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Anti-HIV activities of extracts from Pu-erh tea

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[ABSTRACT] AIM: To evaluate the anti-HIV activities of extracts from two commonly forms of Pu-erh tea (fermented), green tea-like (unfermented). **METHODS:** The cytotoxicities of extracts on C8166, MT4 and PBMC were assessed by MTT and the anti-HIV activities of extracts were evaluated by syncytium reduction and p24 antigen assay. **RESULTS:** All extracts had low cytotoxicities with the CC_{50} from 120.63 to 524.95 μg·mL^{-1}. All extracts can inhibit HIV-induced cytopathic effects, with EC_{50} various from 11.13 to 67.49 μg·mL^{-1}. The WEPT's were better than EEPTs, moreover the FPT's water extracts were better than the GTLPT's especially YYP-31 with SI value 42.40. YYP-31 also inhibited HIV-1_{RF} and HIV-2_{CBL-20} infection with EC_{50} of 30.82 and 39.79 μg·mL^{-1}, respectively. YYP-31 reduced p24 antigen expression in HIV-1_{IIIB} acute infected C8166 and in HIV-1_{KM018} infected PBMC with EC_{50} values of 14.95 and 74.63 μg·mL^{-1}, respectively. YYP-31 blocked the fusion between normal C8166 cells and HIV-1 chronically infected H9 cells with EC_{50} values of 234.27 μg·mL^{-1}. It exhibits markedly synergistic anti-HIV activity in combination with AZT but can't inhibit activities of reverse transcriptase. **CONCLUSION:** Pu-erh tea extracts exhibit highly efficient anti-HIV-1 activity and can be considered to be used to as a complementary therapy or a health product to HIV patients.

[KEY WORDS] Tea; *Camellia sinensis*; Pu-erh Tea; Anti-HIV agents

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1 Introduction

Human immunodeficiency virus (HIV) is the etiologic agent of the acquired immunodeficiency syndrome (AIDS), a disease that already claimed the lives of more than 25 million people. The global incidence of HIV infection in 2010 was estimated to be approximately 33.2 million people. The introduction of potent combination therapies in the mid-1990s has significantly improved the prognosis for infected individuals with access to treatment. However, there are the major drawbacks related to drug adherence, tolerability and long-term toxicity that limit the efficacy of those treatments. Therefore, the need and demand has prompted an intense research effort to discover new, selective and safe drugs for the treatment of HIV/AIDS.

Tea is the product of the leaves, leaf buds, and internodes of various cultivars and sub-varieties of the *Camellia sinensis* plant, processed using various methods. Tea has been considered as both a healthy beverage and a medicinal substance since ancient times. Although various health benefits have been attributed to tea consumption and biological effects have been underway for less than 30 years [1]. The tea fall roughly into green tea, white tea, yellow tea, oolong tea, black tea and Pu-erh tea, depending on the processing and the degree of fermentation. Pu-erh tea is the Yunnan characteristic tea and a microbially fermented tea made from the leaves of large-leaf species tea plant. Pu-erh tea could be classified into GTLPT and FPT, according to their processing procedures [2]. Pu-erh tea is gaining much attention for its biologi-

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cal activities for health benefits. Well-known effects associated with Pu-erh tea include slowing or prevention of cancer, heart disease, rheumatoid arthritis, and impaired immune disease [3-5]. Several reports have shown that green tea catechins have an interfered with HIV-1 life cycle: physical integrity of virion, binding to host cells, post-adsorption entry and replication of genome by inhibiting reverse transcriptase [6-9]. Liu et al found black tea catechin derivatives disrupt conformation of HIV-1 gp41 six-helix bundle fusion-active core, resulting in the failure of viral membrane fusion [10]. Here, we describe the anti-HIV activity of extracts from both GTLPT and FPT.

2 Materials and Methods

2.1 *Reagents and chemicals*

MTT and AZT (3'-azido-3'-deoxythymidine) were purchased from Sigma. Horseradish peroxidase (HRP) labeled goat anti-human IgG was purchased from Dingguo Biotechnology Company (China). The p5F1 and monoclonal antibody (McAb) against HIV-1 p24 were prepared by our laboratory [11].

