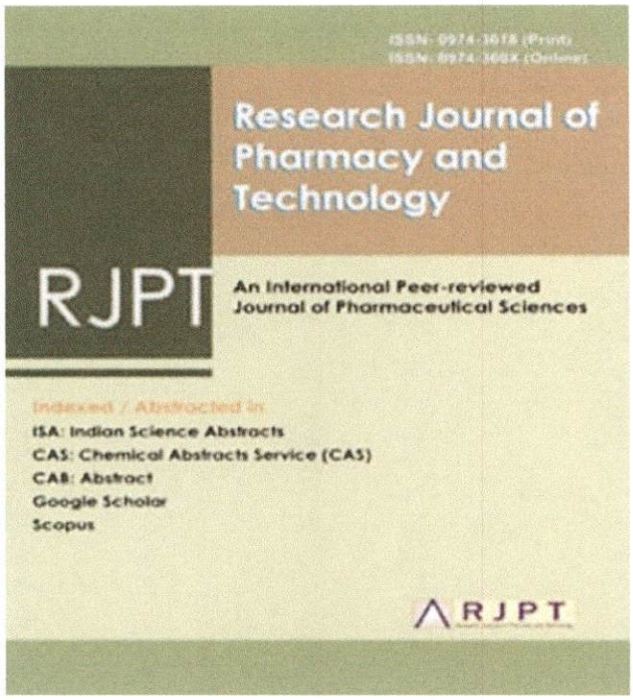
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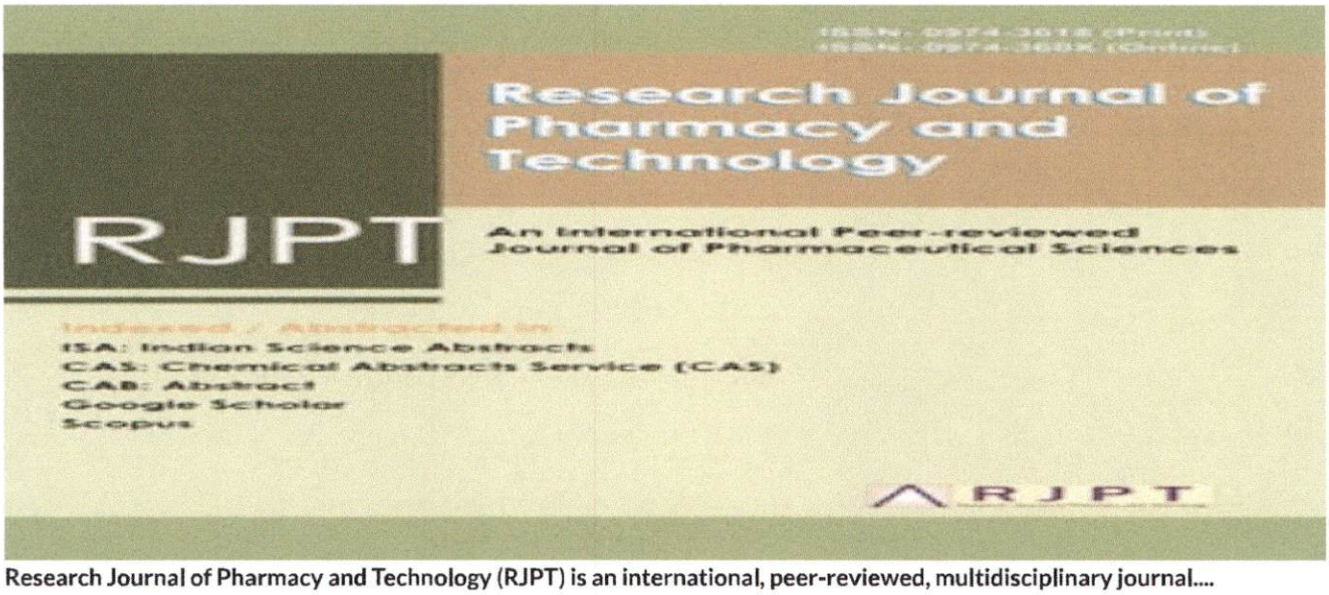

