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Screening of anti-HIV activities in ethanol extract and fractions from *Ficus fistulosa* leaves

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Abstract

Objectives: Human immunodeficiency virus (HIV) infection is considered as a major immunosuppressive disease linked to malignancies and other opportunistic infections. Recently, the high prevalence of HIV drug-resistant strains required a high demand for novel antiviral drug development, especially in herbal medicine approaches. The objective of this study was to evaluate the possibility of *Ficus fistulosa* leaves can inhibit HIV replication in ethanol extract form as well as its fractions using chloroform, ethyl acetate, and butanol solvents.

Methods: *F. fistulosa* leaves were extracted using ethanol as a solvent and further gradually fractionated in chloroform, ethyl acetate, and butanol solvents. The targeted persistently infected virus (MT4/HIV) cell lines were cocultured with ethanol extract and fractions at different time points. The syncytium formation and cytotoxicity assays were performed to evaluate the potential antiviral activity of *F. fistulosa* leaves.

Results: One of the four tested extract/fractions showed antiviral activity against HIV. The ethanol extract showed weak inhibition with a high level of toxicity (IC_{50} = 8.96 μg/mL, CC_{50} ≥50 μg/mL, and SI = 5.58). Meanwhile, chloroform fraction effectively inhibited the MT4/HIV cell proliferation while keeping the toxicity to a minimal level (IC_{50} = 3.27 μg/mL, CC_{50} = 29.30 μg/mL, and SI = 8.96). In contrast of ethyl acetate fraction and butanol fraction showed no anti HIV activity with a high level of toxicity (CC_{50} ≥50 μg/mL) and low SI value (>2.17 μg/mL and >0.97 μg/mL).

Conclusions: Chloroform fraction of *F. fistulosa* leaves showed effectively as anti-viral activity against MT4/HIV cells.

Keywords: anti HIV; *Ficus fistulosa*; *in vitro*; medicinal plant.

Introduction

Since being discovered in 1983, human immunodeficiency virus (HIV-1) has been infecting over 38 million people worldwide based on WHO global data tracking with over 25 million (>65%) infected individuals reside in Africa [1]. The distinctive characteristic of HIV-1 is the substantial genetic diversity that builds up within and between hosts [2]. The high mutation rate in HIV-1 is due to the absence of DNA repair enzymes, creating approximately one nucleotide mutation per cycle during viral replication [3]. This unique characteristic of HIV-1 has become a big obstacle to researchers in studying the new approach of HIV-1 antiviral drug discovery.

Diverse novel approaches to anti HIV treatments are currently being developed worldwide and mostly focusing on the initial step of viral entry. One approach targeted the gp120 receptor on the HIV-1 envelope to create a novel antiviral drug by using boronic acid materials as an anti-retroviral agent (ART) [4, 5]. Another therapeutic method, frequently named "shock and kill" used latency-reversing agents (LRAs) to stimulate pro-viral expression ("shock"), and afterward the latent HIV-infected cells could be exterminated by viral cytopathic effects or host immune responses ("kill") [6, 7]. A recent study showed LRAs extracted from untested marine natural products was efficient to induce antiviral activity *in vitro* [8]. One alternative method is polypharmacology in which two or more multitarget or hybrid drugs can be used simultaneously to increase the antiviral activity against HIV-1 [9, 10]. To date, currently available anti HIV drugs remain nonoptimal

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due to complicated procedures, uncontrolled cytotoxicy, and unpredicted side effects.

The failure in HIV-1 treatment especially in low-middle income countries was due to a shortage of drugs and the high cost of treatment [11]. However, the main factor of treatment failure is the lack of patient's discipline to take their prescribed drugs daily in the correct dose or not showing up at doctor's appointments for follow-up checks. The patient's social behavior could impact the development of drug-resistant therapy as well as increasing the fatality rate. The urge for *de novo* anti HIV drug development that is cost-efficient and has fewer side effects has become the priority in AIDS pharmacological research, preferably compounds extracted from local natural sources.