2.2 *Pu-erh tea samples extract preparation*

The Pu-erh Tea samples were purchased from Simiao Tianfu Economic and Trade Co., Ltd. (SMTF) and Simiao Pingyi Pu-erh Tea House (SMPY) in the Yunnan. Pu-erh tea (30 g) was infused in solvents (water and 95% ethanol, respectively) for 24 h, and solvents were vacuumly evaporated, then water extracts (WEPTs) and ethanol extracts (EEPTs) of Pu-erh tea were obtained (Table1).

Table 1 Summary of the extracts of Pu-erh tea

Samples	extractions	Pu-erh Tea	Company
YYP-11	water	FPT	SMTF
YYP-12	ethanol		
YYP-21	water	FPT	SMTF
YYP-22	ethanol		
YYP-31	water	FPT	SMTF
YYP-32	ethanol		
YYP-41	water	GTLPT	SMTF
YYP-42	ethanol		
YYP-51	water	GTLPT	SMPY
YYP-52	ethanol		

2.3 *Cell lines and viruses*

Human T lymphocyte C8166, MT4 cells and chronically infected H9 cells (H9/HIV-1_{IIIB}) were kindly donated by the Medical Research Council, AIDS Research Project (UK). Cell lines were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Gibco). Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors (Kunming Blood Center, Yunnan) and incubated in complete medium containing 5 μg·mL^{-1} phytohemagglutinin (PHA) (Sigma) and 50 U·mL^{-1} human recombinant IL-2 (Sigma) for 3 d prior to antiviral assays. The laboratory-derived viruses HIV-2_{CBL-20}, and HIV-2_{ROD} were obtained from the NIH AIDS Research and Reference Reagent Program (USA). The HIV-1 clinically isolated strain HIV-1_{KM018} was isolated and cultured in our laboratory from a Yunnan HIV/AIDS patient as described [12]. The 50% HIV tissue culture infectious doses ($TCID_{50}$) were determined and calculated by the Reed and Muench method. Viruses were stored at −70℃ in aliquots.

2.4 *Cytotoxicity assays*

The cytotoxicities of extracts on C8166, MT4 and PBMC were assessed by MTT colorimetric assay as described previously [13]. The absorbance at 595 nm/630 nm 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.

2.5 *Syncytium reduction assay*

In the presence of 100 μL various concentrations of extracts, C8166 cells (4×10^5/mL) were infected with viruses (HIV-1_{IIIB}, HIV-1_{RF}, HIV-2_{ROD}, HIV-2_{CBL-20}) at a multiplicity of infection (MOI) of 0.03, 0.08, 0.08 and 0.1, respectively. The final volume per well was 200 μL. AZT was used as a positive control. After 3 d of culture, the number of syncytia (multinucleated giant cells) was scored under an inverted microscope; 50% effective concentration to blocking syncytia formation (EC_{50}) was calculated [14].

2.6 *Inhibition of HIV-1 p24 antigen production in acute infection*

The inhibitory effect of compound on HIV-1 replication in vitro was further examined by quantification of p24 expression using capture ELISA as previously described. Briefly, C8166 cells were inoculated with HIV-1_{IIIB} (MOI = 0.03) in the absence or presence of various concentrations of extracts at 37 °C for 2 h to allow for viral absorption. It was then washed three times with PBS. After 4 d, the supernatants were harvested and HIV-1 p24 expression in cell-free supernatants was assayed by ELISA. The absorbance at 490 nm/630 nm ($A_{490/630}$) was read in the ELISA reader.

2.7 *Inhibition of HIV-1 p24 antigen production in PBMC*

Adequate numbers of PHA-activated normal PBMC were incubated with HIV-1_{KM018} (M.O.I.=0.08) in presence of various concentration of extracts at 37 °C. After 2 h the cells were washed twice with PBS and then incubated in culture medium supplemented with 50 U·mL^{-1} human recombinant IL-2 for 7 d. At 7 d post-infection, the supernatants were harvested and lysed. HIV-1 p24 antigen in the culture supernatants was analyzed by ELSIA. The absorbance at 490 nm/630 nm was read in the ELISA reader [12].