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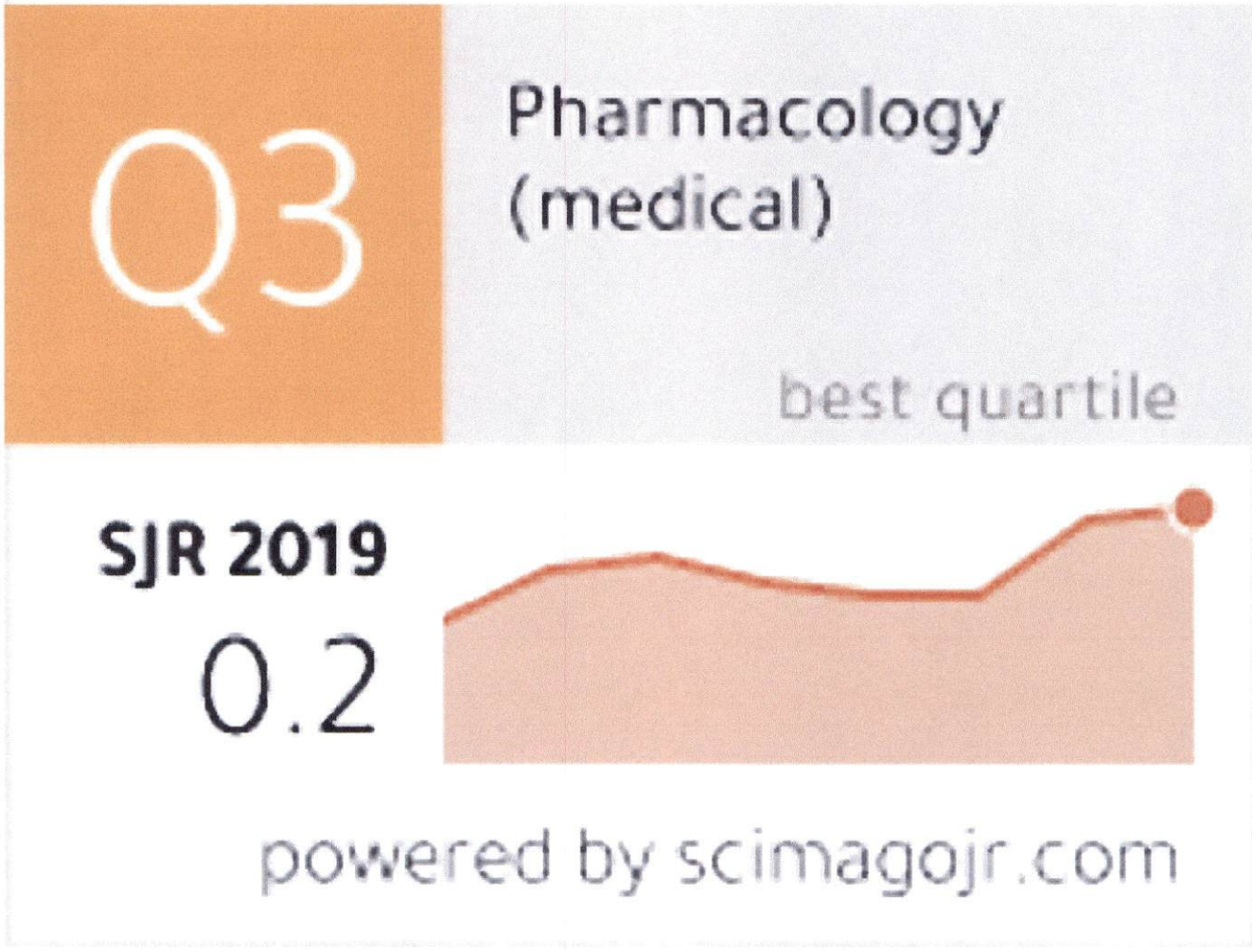