The research about natural products in drug development has been used since centuries ago, pioneered by the Egyptians in the discovery of Penicillin from *Penicillium chrysogenum* recorded in 1928 and was clinically accessible in the 1940s [12, 13]. The medicinal properties of active compounds from plants were derived from the anti inflammatory and protective defense mechanisms of these products [14, 15]. Calanolides from *Calophyllum lanigerum* was one of the first plant-derived compounds discovered to have antiviral activity against HIV-1 [16, 17]. Betulinic acid extracted from the Chinese herb *Syzygium claviflorum* was succeeded to be synthesized as drug, Bevirimat, and continue in clinical trials since 2007 [18]. Other plant-based antiviral candidates have been studied intensively over the years and the results are promising. Additionally, the utilization of locally sourced natural products could be beneficial economically in the development of more affordable medicines.

The genus Ficus has approximately 725 species worldwide and three species namely *Ficus hispida, Ficus septica,* and *F.fistulosa* have been studied, and they exhibited anticancer, anti inflammatory, and antiviral activities [19–21]. However, only Indriati et al. reported the potential anti HIV activity, and this recent study will investigate further the hidden anti-viral activity of *Ficus fistulosa* against HIV-1. In the present study, four samples derived from *F. fistulosa*, ethanol extract, ethyl acetate, chloroform, and butanol fractions were used in *in vitro* assay to assess their antiviral activity.

Materials and methods

Cells and viruses

The human acute T-lymphoblastic leukemia cells (MOLT-4/MT-4) were cultured in RPMI-1640 medium and DMEM medium (GIBCO, USA) with the addition of 10% fetal bovine serum (Sigma, USA), 100 U/mL penicillin G, and 100 µg/mL streptomycin. The cells were incubated at 37 °C and 5% CO_2 humidity for three days prior to further usage. In addition, the persistently infected cells were previously made from coculture of peripheral blood mononuclear cells (PBMC) from HIV-1 patients and healthy blood donors. Initially, the PBMC were pre activated with 10 µg/mL phytohemagglutinin (PHA), mitogen, and later on, induced with interleukin-2 (IL-2) [22]. The mix of MT-4 and the in-house persistently infected cells HIV-1 (MT-4/HIV-1) were cocultured according to the protocol from Gyuris et al. [23]. All HIV-1 isolates derived from the stock were collected from Surabaya, Indonesia.

Plant materials

The leaves from *F. fistulosa* were obtained from Cangar Conservation Forest, Malang, West Java, Indonesia. The verification and identification of the plant were supervised by Purwodadi Botanical Garden – Indonesia Institute of Science, East Java, Indonesia. All plant specimens were stored in Natural Product Medicine Research and Development Laboratorium (NPMRD), Institute of Tropical Disease (ITD), Universitas Airlangga, Surabaya, Indonesia.

Plant extraction and fractionations

F. fistulosa leaves as much as 2.5 kg were drying at room temperature and were powdered into approximately 250 g dry stocks. Later on the 250 g of dry stocks were soaked in 1,250 mL of 80% ethanol and sonicated for 2 min (three times) to extract the constituents. The extract was filtered and residue was extracted again using 1,250 mL of 80% ethanol by the same procedure. The extraction of residue was repeated once again. The extraction was conducted by ultrasonic assisted extraction method for three times and using 3,750 mL of 80% ethanol (3 × 1,250 mL) as a solvent in total. The ethanol extract was further evaporated to obtain 13 g of dried ethanol extract (E). The 10 g of dried ethanol extract was suspended in 100 mL of distilled water and liquid–liquid partitioned successively with chloroform, ethyl acetate, and butanol solvent as much as 3 × 100 mL for each solvent to obtain 4.03 g of chloroform fraction (C), 0.19 g of ethyl acetate fraction (EA) and 0.80 g of butanol fraction (B). The residue was dried to obtain 4.71 g of aqueous fraction (A). All samples were tested for the antiviral activity against HIV-1 except for aqueous fraction was not tested. The stock solutions were stored in –20 °C freezer until being used further.

