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Xanthohumol, a novel anti-HIV-1 agent purified from Hops *Humulus lupulus*

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Abstract

Xanthohumol, prenylchacone flavonoid, is a natural product with multi-biofunctions purified from Hops *Humulus lupulus*. Its anti-HIV-1 activity was tested in the present study. Results showed that xanthohumol inhibited HIV-1 induced cytopathic effects, the production of viral p24 antigen and reverse transcriptase in C8166 lymphocytes at non-cytotoxic concentration. The EC_{50} values were 0.82, 1.28 and 0.50 μg/ml, respectively. The therapeutic index (TI) was about 10.8. Xanthohumol also inhibited HIV-1 replication in PBMC with EC_{50} value of 20.74 μg/ml. The activity of recombinant HIV-1 reverse transcriptase and the HIV-1 entry were not inhibited by xanthohumol. The results from this study suggested that xanthohumol is effective against HIV-1 and might serve as an interesting lead compound. It may represent a novel chemotherapeutic agent for HIV-1 infection. However, the mechanism of its anti-HIV-1 effect needs to be further clarified.

Keywords: Xanthohumol; Anti-HIV-1; Flavonoid; Reverse transcriptase; HIV entry

1. Introduction

Although anti-HIV-1 drugs now available have improved the quality of the lives of HIV/AIDS patients, the appearance of drug resistant virus urged the search for new anti-HIV-1 agents and targets. Many compounds with anti-HIV-1 effect have been screened out from natural products. They include alkaloids, sulfated polysaccharides, polyphenolics, flavonoids, coumarines, phenolics, tannins, triterpenes, lectins, alkaloids, phloroglucinols, lactones, iridoids, depsidones, *O*-caffeoyl derivatives, lignans, ribosome inactivating proteins (RIPs), saponins, xanthones, naphthodianthrones, photosensitisers, phosholipids, quinines and peptides (Ng et al., 1997; Vlietinck et al., 1998). Natural products provide a large reservoir for screening of anti-HIV-1 agents with novel structure and anti-viral mechanism because of their structural diversity. Xanthohumol is a constituent of beer, the major dietary source of prenylated flavonoids and a natural product with multi-biofunctions purified from the hop *Humulus lupulus*. Xanthohumol showed antiproliferative activity in cancer cell lines derived from human breast cancer, colon cancer and ovarian cancer in vitro (Miranda et al., 1999). The anti-cancer activity of xanthohumol may be due in part to the inhibition of cytochrome P450 enzymes that activate carcinogens such as the heterocyclic amine 2-amino-3-methylimidazo[4,5-f] quinoline (Henderson et al., 2000; Miranda et al., 2000a,b). In addition to its anti-cancer activity, xanthohumol showed antioxidant activity in inhibiting low-density lipoproteins oxidation (Miranda et al., 2000a,b). The xanthohumol-enriched hop extract, which contained 8.4% xanthohumol, displayed weak to moderate antiviral activity against bovine viral diarrhea virus (BVDV), herpesviruses HSV-1 and HSV-2. The xanthohumol alone appeared to account for all of the antiviral activity of the xanthohumol-enriched hop extract for the

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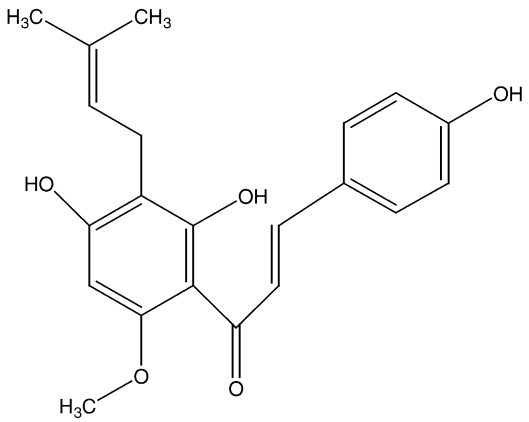
therapeutic index (TI) of xanthohumol against BVDV, HSV-1 and HSV-2 were similar to those of xanthohumol-enriched extract (Buckwold et al., 2004). Xanthohumol is also an inhibitor of diacylglycerol acyltransferase (DGAT) which catalyses the reaction of triacylglycerol formation (Tabata et al., 1997). Xanthohumol is classified as sprenylchacone flavonoid. Flavonoids have showed anti-HIV-1 activity with emphasis on inhibiting HIV-1 reverse transcriptase (RT) (Ng et al., 1997; Vlietinck et al., 1998; Matthee et al., 1999). In the present study, the anti-HIV-1 activity of xanthohumol was investigated. Our results demonstrated that xanthohumol showed anti-HIV-1 activity in addition to its other biological and pharmacological properties and a China patent was applied (Liu et al., 2002).

