INTRODUCTION

Medicinal plants with anti-HIV-1 activity have been studied intensively (Cheenpracha, Karalai, Ponglimanont, Subhadhirasakul, & Tewtrakul, 2006; Suedee, Tewtrakul, & Panichayupakaranant, 2014; Tewtrakul, Itharat, & Rattanasuwan, 2006; Wang et al., 2008). Ellagic acid is a naturally occurring polyphenol compound found in pomegranates, blackberries, raspberries, strawberries, and walnuts (Cerda, Tomas-Barberan, & Espin, 2005; Vattem & Shetty, 2005). A wide range of biological activities have been demonstrated for ellagic acid, including anti-allergy, anti-inflammation, antibacterial, and antitumor properties (Panichayupakaranant, Tewtrakul, & Yuenyongsawad, 2010). However, it is not clear if ellagic acid can inhibit HIV-1 infection in target cells. Therefore, the purpose of this study was to investigate anti-HIV-1 infection of ellagic acid in HIV-1 target cells. The effects on HIV-1 integrase and protease enzymes have also been assessed as well as the cytotoxicity on host cells.

Objective: To investigate the in vitro effects of ellagic acid on HIV-1 replication.

Methods: Anti-HIV-1 activity of ellagic acid was determined in vitro using X4-tropic HIV-1NPO3 and R5-tropic pBaL Env-recombinant virus. Anti-HIV-1NPO3 activity of ellagic acid was investigated at a multiplicity of infection (MOI) of 0.01. Anti-HIV-1 integrase and protease activities of ellagic acid were tested using in vitro integration and proteolytic cleavage assays.

Results: Ellagic acid, added either before or after HIV-1NPO3 exposure, suppressed replication of the virus in C8166 cells up to 34%. Ellagic acid showed an anti-integrase IC50 of 8.7 μM. No cytotoxicity of ellagic acid at concentrations ranging from 12.5 to 100 μM was observed.

Conclusion: We conclude that ellagic acid can inhibit HIV-1 infection without cytotoxicity. Thus, it may be a new effective agent that has potential to be developed as a novel microbicide against HIV-1.

KEYWORDS
ellagic acid, HIV-1, infection, integrase, microbicide, protease
2 | MATERIALS AND METHODS

2.1 | Ellagic acid preparation

Ellagic acid was purchased as a commercial powder from Chestnut bark (Sigma, Saint Louis, MO, USA). It had purity greater than 95% by high-performance liquid chromatography (HPLC). The compound was dissolved in 1 M sodium hydroxide (NaOH).

2.2 | Cells used and cytotoxicity of ellagic acid

Two cell lines were used in this study: C8166 cells, a human CD4+ T-lymphocyte cell line expressing the HIV-coreceptor C-X-C chemokine receptor type 4 (CXCR-4) for anti-HIV-1 activity study; H9 cells, a human CD4+T-lymphocyte cell line for the virus propagation. The cytotoxicity of ellagic acid on C8166 cells was assessed by MTT assay (Sigma).

2.3 | Viral construct used

HIV-1_{NPO3} an X4, subtype E (circulating recombinant [CRF01_AE]) virus was grown in H9 cells. The 50% tissue culture infective dose (TCID_{50}) was 2.38 × 10^{4} TCID_{50} per ml, as determined in C8166 cells according to the Reed and Muench method (Reed & Muench, 1938). HIV-1_{NPO3} was obtained from the Armed Forces Research Institute of Medical Sciences in Bangkok, Thailand. A multiplicity of infection (MOI) of 0.01 was used to determine if ellagic acid could inhibit HIV-1 infection.

2.4 | Effects of ellagic acid on HIV-1 replication

Ellagic acid at concentrations ranging from 12.5 to 100 μM was added in two conditions either before or after the cells were exposed to the virus: 1) Before the exposure, C8166 cells were treated with ellagic acid for 1 hr (called "pretreated cells"), then exposed to HIV-1_{NPO3} for 18 hr, washed three times, and incubated for 72 hr; 2) After exposure, the cells were exposed to HIV-1_{NPO3} for 18 hr, washed three times, then added ellagic acid, and incubated for 72 hr (called "post-treated cells"). Azidothymidine (AZT) served as a positive control. Untreated cells and the cells added with the same volume of NaOH were used as negative controls. HIV-1_{NPO3} replication was detected by p24 antigen ELISA in the cell-free supernatants (ABL), according to the manufacturer’s instructions.

2.5 | HIV-1 integrase and protease activity assays

The effects of ellagic acid on HIV-1 integrase and protease activities were determined using an in vitro integration and proteolytic cleavage assays, respectively (Tewtrakul et al., 2006).