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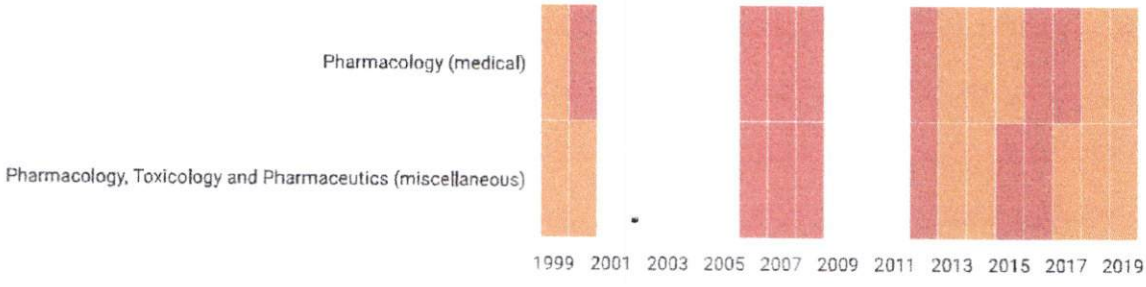

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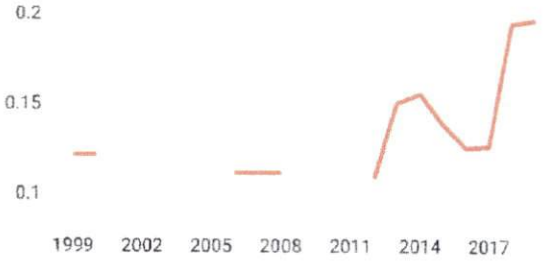

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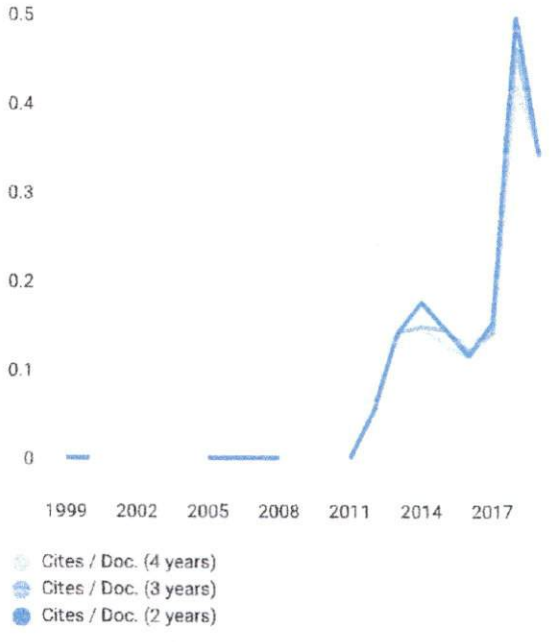

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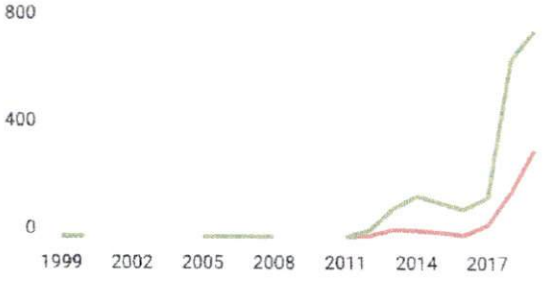

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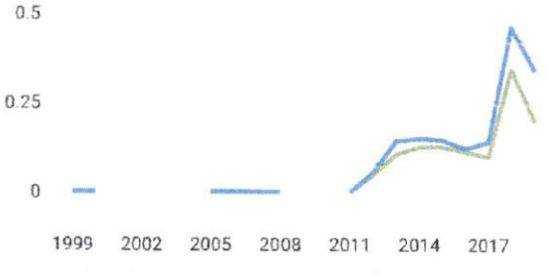

