RESEARCH PAPER

Effect of crude extracts and fractions of *Cuscuta campestris* and two different hosts on peripheral blood mononuclear cells and HIV replication

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Abstract

In this study, the effect of *Cuscuta campestris* and two different hosts *Alhagi maurorum and Calendula officinalis* on peripheral blood mononuclear cells (PBMC) proliferation and HIV replication were investigated. The activity of these plant extracts on HIV-1 replication and PBMC proliferation was performed respectively by use of real time PCR and MTT assay. The results showed that crude methanol extracts of *C. campestris* and two hosts significantly induced PBMCs proliferation in a dose dependent manner up to 1000 μg/ml. The most active fraction of *C. campestris* growing on *A. maurorum and C. officinalis* were also detected by nuclear magnetic resonance as Lupeol epoxid and Lutein. These results demonstrate that Lupeol epoxid and Lutein isolated from *C. campestris* are important agents in PBMCs proliferation. The results also have proved that Lutein hasn't any effect on HIV replication, but Lupeol epoxid showed week anti HIV activity. In conclusion, the results demonstrated that lutein and lupeol are good candidates for PBMC proliferation.

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Introduction

The genus *Cuscuta* (Convolvulaceae) also known as dodder is an obligate stem parasite (Parker and Riches, 1993; Hibberd *et al.*, 1998). About 270 species of *Cuscuta* spp. have been reported throughout the world (Holm *et al.*, 1997). *Cuscuta campestris* is one of the most common species of *Cuscuta* (Dawson *et al.*, 1994). The extract of *C. campestris* proved to have analgesic, antipyretic and anti- inflammatory activities (Agha *et al.*, 1996). This parasite has a wide range of host species (Yuncker, 1932; Parker and Riches, 1993). It mainly parasitizes some horticultural crops such as legumes and broad leaved weeds (Parker and Riches, 1993; Dawson *et al.*, 1994). Many of the primary as well as secondary metabolites have been purified from *C. campestris* that transfers from host to parasite**.** The relationship between medical properties of *C. campestris* and its host" *Nerium indicum*" has been reported previously (Ghule *et al.*, 2011).

Calendula officinalis and Alhagi maurorum are the common host plants for *C. campestris* in Iran. *Calendula officinalis*, Linn of Family Asteraceae has been widely used in medicine for the treatment of many diseases (Zitterl-Eglseer *et al.*, 1997). It has been reported to possess many pharmacological activities, which include anti-inflammatory (Della Logia *et al.*, 1994), antibacterial (Dumenil *et al.*, 1980), antifungal (Kasiram *et al.*, 2000), antiviral (Barbour *et al.*, 2004) and tumor reducing potential (Boucaud-Maitre *et al.*, 1988). Chemical constituents of *C. Officinalis* include some triterpenes, triterpene oligoglycosides and flavonol glycosides (Yoshikawa *et al.*, 2001; Neukirch *et al.*, 2004).

Alhagi maurorum belongs to the Fabaceae family native to desert ecoregions. *A.maurorum* (camelthorn) is considered a medicinal plant with its prospective potent flavonoids. The extracts of alhagi species are used in folk medicine as a diaphoretic, laxative, purgative and expectorant. The essential oils of *A. maurorum* are used as row materials in different fields, including aromatherapy, phytotherapy, nutrition and perfumes (Samejo *et al.*, 2012).

In the present study, the effect of methanol extract and different fractions of *C. campestris* and two its hosts *"A. maurorum and C. officinalis"* on Peripheral Blood Mononuclear Cells (PBMC) proliferation and HIV replication were investigated.

Materials and methods

Plant materials and extraction

The aerial parts of *A. maurorum*, *C. officinalis* and their parasite *C. campestris* were separately collected from University of Isfahan herbarium, Iran in July 2012. *C. campestris* was separated from hosts and dried. Then methanol extracts of two hosts and their parasite *C. campestris* were prepared. Air dried and powdered plant materials were extracted with methanol by shaking for 3×48 h at room temperature. Then, the extracts were collected, filtered and evaporated by a rotary evaporator (Stroglass, Italy) at 45°C and dried using a freeze dryer (Zirbus, Germany).