2. Materials and methods

2.1. Extraction and isolation of xanthohumol

Xanthohumol (M_w 410, Fig. 1) was extracted and purified from the hop *H. lupulus*. Hops were grown in northwest of China (Xinjiang) and harvested in 2001. A voucher specimen is deposited at the Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences.

Hop cones (10 kg) were exhaustively extracted with 70% ethanol (five times) at room temperature. The solutions were combined and the solvent was evaporated in vacuo to afford a residue, which was treated with water (5 L) and extracted with petroleum ether (10 L, five times) and EtOAc (10 L, five times), respectively. EtOAc was removed in vacuo and the residue (540 g) was subjected to silica gel column chromatography using a chloroform–methanol gradient (98:2 v/v, 65× 1000 ml; 95:5 v/v, 60× 1000; 90:10 v/v, 30× 1000 ml). Total 155 fractions were obtained. The fractions 10–20 eluting with chloroform–methanol (98:2 v/v) were purified by repeated column chromatography (chloroform:EtOAc, from 90:10 to 80:20 v/v) to afford xanthohumol (4.66 g). Comparison of the physicochemical properties with the reported data was allowed to identify xanthohumol (Sun et al., 1989; Tabata et al., 1997). Xanthohumol was ultrapure (purity >99%, HPLC analysis) and dissolved in DMSO (Sigma).



Xanthohumol (Mw: 410)

Fig. 1. The molecular structure of xanthohumol.

2.2. Cells and virus

C8166, H9 cells and HIV-1_{IIIB} strain were kindly donated by Medical Research Council (MRC), AIDS Reagent Project, UK. The cells were maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (Gibco), and HIV-1_{IIIB} was obtained from the culture supernatant of H9/HIV-1_{IIIB} cells. The 50% HIV-1 tissue culture infectious dose ($TCID_{50}$) in C8166 cells was determined and calculated by Reed and Muench method. Virus stocks were stored in small aliquots at −70 °C. The titer of virus stock was 9×10^5 $TCID_{50}$/ml.

2.3. Cytotoxicity assay

The cytotoxicity of xanthohumol was determined by MTT assay as described previously (Zheng et al., 1995). The absorbance at 595 nm/630 nm ($A_{595/630}$) was read in an ELISA reader (Elx800, Bio-Tek Instrument Inc., USA). The minimum cytotoxic concentration that caused the reduction of viable cells by 50% (CC_{50}) was determined from dose response curve.

2.4. Assays for anti-HIV-1 activities

In the presence or absence of various concentrations of xanthohumol, 3×10^4 C8166 cells were exposed to HIV-1_{IIIB} at a multiplicity of infection (M.O.I.) of 0.01. The cells were incubated in 96-well plates or 24-well plates at 37 °C in 5% CO_2 for 3 days. AZT (3′-azido-3′-deoxythymidine) was used as a positive control. At 3 days post-infection, the cytopathic effect (CPE) was measured by counting the number of syncytia (multinucleated giant cells) in each well of 96-well plates was counted under an inverted microscope, and HIV-1 p24 antigen in the culture supernatants was determined by an enzyme-linked immunosorbent assay (ELISA) (Wang et al., 2003). The virus particles in the culture supernatant of each well of 24-well plates were precipitated by 30% PEG (M_w 8000), lysed with lysis buffer and the activity of reverse transcriptase (RT) was measured by a commercial RT kit (Roche Molecular Biochemicals). The minimum inhibitory concentrations that reduced CPE, HIV-1 p24 antigen and RT production by 50% (EC_{50}'s) were interpolated from plots generated from the data. The TI was calculated from the ratio of CC_{50}/EC_{50}.