Phytochemical analysis

The phytochemical analysis of ethanol extract (E), chloroform fraction (C), ethyl acetate fraction (EA), butanol fraction (B), and aqueous fraction (A) was conducted by thin layer chromatography (TLC) method. Extract and fractions as much as 10 mg were dissolved in 1 mL of methanol. Extract and fractions as much as 3 µL then spotted on silica gel F254 plate (Merck) as stationary phase and chloroform: methanol (Merck) (9:1 v/v) as mobile phase. The spots were identified using H_2SO_4 10% as a spray reagent and observed under UV 254 and 365 nm.

Analysis of anti HIV potentials of plant extract and fractions

Anti-viral activity (syncytia formation assay): After coculturing the MT-4 cells and persistently infected HIV-1 cells derived from PBMC (MT-4/HIV-1), the syncytia formation occurred on the cultured cells. The next step was to apply the coculture cells into syncytia inhibition assay. Two-fold serial dilution of extract/fractions was prepared (50; 25; 12.5; 6.25; 3.13 µg/L). Upon 30 min incubation at 37 °C, MOLT-4 cells (acute lymphoblastic human leukemia cell line) were added into the culture (2.105 cells/mL for a multiplicity of infection (MOI) of 1/20), and the cocultured cells were incubated at 37 °C for one week (seven days). As a negative control (NC), the MT-4/HIV-1 cells were mixed on cultured MOLT-4 cells and incubated at 37 °C for one week (seven days). After incubation, the number of produced syncytia was microscopically counted. The data were furnished in the percent of inhibition compared with negative control by the below equation.

$$\% \text{ Inhibition} = \left[100 - \left(\frac{\text{NC} - \text{Syncitia on sample}}{\text{NC}}\right)\right] \times 100\%$$

Inhibition activity was also evaluated on half concentration of inhibition (IC_{50}) value. This assay was also done in duplicate replicates to ensure the valid result of the toxicity effect of these antiviral subtances.

Cytotoxicity assay: Follow up the Syncytia assay; the cytotoxicity assay was conducted to measure how potent the tested compound was in terms of antiviral activity against HIV-1. This test only targeted the viable infected cells, not the healthy cells. WST-1 Cell Proliferation Reagent (Roche Applied Science, Switzerland) assay was used in this test by converting tetrazolium salt into a formazan product by viable cells. Serial dilution containing *F. fistulosa* extract/fractions were added into MOLT4 cell culture, using only cell culture without extract/fraction as a positive control and plain RPMI medium as a negative control. The cell culture containing extract/fractions were incubated at 37 °C for seven days. Following the incubation period, the MTT reagent was added into the cell culture and the absorbance was read at 450 nm, measured at two-time points of pre incubation and postincubation at 37 °C for 2 h. The cytotoxicity concentration (CC_{50}) score was measured by assessing in which virus dilution that the 50% viable cells detected. This assay was also done in duplicate replicates to ensure the valid result of the toxicity effect of these antiviral subtances.

Statistical analysis: In this study, we were used randomized complete block design (RCBD), a design which each experiment unit was divided into randomized block. This design were applied to analyzed the effect of extract and its fractions that were divided into experimental groups (ethanol extract, butanol fraction, ethyl acetate fraction, and chloroform fraction) and the control group on anti HIV effectiveness. Analysis that used for the above design were using two way ANOVA method, which was a type of parametric statistical test that aims to determine whether there were effect differences/effect on two factors (concentration and compound) that cause variations. If the test result showed different effect on each extract and its fraction, further test was needed for the next test step. One of the test methods is using Tuckey test. This test was used when the extract and its fraction were different. IC_{50} and CC_{50} were calculated using probit regression analysis.