2.8 *Protection for HIV-1 induced lytic effects*

The activities of extracts protection of MT-4 cells from HIV-1-induced cytopathic effect were assessed. Briefly, 4×10^4 of MT-4 cells (100 μL) uninfected or infected by HIV-1

at 0.1 MOI was cultured with 100 μL of extracts at different concentrations. After 7 d of incubation at 37 °C, cell viability was determined by MTT assay [15-16].

2.9 *Cocultivation assay*

3×10^4 C8166 cells co-cultured with 1×10^4 HIV-1_{IIIB} chronically infected H9 cells in the presence or absence of the extracts with various concentrations at 37 °C in a humidified atmosphere of 5% CO_2. Enfuvirtide (T-20) was used as positive control. After overnight incubation, the number of syncytia was scored under an inverted microscope [17].

2.10 *Anti-HIV-1_{IIIB} activities of YYP-31 combined with AZT*

In the presence of 100 μL various concentrations of YYP-31 and EC_{50} concentrations of AZT (4 ng·mL^{-1}) 50 μL, C8166 cells (4×10^5/mL) were infected with HIV-1_{IIIB} at a multiplicity of infection (MOI) of 0.03. The final volume per well was 200 μL. After 3 d of culture, the cytopathic effect (CPE) was measured by counting the number of syncytia in each well under an inverted microscope. Percentage inhibition of syncytial cell formation was estimated from the percentage of syncytial cell number in treated culture to that in infected control culture and 50% effective concentration (EC_{50}) was calculated.

2.11 *Inhibition assay of recombinant HIV-1 RT activity*

HIV-1 reverse transcriptase (RT) activity was measured by ELISA RT kit using a commercially available kit (Roche) according to the instructions of the manufacturer. The compounds were incubated with DIG-labeled reaction mixture at 37 °C for 2 h, then anti-DIG-POD solution was added, followed by substrate ABTS. Foscarnet was used as a positive control. The absorbency at 405 nm/490 nm ($A_{405/490}$) was read on Bio-Tek ELx 800 ELISA reader [14].

3 Results

3.1 *Anti-HIV-1_{IIIB} activities of the Pu-erh tea extracts*

The cytotoxicities and anti-HIV-1 activities of all the Pu-erh tea extracts (YYP-11, YYP-12, YYP-21, YYP-31, YYP-32, YYP-41, YYP-42, YYP-51 and YYP-52) are summarized in Table 2. The CC_{50}s of WEPTs (YYP-11, YYP-21, YYP-31, YYP-41 and YYP-51) on C8166 were 519.45, 434.20, 524.95, 120.63 and 229.96 μg·mL^{-1}, respectively. The CC_{50}s of the EEPTs (YYP-12, YYP-22, YYP-32, YYP-42 and YYP-52) and tea polyphenols were 354.58, 328.42, 380.20, 295.94, 146.98 and 59.63 μg·mL^{-1}, respectively. The EC_{50}s of WEPTs (YYP-11, 21, 31, 41 and 51) inhibited HIV-1_{IIIB} induced syncytium formation with 17.36, 19.80, 12.38, 15.36, and 11.13 μg·mL^{-1}, respectively, lower than that of EEPTs (YYP-12, 22, 32, 42 and 52) and tea polyphenol, which with EC_{50}s of 65.40, 67.49, 61.34, 21.95, 19.22 and 19.39 μg·mL^{-1}, respectively. Both the cytotoxicities and anti-HIV-1_{IIIB} results showed YYP-31 of WEPTs showed best ability on inhibiting HIV-1_{IIIB} with TI of 42.40.

3.2 *Protection of YYP-31 on HIV-1 infected cells*

Of all the WEPTs tested and the YYP-31 exhibited the best protection of HIV-1-induced cells lytic effects *in vitro* with the CC_{50} of > 200 μg·mL^{-1} and with the EC_{50} of 14.95 μg·mL^{-1} (Fig. 1).

