxic to MT-4 cells at doses as high as 100 µM when tested on C8166 cells. The MeOH extract of leaves was the most potent than the others extracts (hexane, pet. ether and $CHCl_3$) against MT-4 cell cultures infected with the wild type HIV-1$_{IIIB}$ with an ED$_{50}$ = 46 ± 6 µM. All the other extracts (hexane, pet. ether and $CHCl_3$) of both leaves and seeds were inactive against HIV-1 carrying the triple mutations K103R+V179D+P225 (EFV^R) associated with resistance to the HIV drug Efavirenz (EFV^R) and against both the single mutation Y181C and double mutation K103N+Y181C, which are associated with resistance to non-nucleosides (Joly *et al.* 2004). The $CHCl_3$ extract of the seeds was the most potent against the double mutation K103N+Y181C with an ED$_{50}$ = 57 ± 4 µM than other extracts.

The potency of the leaf MeOH extract of *L. grandiflorum* against MT-4 cell was mainly due to previously isolated flavonoids (**Fig. 2**) (Mohammed *et al.* 2009). Flavonoids are natural products derived from plants that are



3,3`,4`,5,7- pentahydroxyflavone 3,5,7,8- tetrahydroxyflavone

Fig. 3 Basic structural features of flavonoids with high multifunctional activities.

found in many families/genera. These compounds display a variety of biochemical properties including antioxidant activity, inhibition of tyrosine kinases and cAMP phosphordiesterase, and induction of phase II metabolizing enzymes both *in vivo* and *in vitro*. These biochemical interferences elicited by flavonoids in some cell systems have been associated with their capacity to control cell growth or destroy pathogen organisms such as fungi and viruses. One of the most interesting biological properties of flavonoids is their ability to inhibit HIV transcriptase and HIV replication (Jesús and Leonardo 2002). Quantitative structure-activity relationship (QSAR) models are useful in providing a biochemical understanding of the biological activity of natural and synthetic chemicals based solely on molecular structure. Both biological properties were basically dependent on electronic parameters describing charge distribution on the two fused rings of the flavonoid molecule. Atomic charges in C-3 and the carbonyl carbon as well as the dipolar moment were important electronic descriptors to define the studied biological properties of flavonoids (Jesús and Leonardo 2002). Both of the aromatic substituents and the keto-enol functionality (**Fig. 3**) can serve as targets for future structure activity relationship (SAR) studies (Wu *et al.* 2003). The presence of 7-OH e.g. Vicenin-1, -2 and -3 and 3′-OH groups e.g. luteolin-7-*O*-*β*-D-glucopyranoside (glucoluteolin) and luteolin 7-*O*-*β*-D-(6‴-*E*-feruloyl)glucopyranosyl(1→2)-*β*-D-glucopyranoside enhance the inhibitory activity of certain flavones. The substituents at the 3′ and 4′ positions of the phenyl ring B should have electron-donating properties and most probably this part of the flavonoid molecule interacts with the catalytic domain of the enzyme, through hydrogen bonds. In summary, the agreement in description of QSAR in terms of classical and quantum chemical parameters is good. Flavonoids are in general good inhibitors of both enzymes, with their inhibitory potency spreading over 5 orders of magnitude. However, there are specific differences in requirements for their binding to the enzyme sites AR and PTK. For the binding to the enzyme site AR (i) a hydrogen bond donor should be present at position 4′, (ii) larger substituents in 4′ than OH is not favourable, (iii) position 3 requires bulky hydrophobic substituents. To the contrary, in PTK (iv) a hydrogen bond donor at position 3′ or 4′ in the phenyl ring is required and (v) specific orientation of hydrophobic substituents at position 8 is required and steric hindrance at position 3 in the chromone ring is decreasing the inhibitory potency of flavonoids (Alenka *et al.* 2002).

Jesús and Leonardo (2002) reported HIV activity of luteolin with IC$_{50}$ 16 µM and EC$_{50}$ 10 µM, luteolin-7-*O*-*β*-D-glucopyranoside with IC$_{50}$ 25 µM and EC$_{50}$ 7 µM and acetate of luteolin-7-*O*-*β*-D-glucopyranoside with IC$_{50}$ 6 µM and EC$_{50}$ 6 µM, which indicate that the HIV activity of the MeOH extract was mainly due to the isolated luteolin derivatives. It is concluded that the HIV-inhibitory properties of flavonoids are mainly the outcome of electronic interactions between atomic charges within these compounds in both A and B rings and possible receptor-like structures in the HIV or the lymphocyte itself. These agonist-receptor interactions are enhanced by hydrogen bonding contributions and by specific geometrical arrangements associated with each flavonoid. On the other hand, cytotoxicity not only requires electrostatic features on the flavonoid structure but also bulk and shape parameters which could be linked to cell penetration mechanisms (Jesús and Leonardo 2002).

The potency of the $CHCl_3$ extract is mainly due to the previously isolated aryltetrahydro-naphthalene-type lignans (**Fig. 2**): podophyllotoxin, deoxypodophyllotoxin and 5-methoxypodophyllotoxin (Mohammed 2008). Lignans are thought to be byproducts and/or components of the pathway of cinnamate biosynthesis leading to the formation of lignans, and these lignans possess a diverse spectrum of biological properties. Podophyllotoxin and dibenzylbutyrolactone lignans have received considerable attention in recent decades because of their wide-ranging biological activities. Several members of this family and their analogs have been shown to possess potent antiviral properties. For example, the clinically useful anti-cancer drug etoposide has *in vitro* anti-HIV activity with an EC$_{50}$ of 0.03 µM and TI of 42.7 (Zhu *et al.* 2004). Its parent compound podophyllotoxin was toxic at all tested concentrations. Several C-4 modified podophyllotoxins with the methylenedioxy A-ring opened and methylated, and 4′-position demethylated had EC$_{50}$s less than 0.001 µM and TIs greater than 120 (Zhu *et al.* 2004). Podophyllotoxin, etoposide and teniposide are the major lignans which have defined applications in clinical medicine. The role of lignans in the diet, their antiviral properties and their presumed protective roles against certain cancers await clarification. The use of lignans in folk medicine has afforded interesting leads in developing new pharmacological agents (Ayres and Loike 1990).

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