Isolation of active compound from C. campestris

Silica-gel column fractionation chromatographies were carried out with the dried methanol extract of *C. campestris* growing on *A. maurorum and C. officinalis*. 5 g of dried methanol extract of *C. campestris* growing on *A. maurorum* was eluted with Hexane: Acetone: Methanol (10:0:0 – 0:0:10, v/v/v). Fractions 1–24 (0.14, 0.19, 0.19, 0.15, 0.17, 0.20, 0.19, 0.24, 0.17, 0.13, 0.22, 0.18, 0.22, 0.23, 0.19, 0.15, 0.18, 0.21, 0.25, 0.24, 0.27, 0.21, 0.18, 0.19 g) were obtained from *C. campestris*. Fraction 17 was the most active fraction and also evaluate by NMR. Dried methanol extract of *C. campestris* growing on *C. officinalis* (5 g) was eluted with Hexane: Aceton: Methanol: (8:2:0–0:4:6, v/v/v) and 100% methanol. Fractions 1–28 (0.21, 0.21, 0.21, 023, 0.22, 0.21, 0.17, 0.16, 0.19, 0.14, 012, 0.21, 0.27, 0.21, 0.21, 0.25, 0.18, 0.17, 0.13, 0.22, 0.18, 0.19, 0.15, 0.18, 0.21, 0.15, 0.14 and 0.17 g) were obtained. Fraction 16 was found to increase PBMCs proliferation and was also analyzed by NMR.

PBMCs proliferation assay

The proliferation of PHA-stimulated PBMCs was

determined in the presence of methanol extracts of two hosts' *A. maurorum and C. officinalis* and their parasite *C. campestris.* The effect of active fractions isolated from *C. campestris* growing on *A. maurorum and C. officinalis* were also tested on PBMCs proliferation. The solutions to be tested on PBMC proliferation were prepared by dissolving the extracts in Dimethyl sulfoxide (DMSO) and sterilized distilled water. The DMSO has been added to solution at sub toxic concentration (maximum of 0.019%). The effect of different concentrations (10, 100 and 1000 µg/ml) of these methanol extracts on PBMCs proliferation was determined through a modified 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. PBMCs were grown in a 96-multiwell micro plate with 4.0×10^4 cells per well. The cells were cultured in RPMI supplemented with 10% (v/v) Fetal Calf Serum (FCS; Gibco-BRL, Grand Island, NY, USA), 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine and 1 mM Na-pyruvate. After 48 h of incubation at 37 °C, MTT solution (5 mg/ml) was added to each well, and the plate was incubated for 4 h. Finally, 50 µl of PrOH/HCl/TX (0.04M HCl in 2-propanol plus 10% Triton X-100) was added to solubilize the formed formazan crystals. The plate was re-incubated for 24 h and amount of formazan crystal was determined by measuring the absorbance at 492 nm using a micro plate spectrophotometer (Awareness Technology Inc., stat fax 2100).

The PBMC proliferation index (PPI) was calculated as follow:

$$PPI = \frac{OD570\ plant\ extracts\ treated\ cells - OD\ 570\ blank\ solution}{OD570 control - OD\ 570\ blank\ solution} \times 100$$

Percentages of cells proliferation were calculated by using the proliferation indexes as compared with untreated control.

NMR analysis

NMR screening was used to approve structure of active compound. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker 500 MHz spectrometer by use of CDCl$_3$ as residual solvent with chemical shifts expressed in parts per million (ppm).

Antiviral activity

Anti-HIV-1 activity of lupeol epoxide and lutein was studied via Real time PCR. The PBMCs were grown in microtiter tissue culture plates. The cells were infected with 10µl of HIV-1 and supplemented with different concentrations of extract (10 and100 µg/ml) and incubated at 37ºC for 12 hours. Then infected cells were washed and then overlaid with medium. DMSO (0.1%) was used as negative control respectively. After 72 hours of incubation, the overlay medium was used to detect and quantify HIV-1.

Quantitative real-time PCR assay for HIV-1

For real-time PCR, RNA was purified from each specimen by high pure viral nucleic acid kit (Roche Diagnostics, Meylan, France) according to the standard protocol. HIV-1 RNA was detected and quantified by real-time reverse transcription PCR (RT-PCR). As previously described, forward primer NEC152 (5′-CCTCAATAAAGCTTGCCTTGA-3′) and the reverse primer NEC131 (5′-GGCGCCACTGCTAGAGATTTT-3′) and the dually labeled NEC-LTR probe (5′-6-carboxyfluorescein-AGTAGTGTGTGCCCGTCTGTTRTKTGACT-6-carboxytetramethylrhodamine-3′) in the long terminal repeat gene were used [12]. Primers and probes were synthesized by Metabion Co. (Germany). The master mix contained 1× RNA master hybridization buffer, including the Tth DNA polymerase and deoxynucleoside triphosphate mix (containing dUTP instead of dTTP), 2.5 Mn (OAC) 2, and 0.3 µM concentrations of each primer and probe. Cycling conditions were as follows: initial reverse transcription at 61ºC for 30 min, denaturation at 95ºC for 30 s, and 45 cycles of denaturation at 95ºC for 1 s, annealing at 55ºC for 15 s and elongation at 65ºC for 1 min with a ramp of 5ºC/s (with fluorescence acquisition at the end of each elongation stage). The positive control was consisted of culture supernatant of the HIV-1. As a control for cross-contamination, a sample of distilled water was subjected to the RNA extraction procedure, and the resulting extract was amplified in duplicate.