Table 2 Anti-HIV-1 activities and cytotoxicities of the Pu-erh tea extracts

samples	Cytotoxic (CC_{50}, μg·mL^{-1})	Syncytia (EC_{50}, μg·mL^{-1})	Selective index (SI)
YYP-11	519.45	17.36	30.43
YYP-12	354.58	65.40	5.42
YYP-21	434.20	19.80	21.93
YYP-22	328.42	67.49	4.87
YYP-31	524.95	12.38	42.40
YYP-32	380.20	61.34	6.20
YYP-41	120.63	15.36	7.85
YYP-42	295.94	21.95	13.48
YYP-51	229.96	11.13	20.35
YYP-52	146.98	19.22	7.65
Tea polyphenol	59.63	19.39	3.08
AZT	1 289.24	3.87 ng·mL^{-1}	33 3136.95

The data shown in the table are a representative of three independent experiments.

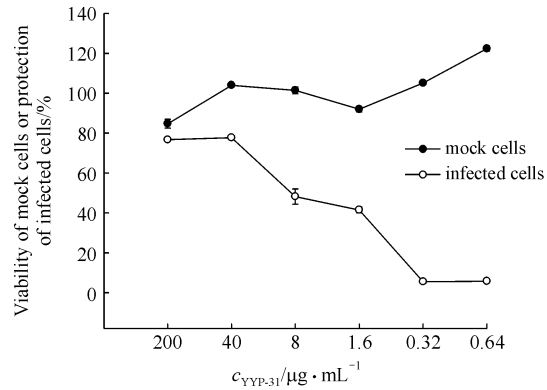

Fig. 1 Protection of WEPT on HIV-1 induced cell lytic effects. Results are expressed as percentages of cellular viability relative to control (100%). The toxicity to MT-4 cells was also measured (Mock). Data are expressed as $\bar{x} \pm s$

3.3 *Anti-HIV-1_{RF} activity of YYP-31*

HIV-1_{RF} strain, another HIV-1 B subtype virus, was evaluated in this study. The results showed that YYP-31 with highly potent activity against HIV-1 replication. The EC_{50} is 30.82 μg·mL^{-1} (Fig. 2A).

3.4 *Anti-HIV-1 clinical strains HIV-1_{KM018} activities of YYP-31*

To test YYP-31 on HIV-1 inhibitory activity of clinical isolates, we successfully isolated and cultured clinical isolates of HIV-1_{KM018} from HIV-1 infected patients of Yunnan Province. The results showed that YYP-31 can inhibit HIV-1_{KM018} with the EC_{50} of 74.63 μg·mL^{-1} (Fig. 2B).

3.5 *Anti-HIV-2 activities of YYP-31*

HIV-1 as the main cause of the AIDS pandemic virus, HIV-2 spread ability and pathogenicity are much lower than HIV-1. HIV-2 and HIV-1 homology sequence is only 55% −60%. We also detected the WEPTs' inhibition activities on HIV-2_{ROD} and HIV-2_{CBL-20} replication. The YYP-31 exhibited inhibition activities on HIV-2_{CBL-20}. The EC_{50} of YYP-31 was 39.79 μg·mL^{-1} (Fig. 2C) but did not show effective inhibition activities on HIV-2_{ROD} with EC_{50} > 200 μg·mL^{-1} (Fig. 2D).

3.6 *Anti-HIV-1_{IIIB} activities of YYP-31 combined with AZT*

The result showed that YYP-31 combined with half EC_{50} of AZT (4 ng·mL^{-1}) did not increase toxicity on C8166 cells, with the CC_{50} 551.80 μg·mL^{-1} (Fig. 2E), meanwhile strikingly increased inhibitory activity with the EC_{50} of 2.80 μg·mL^{-1} which decreased about 4−5 folds compared to without AZT (Fig. 2F).