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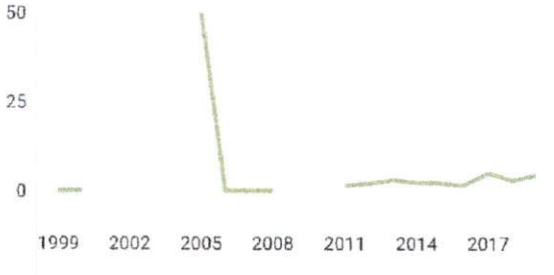

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RESEARCH ARTICLE

The activities of Methanol extract, Hexane and Ethyl Acetate Fractions from *Ficus fistulosa* in HIV inhibition *In Vitro*

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ABSTRACT:

Background: Human Immunodeficiency Virus infection can lower the immune system in HIV patient and makes it more vulnerable to opportunistic infection. **Objectives,** this study aims in provide alternative therapy since recent trends showed that there is a drug resistance therapy from existing antiretroviral treatment. **Material and Methods:** *Ficus fistulosa* extract were collected using methanol as a solvent. Further fractionation was done with n-hexane and other using ethyl acetate resulting in 4 fractions (F1, F2, F3, ethyl acetate). The persistently infected cells and herbal extract (7.8-1000 ppm) were co-cultured to observe the extract`s potential in inhibiting HIV replication. Toxicity assay was also performed in this study. **Results**: n-hexane fraction were showing an active inhibition to HIV replication and not toxic for healthy cells (IC_{50}=27.2 ug/ml; CC_{50}= 377.9 ug/ml and SI =13.89). While other fraction showing potentially active compound were ethyl acetate fraction and F1 (ethyl acetate; IC_{50}=22 ug/ml; CC_{50}= 214.47 ug/ml and SI =9.75; F1; IC_{50}=17.32 ug/ml; CC_{50}= 86.8ug/ml and SI =5). The Thin Layer Chromatography result showed that n-hexane contained chlorophyl (dominant), terpenoid and flavonoid. **Conclusion:** The n-hexane fraction was proved to be active and potential as anti-HIV. But further fractionation to separate real active compound for inhibiting HIV replication are required, to provide potential herbal therapy for anti- HIV.

KEYWORDS: *Ficus fistulosa*, HIV, in vitro, herbal extract.

1. INTRODUCTION:

Since antiretroviral therapy has emerged as main tools against Human Immunodeficiency Virus (HIV), the prevalence and incidence of HIV were significantly reduced except for several countries in Europe and Central Asia[1]. HIV infection can lead to severe opportunistic infection since the immune system was significantly disrupted. Thus, immediate therapy for a newly diagnosed patient was recommended. In recent years, antiretroviral therapy has found its limitation though it professes stable viral suppression it has undesirable side effects namely drug resistance mutation. Our previous studies also showed the emergence of a drug resistance mutation in HIV patient which have started therapy for two years after first diagnosis[2,3]. Drug resistance mutation also can occur due to transmitted drug resistance mutation strain in newly diagnosed HIV[4,5].

Recent studies have now taken an effort to find an alternative therapy which has less side effects. Nature product was considered suitable for this purpose. Nature can provide resources such as medicinal plants that have an anti-HIV property with a low level of toxicity. Indigenous knowledge of medicinal plants, screening based on ethnopharmacological data also an effort to isolate the active compound from plants and other natural product can enrich a drug discovery journey for anti-HIV[6–8].

Several compounds from herbal extract have been reported to have an anti-HIV effect such as alkaloids, lignans. Those compounds attack the replicative cycle of HIV. Replicative cycle of HIV consists of ten different steps which can be a potential target for chemotherapeutical. Those ten steps are adsorption, fusion, uncoating, reverse transcription, integration, DNA replication, transcription, translation, maturation and budding (assembly and release)[9]. The mostly plant-based compound can be assigned as anti-HIV in one among those ten steps of the replicative cycle.