2.5. HIV-1 p24 detection assay

McAb to HIV-1 p24 (kindly provided by Prof. H. Hoshino, Gunma University School of Medicine, Japan) dissolved in carbonate buffer was used to coat a 96-well microtiter plate

(Greiner), which was then blocked with phosphate-buffered saline (PBS) containing 5% milk and 0.1% BSA. The 0.5% Triton X-100 treated culture supernatants were added to the wells and incubated at 37 °C for 2 h. Then purified antiserum of HIV-1 positive individuals, horseradish peroxidase (HRP)-labeled-goat-anti-human IgG (Sino-America Biotech. Co., China) and the substrate OPD were added sequentially (Wang et al., 2003). The absorbance at 490 nm/630 nm ($A_{490/630}$) was read in the ELISA reader.

2.6. RT assay

HIV-1 RT activity was measured using an ELISA RT kit according to the instructions of the manufacturer. The samples were incubated with DIG-labeled-reaction mixture at 37 °C for 15 h, then anti-DIG-POD solution was added, followed by substrate ABTS. Foscarnet was used as a positive compound. The absorbance at 405 nm/490 nm ($A_{405/490}$) was read in the ELISA reader.

2.7. Inhibition of HIV-1 replication in PBMC

The 5 μg/ml PHA activated peripheral blood mononuclear cells (PBMC) were pretreated with xanthohumol for 1 h, then the cells were infected with HIV-1_{IIIB} at a M.O.I. of 1.0 for 2 h. After washing with PBS, the cells were incubated in culture medium supplemented with xanthohumol and 50 U/ml of human recombinant IL-2. Fresh medium was added at the third day post-infection and the supernatant was collected at the sixth day post-infection for HIV-1 p24 antigen detection. The viability of PBMC was determined by the Trypan blue dye exclusion test.

2.8. HIV-1 entry assay

C8166 cells were pretreated with or without xanthohumol at 37 °C for 1 h before being infected at a M.O.I. of 0.01. Dextran sulfate (DS) was used as positive control. The infected cells were trypsinized and washed with PBS completely to