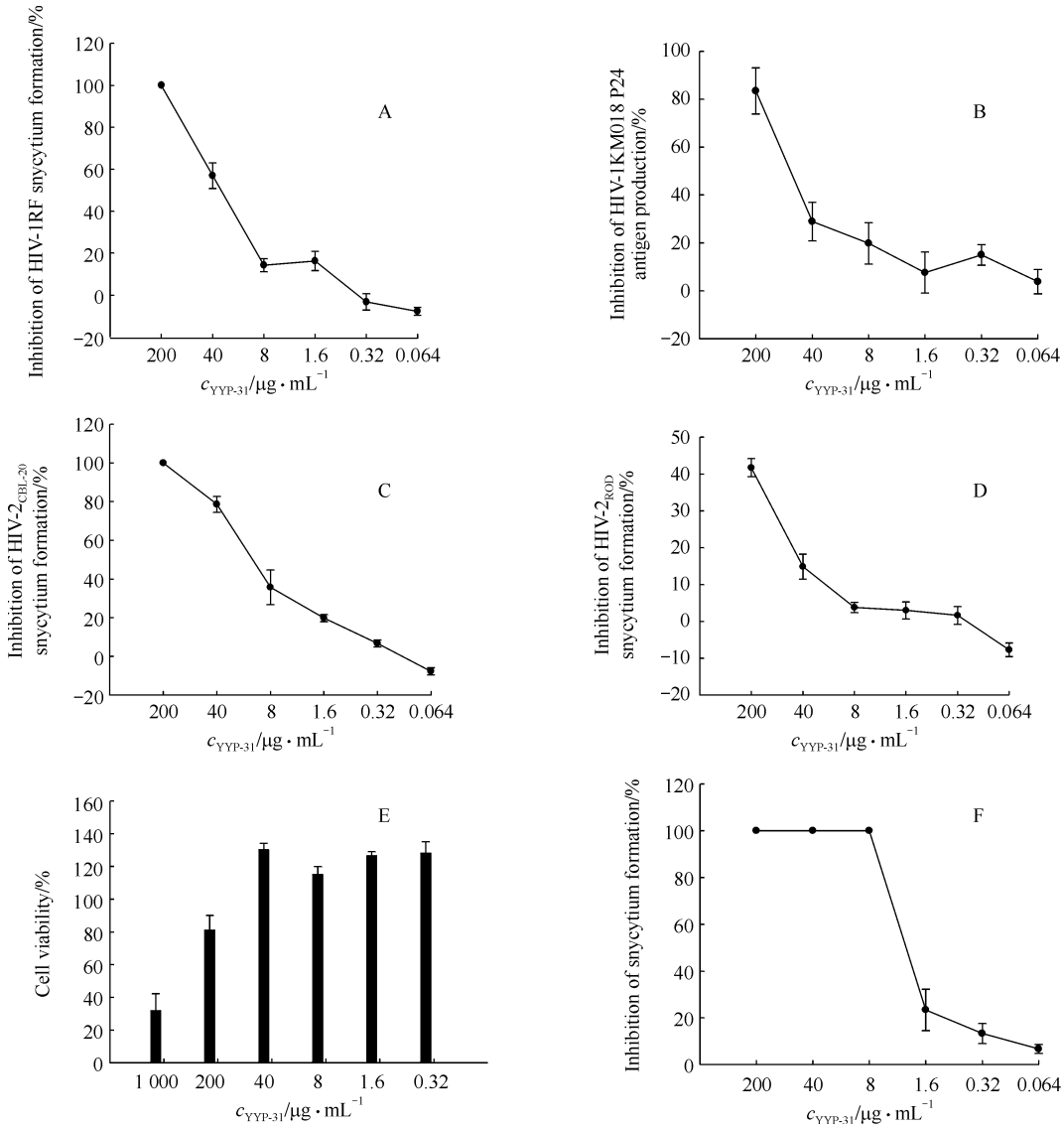

Fig. 2 Inhibitory activities of YYP-31 on HIV replication. Replication of HIV-1KM018 p24 antigen in PBMC (B) were measured by ELISA; inhibiton of HIV-1RF (A), HIV-2CBL-20 (C) and HIV-2ROD (D) induced CPE in C8166 cells, were measured by counting the syncytia formation under inverted microscope. Assay of YYP-31 combined with AZT on cytotoxicities (E) and anti-HIV-1 activities (F). Cytotoxicity on C8166 cells was measured by MTT assay. Anti-HIV-1 activity was determined by syncytia reduction assay. Data are expressed as $\bar{x} \pm s$

3.7 *Anti-HIV mechanism of WEPT*

3.7.1 *Co-cultivation assay of YYP-31*

To address the action mechanisms, uninfected C8166 cells were co-cultured with infected H9/HIV-1_{IIIB} cells in presence of compound. The results suggest that YYP-31 can inhibit cell-to-cell transmission of HIV-1_{IIIB}. The EC_{50} of YYP-31 was 234.27 μg·mL^{-1} (Fig. 3).