Indonesia has widely known to have varieties of natural resources. One example of those plants that garnered attention to have anti -HIV is coming from the Moraceae family. The previous study stated that Moraceae family contain three first compound (from flavonoid) that showed anti-HIV, mulberrin, morusin and sanggenol N[10]. Other *Ficus* species such as *Ficus glomerate* extracted from wood (ethanol extraction) also showed a potent anti-HIV with IC_{50} 7.8µg/ml[11]. Thus, this study wants to evaluate the potential of other *Ficus* namely *Ficus fistulosa* which found in the tropical area including Indonesia as anti-HIV.

2. MATERIAL AND METHODS:

2.1 Plant material:

Ficus fistulosa leaves were collected from Salak Mountain (900 masl), West Java, Indonesia. Authentication, identification and determination of plant were carried out by Purwodadi Botanical Garden-Indonesia Institute of Science, East Java. All samples were deposited in Natural Product Medicine Research and Development Laboratorium, Institute of Tropical Disease, Universitas Airlangga, Surabaya.

2.2 Extraction and fractionation:

Ficus fistulosa leaves were dried at room temperature and gound (0.5 kg) were extracted using methanol as solvent (totally 4 Liters) by ultrasonic assisted extraction (UaE) for two minutes to three times replications. The methanol extract obtained was concentrated using rotary evaporator until the volume remains 0.8 ml. The extract was fractionated by liquid-liquid fractionation using n-hexane to be obtained n-hexane fraction (3g; % yield = 6% w/w). The residue was further fractionated by the same method using ethyl acetate to obtain ethyl acetate fraction (6.06 g; % yield = 12% w/w). Further separation of n-hexane fraction by vacuum liquid chromatography (VLC) using silica gel as a stationary phase and n-hexane: ethyl acetate (9:1; 8:2; 7:3 and 1:1 v/v) as a mobile phase. Fractions with similar thin layer chromatography (TLC) profiles were combined so as 3 fractions was obtained (F1 (607.5 mg; % yield = 20.25% w/w); F2 (1.4 g; % yield = 46.67% w/w); and F3 (837 mg; % yield = 27.9 % w/w). N-Hexane fraction, F1, F2, F3 and Ethyl acetate fraction were further tested for their anti-HIV activity.

2.3 Syncytia assay:

The persistently infected cell was initially made from co-culture peripheral blood mononuclear cells (PBMC) from HIV patient and a healthy donor. Persistently infected cell-MT4 were made by infecting MT4 cell line with HIV from HIV patient`s PBMC. The PBMC were preactivated with mitogen, phytohemagglutinin (PHA) and activated with interleukin-2 (IL-2)[12]. These virus stock will be infected to MT4 cell line resulting in MT4 cell line persistently infected with HIV, similar to other protocol used in the previous study[13]. Then finally co-culturing with cell line MT4 (derived from human T cell leukaemia) to produce a persistently infected cell which showing syncytia formation on culture cells. The MT4/HIV cells then used for syncytia inhibition assay. This persistently infected cells will be incubated together with two-fold serial dilution of extract/fraction (1000; 500; 250; 125; 62.5; 31.25; 13.625; 7.8125ug/L). After 30 min incubation at 37°C, another human T lymphoblast cell, acute lymphoblastic leukemia cell line (MOLT4 which were originated from acute lymphoblastic leukaemia[14]) was added into the culture (2.10^5 cells/ml for multiplicity of infection (MOI) 1/20). These cultures will be incubated at CO_2 incubator at 37°C for 7 days. After 7 days, syncytia inhibition will be observed with Viral ToxGlo Assay (Promega, Wisconsin, USA). Viral ToxGlo assay measure cellular adenosine triphosphate (ATP) which correlated with a number of viable host cells in culture. Thus, this method can quantify the viral-induced cytopathic effect (CPE). Inhibition concentration (IC_{50}) can be determined in which virus dilution that produce cytopathic endpoint effect (cytopathic effect in 50% of inoculated tissue culture). The syncytia assay was done in three replicates to confirm the inhibition effect of antiviral.