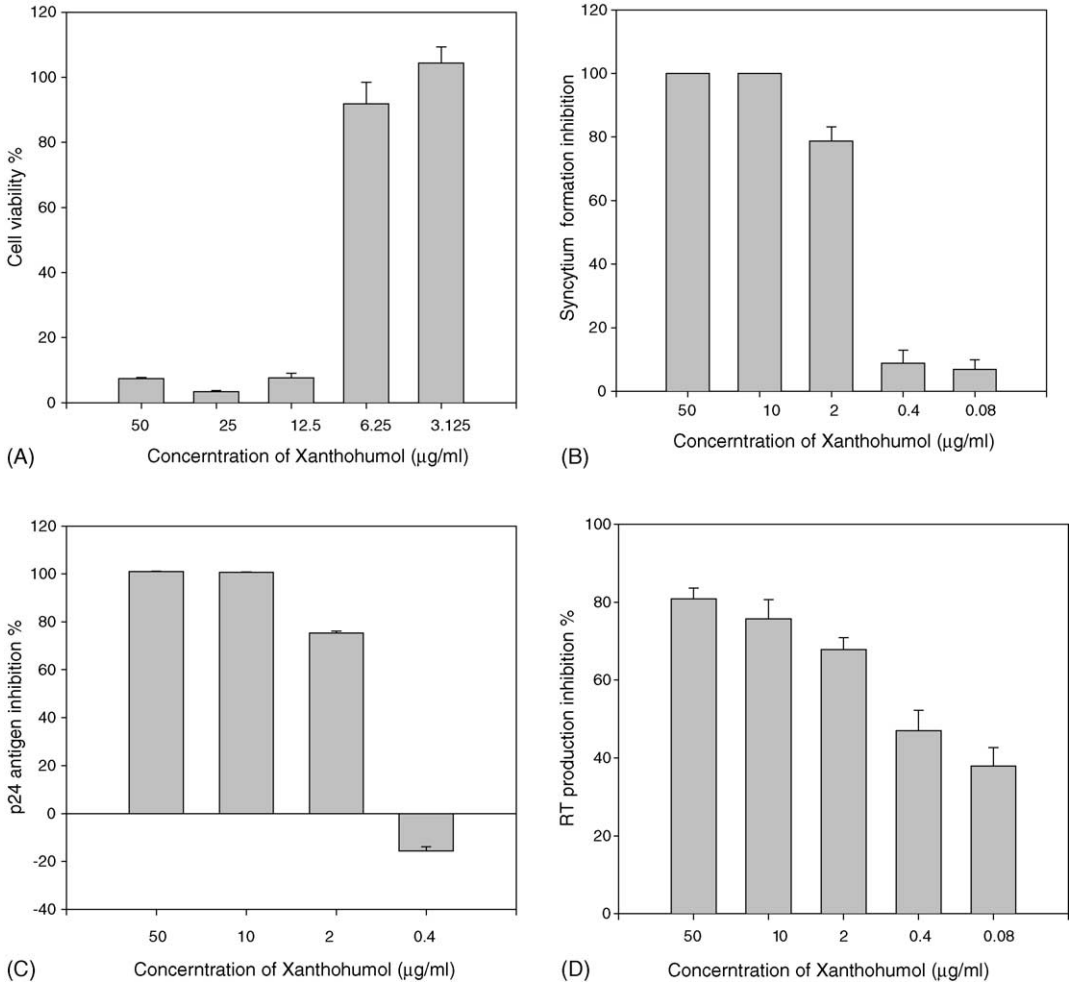


Fig. 2. Cytotoxicity and anti-HIV-1 activities of xanthohumol. Xanthohumol was dissolved in DMSO with a concentration having no effect on the results. Cytotoxicity on C8166 cells (A), HIV-1_{IIIB} induced syncitium formation inhibition (B), HIV-1_{IIIB} p24 antigen in culture supernatants inhibition (C) and HIV-1_{IIIB} RT production in supernatants inhibition (D). The RT concentration of the samples was calculated according to an absorbance–concentration calibration curve.

remove the unattached virus. Total RNA and the internalized HIV-1 RNA were extracted and amplified with a commercial HIV-1 RT-PCR kit (Sino-America Biotech. Co.) on a Cycler (Biometra, Germany). The products were detected on a 2% agarose gel.

3. Results

3.1. Xanthohumol was a novel inhibitor of HIV-1

The cytotoxicity of xanthohumol was shown in Fig. 2A. The CC_{50} of xanthohumol was 8.82 μg/ml (21.51 μM) and the EC_{50} of xanthohumol on inhibiting the cytopathic effects was 0.82 μg/ml (2.00 μM). The TI of xanthohumol was about 10.8. The EC_{50}'s of xanthohumol on inhibiting HIV-1 p24 antigen and RT production were 1.28 μg/ml (3.21 μM) and 0.50 μg/ml (1.22 μM), respectively. The inhibitory activity of xanthohumol on inhibiting HIV-1 induced syncytium formation (cytopathic effects), HIV-1 p24 antigen and HIV-1 RT production was shown in Fig. 2. Xanthohumol also inhibited HIV-1 replication in PBMC infected with $HIV\text{-}1_{IIIB}$ (Fig. 3), the EC_{50} of xanthohumol for inhibiting p24 antigen production was 20.74 μg/ml (50.55 μM). 54.2% p24 antigen production in $HIV\text{-}1_{IIIB}$ chronically infected H9 cells was inhibited by xanthohumol at dosage of 50 μg/ml (data not shown). The results showed that xanthohumol had no obviously cytotoxic effects on PBMC. The viable percent of PBMC was above 60% for xanthohumol at concentration of 50 μg/ml (Fig. 3).