3.7.2 *Recombinant HIV-1 RT activity assay of YYP-31*

YYP-31 showed no activity on inhibition HIV RT. The inhibition of YYP-41 was only 8.9% at concentration of 250 μg·mL^{-1}.

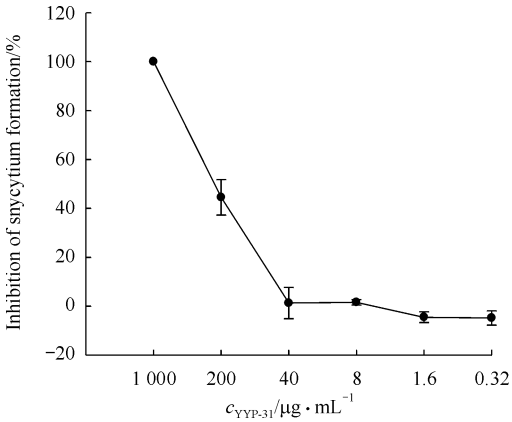

Fig. 3 Inhibitory activities of YYP-31 on blocking fusion. Inhibitory effect on cell-to-cell fusion between normal C8166 cells and HIV-1_{IIIB} infected H9 cells were measured by counting the syncytia formation under inverted microscope. Data are expressed as $\bar{x} \pm s$

4 Discussion

The identification of nutrients or food factors for the prevention or treatment of diseases is an evergreen topic. Pu-erh tea is a unique resource in Yunnan province and it has a long history in China and abroad. Moreover Pu-erh tea is not only a popular, economical and safe drink beverage, later it was found many kinds of bioactivities. There are a number of literatures documenting the anti-HIV activity of green tea [9, 18-20]. However, there is a few reports, to the best of our knowledge, showing that Pu-erh tea have inhibitory activity on HIV-1 infection.

In the present study, we tested the anti-HIV-1 activities of Pu-erh Tea extracts including WEPTs and EEPTs that came from the different kind products and found that most of these extracts had inhibitory activity on HIV-1 replication at low microgram levels. Meanwhile WEPTs were better than EEPTs and the FPTs were better than GTLPT but tea polyphenols was very poor on inhibiting HIV-1 repilcation. Polyphenols are the major components of tea leaves, including the Pu-erh variety [21]. However, the fermentation process lowers the polyphenol levels in tea; conversely, levels of polysaccharides and statins greatly increase after the fermentation process [2]. We all know that the major pharmacologically active ingredients such as tea polyphenols, tea polysaccharide, caffein is water-soluble substances. Based on these we hypothesized that except polyphenol it is also possible that there are other less abundant constituents in Pu-erh tea extract, such as free amino acid and unsaturated fatty acids, may have significant effects on inhibition HIV-1 replication.

The results showed that YYP-31 can markedly inhibit HIV-1_{IIIB} replication and also had effect on protecting the infected cells of MT-4 cell line with the EC_{50} 14.95 μg·mL^{-1}, so the anti-HIV activities of YYP-31 were further evaluated. The results suggest that it inhibited HIV-1_{RF} and clinical isolate HIV-1_{KM018} potently. Similar results were obtained when anti-viral activities on HIV-2_{CBL-20} strains were tested. Research reports suggest that EGCG (the main polyphenol in green tea) inhibits HIV-1 infection of lymphocytes in multiple step, one of the mechanism is binding to CD4 and interfering with gp120 binding [18]. The anti-HIV mechanisms of YYP-31 were investigated in this research, too. YYP-31 inhibited cell-to-cell transmission of HIV-1 potently indicated that it interfered in the early stage of HIV life cycle, but the EC_{50} of fusion assay was higher than the EC_{50} of HIV-1_{IIIB} p24. This suggested that YYP-31 had other targets on the HIV-1 replication cycle. To identify major targets of YYP-31, more tests were carried out. In the enzymatic assay, YYP-31 can't suppresse HIV-1 RT activity by only 8.9% at concentration of 250 μg·mL^{-1}. In summary, these results suggested that YYP-31 may interfere in early steps of HIV-1 life cycle. The molecular mechanisms of it deserve further investigation.