Results

Phytochemical properties from *F. fistulosa* are known to have anti-viral activity, and those subfractions contained flavonoids, terpenoids, and chlorophyll compounds. Genus Ficus have several phytochemical compounds such as triterpenoid; flavonoids; sterols; coumarin; and anthocyanins in every part of the plant [24]. Previous study reported that ethanol extract of *F.fistulosa* leaves was exhibited anti hepatitis C virus activity against JFH1a. The phytochemical identification revealed that the ethanol extract was contained flavonoids, terpenoids, and chlorophyl compounds [25]. Meanwhile, alkaloids compounds as antifungal against *Aspergillus fumigatus* and *Candida albicans* were isolated from *F.fistulosa* stem bark [26]. Phytochemical analysis was conducted in this study by TLC analysis. The result showed that *F. fistulosa* leaves was contained flavonoids, terpenoids and chlorophyl compounds as previous reported [25].The ethanol extract and chloroform fraction showed a similar TLC profile. Chlorophyll was detected in ethanol extract and chloroform fraction under UV light 365 nm which was indicated by red spots. Terpenoids and flavonoids were detected as well after sprayed with H_2SO_4 10% which was indicated by violet spots and yellow–dark brown spots. Meanwhile, ethyl acetate fraction was dominated with flavonoids and contains minor terpenoids. Butanol fraction and aqueous fraction were not eluted well on silica gel due to polar compounds as a dominant content (Figure 1).

In the present study, one extract and three fractions; ethanol extract, chloroform, ethyl acetate, and butanol fractions extracted from *F. fistulosa* leaves were examined for their anti HIV activities *in vitro* (Table 1). Of four samples, only one sample namely chloroform fraction exhibited the antiviral activities against HIV-1. The ethanol extract showed a low IC_{50} value calculated as 8.96 µg/mL and a high CC_{50} of >50 µg/mL while these extract exhibited weak anti HIV activity. Additionally, the chloroform fraction displayed interesting values analyzed as 3.27 and 29.30 µg/mL for IC_{50} and CC_{50}, respectively. These high values of SI (8.96) detected in these fraction suggested that the chloroform fraction could be utilized safely under the parameters applied in this study. In contrast of ethyl acetate fraction and butanol fraction showed no anti HIV activity, while we detected the high value of CC_{50} in both fractions (>50).

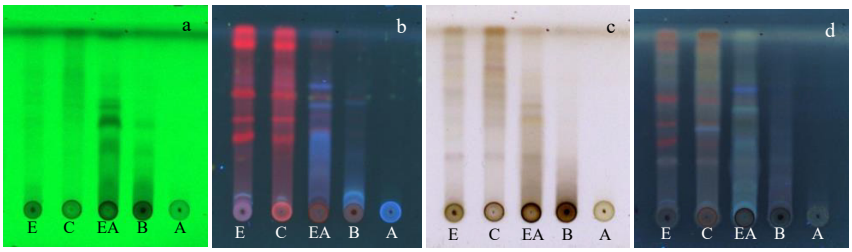

Figure 1: Thin layer chromatography (TLC) profile of ethanol extract (E), chloroform fraction (C), ethyl acetate fraction (EA), butanol fraction (B), and aqueous fraction (A) of *Ficus fistulosa* leaves using silica gel as a stationary phase and chloroform-methanol (9/1 v/v) as a mobile phase. The TLC was observed under UV light 254 nm (a), 365 nm (b), white light (c), and UV light 365 nm after sprayed with H_2SO_4 10% and heated at 105 °C for 5 min (d).

Table 1: The results of syncytium formation and cytotoxicity assays depicted in calculated IC_{50}, CC_{50} and SI values from *Ficus fistulosa* leaves.

Samples	IC_{50}, µg/ml	CC_{50}, µg/ml	SI*
Ethanol extract	8.96	>50	>5.58
Chloroform fraction	3.27	29.30	8.96
Ethyl acetate fraction	23.04	>50	>2.17
Butanol fraction	53.98	>50	>0.97

*SI, selectively index; CC_{50} value was divide by the IC_{50} value.