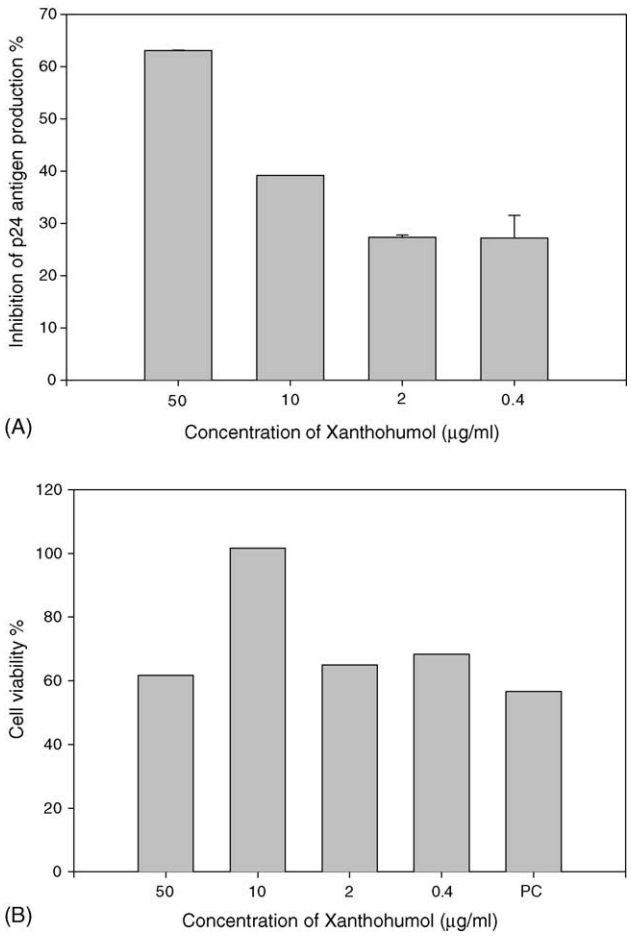


Fig. 3. Inhibition of HIV-1 replication in PBMC of xanthohumol. Inhibition of p24 antigen production (A). Viable percent of PBMC detected by Trypan blue dye exclusion test (B). PC represented positive control (HIV-1 infected PBMC without compounds).

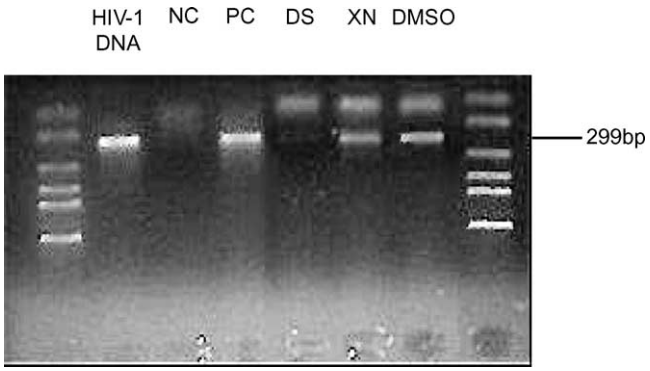


Fig. 4. Effects of xanthohumol on HIV-1 entry. The lanes from left to right were DNA markers, HIV-1 DNA, normal cells (NC), infected cells (PC), DS (50 μg/ml), Xanthohumol (XN, 50 μg/ml), DMSO (0.2%), DNA Markers. DNA Markers from bottom to top were 2000, 1000, 750, 500, 250, 100 bp. The amplified HIV-1 DNA fragment was 299 bp.