Anti-HIV-1_{IIIB} activities assays indicated that YYP-31 exhibits synergistic anti-HIV activity in combination with AZT at 4ng/mL, the inhibitory effects on HIV-1_{IIIB} increased 4−5 times, the EC_{50} from 12.38 μg·mL^{-1} decrease to 2.8 μg·mL^{-1} but the CC_{50} did not influence. This suggests that YYP-31 could be used as a safe complementary therapy to HIV patients.

In conclusion, the Pu-erh tea extracts especially YYP-31 of WEPTs can effectively inhibit HIV replication and Pu-erh tea extracts can be used to as a complementary therapy or a health product to HIV patients. It appeared that polyphenol in Pu-erh tea didn't play an important role in Pu-erh tea-induced anti-HIV activity. It will be beneficial to identify the responsible components of this crude fraction. More studies on anti-HIV mechanisms of this Pu-erh tea extracts are in progress.

Abbreviations

AIDS: acquired immunodeficiency syndrome; CC_{50}: 50% cytotoxic concentration; EC_{50}: 50% effective concentration; HIV: human immunodeficiency virus; HRP: horseradish peroxidase; M.O.I: multiplicity of infection; MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; $TCID_{50}$: 50% HIV-1 tissue culture infectious dose; WEPT: water extracts of Pu-erh tea; EEPT: ethanol extracts of Pu-erh tea; FPT: fermented Pu-erh tea; GTLPT: green tea-like Pu-erh tea.

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普洱茶提取物的抗 HIV 活性

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【摘　要】 **目的：**研究普洱熟茶(发酵)和普洱绿茶(未发酵)这 2 种常见普洱茶的抗 HIV 作用。**方法：**采用 MTT 比色法检测普洱茶提取物对 C8166, MT4 和 PBMC 细胞的毒性作用; 细胞病变法检测化合物对 HIV-1 急性感染的抑制活性。采用细胞病变法和 HIV-1 p24 抗原 ELISA 方法检测普洱茶提取物的抗 HIV 活性作用; **结果：**所有普洱茶提取物都具有较低的细胞毒性 CC_{50} 为 120.63−524.95 μg·mL^{-1}。所有普洱茶提取物均能抑制 HIV 诱导细胞形成合胞体其 EC_{50} 为 11.13−67.49 μg·mL^{-1}。普洱茶水提物的抑制作用比醇提物的抑制作用要好, 并且普洱熟茶的抑制作用好于普洱生茶, 尤其是 YYP-31, 其 SI 值为 42.40。YYP-31 对 HIV-$_{RF}$ 和 HIV-2$_{CBL-20}$ 也有很好的抑制作用, 其 EC_{50} 分别为 30.82 和 39.79 μg·mL^{-1}。YYP-31 还能很好地抑制 HIV-1$_{IIIB}$ 急性感染 C8166 细胞和 HIV-1$_{KM018}$ 感染 PBMC 细胞 p24 抗原的产生, 其 EC_{50} 分别为 14.95 和 74.63 μg·mL^{-1}。YYP-31 能阻止正常细胞 C8166 与 HIV-1 慢性感染细胞 H9/HIV-1 $_{IIIB}$ 之间的融合, EC_{50} 为 234.27 μg·mL^{-1}。提取物 YYP-31 与 AZT 联合用药具有显著的协同抗 HIV 作用, 但是对 HIV 逆转录没有抑制作用。**结论：**普洱茶提取物具有很好的抗 HIV 的活性作用, 可以作为一种 HIV 辅助治疗的产品或保健品用于 HIV 患者的治疗过程中。

【关键词】 茶; 茶树; 普洱茶; 抗 HIV 药物

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