Statistical analysis

Data from three independent experiments are presented as mean ± SD. One-way analysis of variance (ANOVA) test was used to assess significance between solvent control and the test sample. P-value <0.05 was considered to be statistically significant.

Results

NMR analysis

The most active fractions on lymphocyte proliferations obtained from *C. camperstris* growing on *A. maurorum* and *C. officinalis* were respectively fractions 17 and 16. Both fractions were analyzed by ^{1}HNMR and ^{13}CNMR experiments as lupeol epoxid and lutein.
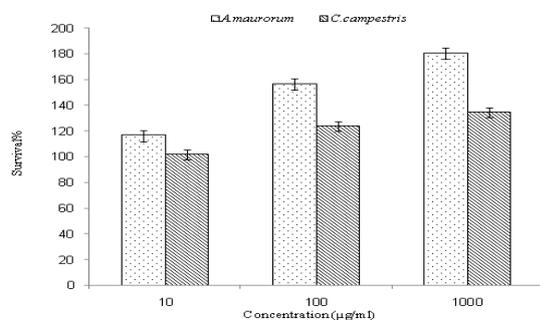

Fig. 1. Effect of A. *maurorum* and *C. campestris* on proliferation of PBMC. Each value is the result of mean ± SD of three independent experiments. P value <0.05 was considered to be statistically significant.

lupeol epoxid NMR Data

^{1}HNMR (CDCl$_3$, 400 MHz): δ 3.23 (1H, m), 2.59 (d, 1H, *J* = 4.8 Hz), 2.42 (m, 1H), 2.28 (d, 1H, *J* = 4.8 Hz), 1.80 (m, 1H), 1.70 (t, 1H, *J* = 5.6Hz), 1.63 (s, 3H), 1.57 (m, 2H), 1.40 (m, 2H), 1.36 (m, 2H), 1.20 (m, 1H), 1.03 (m, 1H), 0.99 (s, 3H), 0.97 (s, 3H), 0.95 (s, 3H), 0.83 (s, 3H), 0.79 (s, 3H), 0.75 (s, 3H).

^{13}CNMR (CDCl$_3$, 100 MHz): δ 79.42, 64.32, 58.14, 53.21, 50.36, 48.65, 48.01, 42.47, 42.01, 41.74, 40.59, 39.84, 38.94, 38.25, 37.34, 36.21, 34.56, 32.12, 30.15, 28.16, 28.02, 24.03, 22.13, 19.86, 19.03, 18.56, 17.17, 16.78, 15.27, 14.42.

Lutein NMR Data

^{1}HNMR (DMSO, 400 MHz): δ 6.21-6.76 (m, 13H), 5.52 (m, 1H), 3.82 (m. 1H), 3.41 (m, 1H), 3.68 (s, 1H), 2.95 (s, 1H), 2.71 (d, 1H, *J* = 9.2Hz), 1.58-2.36 (m,

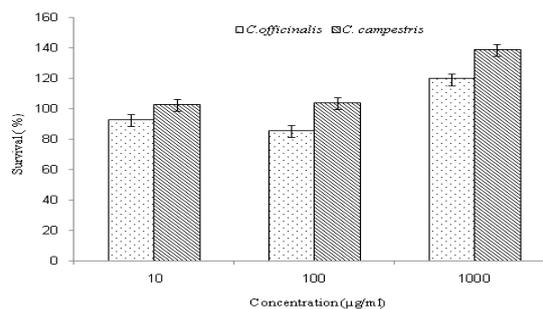6H), 1.69 (s, 12H), 1.71 (s, 6H), 1.01 (s, 6H), 0.85 (s, 6H).

Fig. 2. Effect of *C. officinalis* and *C. campestris* on proliferation of PBMC. Each value is the result of mean ± SD of three independent experiments. P value <0.05 was considered to be statistically significant.

^{13}CNMR (DMSO, 100 MHz): δ 138.11, 137.82, 137.65, 136.81, 136.50, 135.06, 131.24, 131.01, 130.65, 130.01, 129.85, 128.06, 126.66, 125.02, 68.35, 65.01, 57.32, 48.02, 43.91, 42.98, 37.65, 32.34, 28.01, 26.62, 24.31, 21.93, 14.35, 13.78.