Discussion

To date, over 40 currently accessible antiretroviral therapy (ART) drugs have been circulating worldwide converting HIV/AIDS from a lethal infection into a manageable chronic disease [27]. However, due to the high rate of mutations detected in the HIV-1 viral replication cycle, drug resistance mutants have become a big obstacle in HIV-1 treatment accomplishment [28, 29]. The other challenge was to offer more affordable HIV-1 drugs, especially in low-income countries. Therefore, new approaches of using local resources plant-based medicines have been introduced in several Asian countries like Thailand and Indonesia [21, 30].

Indonesia has known for its tropical climate and home of the second-largest biodiversity in the world spreading in over 17,000 islands. Due to its geographical location, the flora of Indonesia displays the Asian, Australian, and native collections. Plant-based medicines using indigenous Indonesian flora have been developed rapidly in recent years. Different studies focusing on antiviral, antibacterial, antimalarial, and antifungal potentials of Indonesian medicinal plants reported promising results to be used further for drugs development Different parts of the plants like stem, leaves, and roots of the following species of native ginger rhizome, *F. fistulosa*, *Garcinia mangostana*, *Melanolepis multiglandulosa*, and *Melicope latifolia* have been studied intensively in the past years [21, 31, 32].

F. fistulosa is a part of the genus Ficus in the family of Moraceae commonly found in Asia and New Guinea. In ancient medicine history, Ficus genus species have been used for traditional remedies to cure headaches, breathing problems, diarrhea, cough, toothache, eye infection, and scabies [33]. The extensive pharmacological properties of Ficus have been reported including antimicrobial, antioxidant, antiviral, antiparasitic, anti inflammatory, and anticancer [34].

Wahyuni et al. reported that *F. fistulosa* was efficient in inhibiting the growth of two hepatitis C virus (HCV) strains *in vitro*. The study showed that the ethanol extract from *F. fistulosa* leaves successfully inhibits the virus growth during the initial step of inoculation. The study conducted by Indriati et al. observed that the n-hexane fraction *of F. fistulosa* showed a potent activity as an anti HIV drug candidate. This study result was in accordance with a previously reported study in which n-hexane fraction was exhibited anti HIV activity. Furthermore, crude extract (ethanol extract) showed the highest activity among other samples. The phytochemical content of ethanol extract and chloroform fraction was probably the same due to their similar TLC profile. Both ethanol extract and chloroform fraction contained chlorophyll, terpenoids, and flavonoids compounds which were possible to take a role in their anti HIV activity.

Flavonoids inhibit HIV replication in PBMC in dose dependent manner as it is shown in other herbal medicine known as *Sctellaria* baicalemsis [35]. Other type of flavonoids, gallate ester and quercetin 3-O-(2-galloyl) a-L arbinopyranose inhibit the activity of integrase enzyme. Flavonoids also showed inhibition to RT activity [36]. While triterpenoid acts as anti HIV in several step of HIV cycle such as entry inhibitor which block membrane fusion, inhibit HIV enzymes (protease, reverse transcriptase (RT), and integrase), and viral maturation [37]. Further study needs to be conducted for isolation and identification of active compounds from ethanol extract and chloroform fraction.

Conclusions

In conclusion, our recent study reported the novel potential of chloroform fraction from *F. fistulosa* as antiviral against HIV-1 in terms of HIV-1 growth inhibition *in vitro* and proven nontoxic to healthy cells since these e fractions contain chlorophyll, terpenoid, and flavonoid which can act as anti HIV.

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Author contributions: SQK performed HIV *in vitro* analysis and drafted the manuscript, as well as involved in the design and coordination of the study; DWI performed HIV *in vitro* analysis and helped in drafting the manuscript; LT performed plant extraction and phytochemical analysis; AW and N were involved in the design and coordination of the study. All authors read and approved the final version of the manuscript.
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Informed consent: Informed consent was obtained from all individuals included in this study.
Ethical approval: The local Institutional Review Board deemed the study exempt from review.

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