2.4 Toxicity assay:

The effectiveness of extract/fraction in syncytia inhibition need to be verified with toxicity assay. The effective extract/fraction will kill cell infected virus but not healthy cells. Toxicity assay was conducted with MTT (3-(4,5-Dimethylthiazol-2-)-2.5-Diphenyltetrazolidum Bromide) assay (Promega, Wisconsin, USA). This method is based on the cellular conversion of a tetrazolium salt into a formazan product by viable cells. Serial dilution of extract/fraction was added to MOLT4 cell culture. Positive control used for this assay was MOLT4 culture without extract/fraction while negative control used only RPMI (Roswell Park Memorial Institute) medium only. MOLT4 and extract/fraction culture should be incubated at 37°C for 7 days. After 7 days, MTT reagent will be added and read the absorbance in 450 nm, before and after incubation

37°C for 2 hours. The cytotoxicity concentration (CC_{50}) value will be determined in which virus dilution that cause death to 50% of viable cells. The cytotoxicity assay was done in three times replications to ensure the toxicity effect of this antiviral.

2.5 Phytochemical analysis:

Thin Layer Chromatography (TLC) profile of *Ficus fistulosa* leaves fractions: Hexane fraction (H), F1 (1), F2 (2), F3 (3), ethyl acetate fraction (EA) using solid phase silica gel F254 plate (Merck 1.05554) as stationary phase and chloroform (Merck 1.02445), methanol (Merck 1.06009) as mobile phase. The TLC profile was observed under UV 254 nm (A), UV 365 nm (B), after sprayed using H_2SO_4 10% dan heated at 105°C for 5 min (C), after sprayed using H_2SO_4 10% dan heated at 105°C for 5 min then observed under UV 365 nm (D) (Figure 1).

3. RESULT AND DISCUSSION:

The potential of *Ficus fistulosa* as anti-viral already shown in other studies by our group, which showed its potential as anti-hepatitis C virus (HCV), the ethanol extract of *Ficus fistulosa* showed IC_{50} value 20.43±4.51 µg/ml and CC_{50} value > 200 µg/ml[15]. In this study, we want to study methanol extracts, n-hexane fractions and ethyl acetate fractions from *Ficus fistulosa* leaves and analyze its potential as anti-HIV in vitro.

Syncytia assay was conducted to evaluate the effectivity of fraction in inhibiting HIV. The half maximal inhibitory concentration (IC_{50}) was measured among 5 fractions studied. Overall, each fraction has a low value of IC_{50} with a range from 17.3 µg/ml to 46.7µg/ml. High IC_{50} value was found in F3 fractions 46.7µg/ml while the lowest IC_{50} value was found in F1 fractions 17.3µg/ml (Table 1).

Toxicity assay was also performed to confirm the effectiveness of those fractions to inhibit HIV replication and to reassure that fractions will not be toxic to healthy cells. The higher value of CC_{50} compare to IC_{50} value means that this fraction will not be harmful to a healthy cell and the fraction is active for inhibiting HIV. While the lower value of CC_{50} compares to IC_{50} value means that this fraction is toxic and will not be useful for further anti-HIV analysis. The CC_{50} value and Selectivity Index (SI) are shown in table 1. The n-hexane and ethyl acetate fractions are shown high CC_{50} value with 377.9µg/ml and 214.5µg/ml, respectively. Among the other three fractions are showing moderate cytotoxicity range between 24.7-86.8µg/ml. High SI value is desirable to give maximum antiviral inhibition and with minimal cell toxicity (Table 1). Based on high CC_{50} value n-hexane and ethyl acetate fraction show high SI value compare to other fractions (13.9 and 9.8, respectively).

The CC_{50} value from n-hexane and ethyl acetate fraction showed that this fraction is not toxic for healthy cells (MOLT4) but showed lower IC_{50} value, confirming their effectiveness as an active fraction in inhibiting HIV. Other species from genus *Ficus, Ficus glomerate* showed an IC_{50} 7.8µg/ml showing remarkable potential as HIV-1 integrase inhibitory activity[11]. While other *Ficus* species, *Ficus polita* showed inhibition of HIV-1 proviral DNA copying as determined in a polymerase chain reaction[16].