2.6 | Statistical analysis

The data were analyzed using one-way ANOVA and/or Kruskal-Wallis H test for the differences between groups and considered statistically significant at p-values of <.05.

3 | RESULTS

3.1 | Ellagic acid shows no cytotoxicity

No cytotoxicity on C8166 was observed for up to 72 hr in the presence of ellagic acid ranging from 12.5 to 100 μM (Figure 1).

3.2 | Ellagic acid inhibits HIV-1 replication in target cells

Ellagic acid at a concentration of 25 μM significantly inhibited X4-tropic HIV-1_{NPO3} replication by up to 34% in pretreated cells and 33% in post-treated cells (p < .05; Figure 2).

3.3 | Ellagic acid inhibits HIV-1 integrase but not protease in vitro

Ellagic acid inhibited HIV-1 integrase activity in a dose-dependent manner with 50% inhibitory concentration (IC_{50}) value of 8.7 μM (Figure 3). In contrast, ellagic acid at the dilutions used in the experiment had no effect on HIV-1 protease activity (IC_{50} > 100 μM).

4 | DISCUSSION

In this study, we investigated the effects of ellagic acid on HIV-1 enzymes and HIV-1 replication in vitro. Our results demonstrate that ellagic acid inhibited HIV-1 integrase, but not protease. Ellagic acid suppressed replication of X4-tropic HIV-1 in the target cells without cytotoxic effects, when added either before or after the viral challenge. Taken together, these results suggest that ellagic acid possessed potent anti-HIV-1 activity without cytotoxicity, and thus, it may be developed as a novel microbicide. The pronounced
Ellagic acid is a phenolic phytochemical (Vattem & Shetty, 2005). Reports from recent studies that phenolic compounds such as flavonoids showed HIV integrase inhibitory activity in enzyme-based assays (Li et al., 2014) are consistent with our findings. Flavonoids have also been reported to block the interaction between integrase and the lens epithelium-derived growth factor/p75 (LEDGF/p75), a cellular HIV-1 integration cofactor that promotes binding of the pre-integration complex to host chromatin (Li et al., 2014). As expected from its inhibitory effects on the HIV-1 integrase enzyme, ellagic acid inhibited HIV-1 infection in the cell lines tested. Consistent with these findings, flavonoids like ellagic acid have been reported to prevent HIV-1 infection in C8166 cells (Li et al., 2014). Another study reported that tannins potently prevented membrane fusion and HIV-1 entry into target cells by interfering with transmembrane glycoprotein (gp41) core formation, which is a critical step of viral-cell fusion (Lu, Liu, Jiang, & Wu, 2004). Thus, our results and those of other groups suggest that ellagic acid may have multiple mechanisms of action against HIV-1 infection. Further studies should be performed to determine if anti-HIV-1 activity of ellagic acid may also occur indirectly via alteration of the innate response repertoire of the infected target cells. Our study demonstrated that ellagic acid suppressed the replication of X4-tropic HIV-1 (CRF01_AE), which is primarily reported in South and South-East Asia (Hemelaar, Gouws, Ghys, & Osmanov, 2011). Both viral strains used in the study are in HIV group M, which is predominant worldwide (Hemelaar et al., 2011). Interestingly, both pre- and post-treatment with ellagic acid demonstrated inhibitory effects on HIV-1 infection. This may imply that ellagic acid can suppress HIV-1 replication at both early and late stages. However, it seems to be more potent when delivered before the viral challenge than after.

In summary, this study demonstrated that ellagic acid with no toxicity to target cells can effectively inhibit HIV-1 replication, possibly through multiple molecular mechanisms. First, ellagic acid inhibited HIV-1 integrase in vitro. Second, ellagic acid inhibited HIV-1 replication when delivered before or after the viral challenge, suggesting that ellagic acid may play a crucial role against HIV-1 infection at both early and late stages. The results of this study indicate that ellagic acid may be used as a new effective anti-HIV-1 agent.

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CONFLICTS OF INTEREST
None to declare.

AUTHOR CONTRIBUTIONS
Dr. Aornrutai Promsong developed the proposal, performed experiments, analyzed data, and prepared and revised the manuscript. Dr. Thippawan Chuenchitra, Ms. Krongkan Saipin, Assoc. Prof. Dr. Supinya Tewtrakul, and Assoc. Prof. Dr. Pharkphoom Panichayupakaranant advised experiments. Dr. Surada Satthakarn performed experiments. Prof. Dr. Wipawee Nittayananta designed the study, developed the proposal, applied for the research grant, directed the project, interpreted the results, and prepared and revised the manuscript.

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