3.2. The targets of xanthohumol on HIV-1

The inhibition of xanthohumol on the activity of recombinant RT and HIV-1 entry were assayed. The activity of recombinant RT was not inhibited by xanthohumol. At a dosage of 10 μg/ml (24.39 μM), only 3.80% inhibition of activity of recombinant RT was caused whereas the same concentration of xanthohumol inhibited the cytopathic effects completely. Xanthohumol also did not interfere with the entry step of HIV-1. As shown in Fig. 4, xanthohumol did not show any inhibition of $HIV\text{-}1_{IIIB}$ entering into the target cells at a dosage of 50 μg/ml (121.94 μM), the concentration that inhibited the cytopathic effects completely. However, the DS blocked the $HIV\text{-}1_{IIIB}$ entering into cells. The target of xanthohumol on HIV-1 needed to be further studied and it may lie on the steps post reverse transcription.

4. Discussion

The anti-HIV-1 activities and cytotoxicity of xanthohumol were summarized in Table 1. Xanthohumol was a selective inhibitor of HIV-1 and may represent a novel therapeutic agent for HIV-1 infection. Buckwold et al. (2004) reported that xanthohumol-enriched hop extract with antiviral activity against BVDV, HSV-1 and HSV-2 had no anti-HIV-1 activity. It may due to the low content of xanthohumol in the

Table 1
Summary of cytotoxicity and anti-HIV-1 activity of xanthohumol

Cells	Cytotoxicity (CC_{50}, μg/ml)	Anti-HIV-1 activities (EC_{50}, μg/ml)		
		Syncytia	p24	RT
C8166	8.82 ± 0.43	0.82 ± 0.05	1.28 ± 0.03	0.50 ± 0.09
PBMC	>50	ND	20.74 ± 0.12	ND

ND: not determined.

xanthohumol-enriched extract, since the extract only contained 8.4% xanthohumol.

Unlike many other flavonoids with inhibitory activity on HIV-1 RT (Ng et al., 1997; Matthee et al., 1999), xanthohumol did not inhibit the activity of recombinant HIV-1 RT. Xanthohumol completely inhibited HIV-1 induced cytopathic effects at 10 μg/ml (24.39 μM) concentration, but caused only 3.80% inhibition on activity of recombinant HIV-1 RT.

Entry step of HIV-1 was another attractive target for flavonoids. Flavonoids extracted from grape seed showed anti-HIV-1 activities by interfering the HIV-1 coreceptors CCR2b, CCR3 and CCR5 (Nair et al., 2002). Flavonoid compound baicalin, a well-known HIV-1 non-nucleoside reverse transcriptase inhibitor (NNRTI), also showed its anti-HIV-1 activity by inhibiting the HIV-1 coreceptors CCR5 and CXCR4 (Li et al., 2000). However, xanthohumol did not inhibit HIV-1 entering into the target cells whereas the control compound DS did as shown in Fig. 4.

The target of xanthohumol on HIV-1 may lie on the steps post reverse transcription. There is an evidence to show that flavonoids can inhibit HIV-1 activation in models of latent infection (Critchfield et al., 1996). The inhibitory activity may due to the effects of flavonoids on HIV-1 transcription. Flavonoids inhibited the cellular protein casein kinase II (CKII) that was involved in HIV-1 transcription (Critchfield et al., 1997). A flavonoid compound quercetin inhibited the HIV-1 Vpr protein which induced transcription from HIV-1-LTR (Shimura et al., 1999). Flavonoid compound flavopiridol inhibited HIV-1 transcription by inhibiting cellular protein P-TEFb which was a cofactor for HIV-1 transactivator, Tat (Chao et al., 2000). Flavonoids also showed inhibitory activities on HIV-1 protease and integrase (Xu et al., 2000; Fesen et al., 1993). The mechanism of anti-HIV-1 activity of xanthohumol needs to be further studied and will be focused on the steps of HIV-1 post reverse transcription.

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