We can assume that fractions contain chlorophyl as many red spots appear under UV 365 nm observation (profile B and D). Spray reagent H_2SO_4 10% followed by heating was used to identify polyphenol groups. There were violet color spots which indicate terpenoids compounds contain in Hexane fraction, F1 and F2. Meanwhile, yellow-orange-brown colors were signified flavonoids compounds contain in all fractions (Figure 1).

Phytochemical properties from *Ficus fistulosa* is known to have antiviral activity, and those subfractions contained flavonoids, terpenoids and chlorophyl compound. Genus *Ficus* are known to have several phytochemical compounds such as triterpenoid; sterols; flavonoids; coumarin; anthocyanins in every part of the plant[17]. Several important compound in natural products that has been proved to have anti-HIV activity are triterpenoid., alkaloid and polyphenolic[18]. While an example of pentacyclic triterpenes known as betulinic acid and platanic acid which is extracted from *Syzigiu claviflorum* showed a selective virus-cell fusion inhibitor for HIV-1[19], a triterpene from *Ficus fistulosa* is yet to be elucidated as anti-HIV. Another compound such as coumarins, flavonoids are interfering in virus adsorption, reverse transcription and integration[20]. This study was presented initial data showed *Ficus fistulosa* has potential in anti-HIV besides other species from genus *Ficus*. Further fractionation and finding a pure compound that corresponds to anti-HIV from *Ficus fistulosa* leaves is required for the next step.

Table 1. IC_{50}, CC_{50} and SI value among 5 fractions of *Ficus fistulosa*

Name	IC_{50} (ug/ml)	CC_{50} (ug/ml)	SI
F1	17.3	86.8	5
F2	19.7	24.7	1.3
F3	46.7	53.6	1.1
n-hexane	27.2	377.9	13.9
Ethyl acetate	22	214.5	9.8

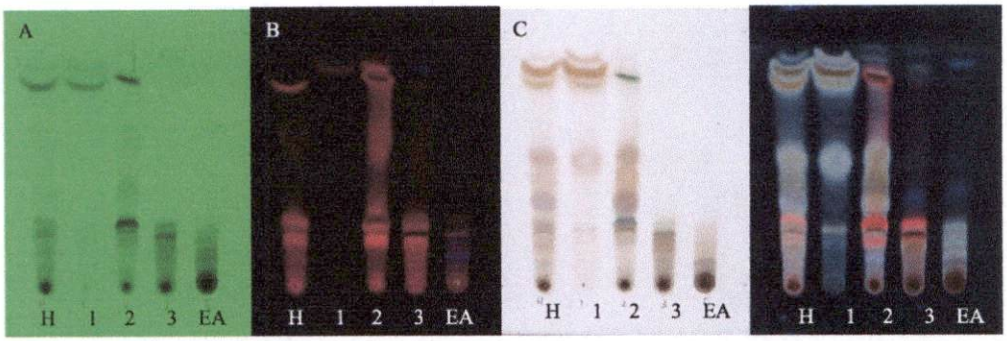

Figure 1. /Thin Layer Chromatography (TLC) profile of *Ficus fistulosa* leaves fractions: Hexane fraction (H), F1 (1), F2 (2), F3 (3), ethyl acetate fraction (EA) using silica gel as stationary phase and chloroform as mobile phase. The TLC profile was observed under UV 254 nm (A), UV 365 nm (B), after sprayed using H_2SO_4 10% dan heated at 105°C for 5 min (C), after sprayed using H_2SO_4 10% dan heated at 105°C for 5 min then observed under UV 365 nm (D).

4. CONCLUSION:

The ethyl acetate and F1 fraction will be a good candidate for anti- HIV. Since These two fractions contain terpenoid and flavonoid which can act as anti-HIV. But further fractionation in order to find pure active compound responsible for inhibiting HIV.

5. CONFLICT OF INTEREST:

The atuhors declare there is no conflict of interest.

6. ACKNOWLEDGEMENT:

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