PBMCs proliferation assay

The activity of crude methanol extracts of *C. camperstris* growing on *A. maurorum* and *C. officinalis* at different concentrations (10, 100 and 1000 μg/ml) were evaluated on *PBMCs proliferation* by using MTT assay. The results showed that crude methanol extracts of all plants significantly induced PBMCs proliferation in a dose dependent manner up to 1000 μg/ml. As shown in Fig. 1-2 the effect of *A. maurorum* on PBMC proliferations was significantly more than its parasite, But *C. camperstris* growing on *C. officinalis* can increase the number of cells more than its host. The most active fractions of *C. camperstris* growing on *A. maurorum* and *C. officinalis* were respectively identified as lupeol epoxid and lutein. PBMC proliferation of these two extracts was further tested at different concentrations (10, 100 and 1000 μg/ml). As shown in fig.3 both fractions can increase lymphocyte proliferation in a dose dependent manner. The effect of *lupeol* epoxide on PBMC proliferations was significantly more than lutein.

Anti HIV-1 activity

Antiviral activity of lupeol epoxide and lutein isolated from *C. camperstris* growing on *A. maurorum* and *C. officinalis* were evaluated by the real time PCR. Results showed that 1000 µg/ml lupeol epoxide and lutein inhibit viral replication respectively 40 and 15 %. The antiviral activity of these two extracts was further examined by different concentrations (Figure 3). The effective dose to reduce virus titers by 50% (EC50) of lutein epoxide was observed > 1000 µg/ml, respectively.
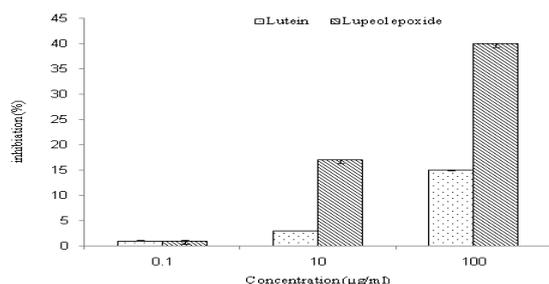

Fig. 3. Effect of lutein and Lupeol epoxide on HIV-1 replication. Each value is the result of mean ± SD of three independent experiments. P value <0.05 was considered to be statistically significant.

Discussion

The results of present study showed that crude methanol extracts of *A. maurorum* and *C. officinalis* and their parasite *C. campestris* significantly induced PBMCs proliferation. *A. maurorum* was found to have higher activity on PBMCs proliferation compared to its parasite. The result also demonstrated that extract of *C. campestris* growing on *C. officinalis* increase PBMCs proliferation more than its host. Some research reported that *A. maurorum* and *C. officinalis* and *C. campestris* extracts could stimulate lymphocytes proliferation (Chew *et al.*, 1995; Bao *et al.*, 2002; Ogechukwu *et al.*, 2011). Lee *et al.* reported that *C. campestris* has strong anti-inflammatory and antiproliferative activities. He also reported that quercetin isolated from *C. campestris* plays an important role in these properties (Lee *et al.*, 2011). But the most active compounds of *C. campestris* growing on *A. maurorum* and *C. officinalis* have not been studied yet. So in the present study column chromatography have been done to purify the active compounds of C. *campestris growing on different hosts*, which increase PBMCs proliferation. The active fractions

obtained from *C. Campestris* growing on *A. maurorum* and *C. officinalis* were also analyzed by spectroscopic experiment as lupeol epoxid and lutein. Lupeol is a bioactive triterpenoid that has been isolated from the root barks of *A. maurorum*. The anti-inflammatory property of *A. maurorum* can be correlated to this compound (Laghari *et al.*, 2011). The previous results demonstrated that the chemopreventive potential of lupeol is probably due to its antioxidant or free radical scavenging property (Palanimuthu *et al.*, 2012). Siddique *et al.* provide mechanism-based evidence that lupeol (Lup-20(29)-en-3b-ol) is a potent inhibitor of androgen receptor (AR) *in vitro* and *in vivo* (Siddique *et al.*, 2011). In recent decade, results confirm the potential of Calendula officinalis investigated hydroalcoholic extracts as medicinal remedies to be used in different inflammatory/allergic diseases. Previously studies demonstrated that lutein increased tumor latency, suppressed mammary tumor growth and enhanced lymphocyte proliferation (Chew *et al.*, 1995). Proliferation effect of *C. campestris* may be due to the transfer from hosts to its parasite. Many of the primary as well as secondary metabolites may transfer from host to parasite. It has been reported that some metabolites transfer from *Nerium indicum* to *C. campestris* and showed potent analgesic effect (Ghule *et al.*, 2011). One study was also demonstrated that Cd and essential metals (such as Zn and Cu) transfer from the host vascular bundles to the parasite (Vurro *et al.*, 2011). This study demonstrated that there is a relationship between lymph proliferative activity of *C. campestris* and their hosts. The results of detection of the bioactive compounds signified that lupeol epoxide and lutein constituted the most active fractions in the crude metabolic extracts of *C. campestris* and displayed a potent lymph proliferative activity.

Acknowledgment

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