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Research Article

Antiretroviral Effect of Combination of Ethanol Extract from Leaves of *Psidium guajava* and *Andrographis paniculata*

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ABSTRACT

Ethanol extract of *Psidium guajava* and *Andrographis paniculata* leaves individually has been reported to have antiviral activities. Combination of both plants in a formulated form to increase the antiretroviral potency have never been reported. Therefore, the objective of this study was to evaluate the *in vitro* effect of combined ethanol extracts of *P. guajava* and *A. paniculata* leaves as antiretroviral to *Simian retrovirus-2* (SRV-2). The source of SRV-2 was from *Macaca fascicularis* inoculated in A549 cells. This virus can be used as a model for human immunodeficiency virus that caused (acquired) immunodeficiency syndrome. Leaves of both plants were macerated in 96% ethanol then dried with rotary evaporator. Formula of both extracts were used in this study with different ratios and analyzed with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method to determine the cytotoxicity on non infected A549 cells. The ratio of *P. guajava* and *A. paniculata* with minimal toxicity was three parts of *P.guajava* and one part of *A. paniculata* at concentrations less than 125 ppm. These concentrations of the extract formula were then added to A549 infected cells compared to lamivudine as control. The supernatants were collected on day-1, 3 and 5 to evaluate the viral replication. Based on the copy number of SRV-2 using real time–Polymerase Chain Reaction, the formula showed highest inhibition (99.96%) at concentration of 125 ppm on the fifth day. This result showed that the *P. guajava* and *A. paniculata* with ratio of 3:1 was most potential as antiretroviral compared to lamivudine, a generic antiretroviral drug.

Key words: *Psidium guajava*, *Andrographis paniculata*, combination, cycle threshold, antiretroviral

INTRODUCTION

Simian retrovirus (SRV) naturally infect long tailed macaque (*Macaca fascicularis*) which is in the same family with Human Immunodeficiency Virus (HIV) that caused Acquired Immune Deficiency Syndrome (AIDS). This SRV can be used as model for HIV studies (Stump and VandeWoude, 2007). These viruses damage and weaken immune system function by affecting $CD4^+$ T cells and macrophages. Continuing efforts to find affordable, accessible and effective cure to combat HIV including the use of medicinal plants are increasing (Gebo *et al*., 2012).

P. guajava as medicinal plant has many beneficial medicinal effects such as antioxidant (Moreno *et al*., 2014), antibacterial (Mundi *et al*., 2014) and antiviral (Kannan *et al*., 2012). Sriwilaijaroen (2011) had reported that the water extract of *P. guajava* leaves contained flavonoids had the ability to inhibit H_1N_1 RNA virus at concentration of 200 µg/ml through the inhibition of *reverse transcriptase* enzyme. Other medicinal plants with antiviral activity was *A. paniculata* which has potency to inhibit the *protease* enzyme of HIV (Elfahmi *et al*., 2014). Effort to increase antiviral activities or potency can be done by combining both plants in a certain formulation. Combination of medicinal plants are widely used for treatment to obtain synergistic effect and gain the desired therapeutic goal (Yang *et al*., 2014, Williamson 2002,). Synergistic effect of formulated of *P. guajava* and *A. paniculata* as antiretroviral have never been reported.

In vitro antiviral testing system could be carried out using A549 cell culture, a human lung adenocarcinoma, which support the replication of virus to evaluate the inhibition activities of medicinal plants (Ehrhardt *et al*.,

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2007, Li *et al*., 2013). The reverse transcriptase real-time polymerase chain reaction (RT-PCR) used to measure the copy number of the virus. The current study investigates the *in vitro* effect of combined ethanol extracts of *P. guajava* and *A. paniculata* leaves as antiviral against SRV.

MATERIALS AND METHODS

Collection and extraction of medicinal plants

Samples were collected from conservation and cultivation unit of Biopharmaca Research Center of Bogor Agricultural University, Indonesia. The sixth to tenth leaves from the top of the tree of *P. guajava* were used as samples and samples of *A. paniculata* leaves harvested from 3 months old plants. Each sample washed and dried at temperature of 50^{o}C for 2-3 days until the leaves turned dry then powdered to the size of 30 mesh.

Extraction of samples was done by maceration method without heating for three days with 96% ethanol. Extracts obtained were stored at 4^{o}C for further evaluation of phytochemical compound content (Tiwari *et al*., 2011).

Formulation of *P. guajava* and *A. paniculata* leaves extracts

Extract of *P. guajava* and *A. paniculata* formulated at various ratios i.e. 1:1, 1:2, 2:1, 1:3, 3:1, 1:4 and 4:1. Each formula extract was made at concentration of 1000 ppm, 500 ppm, 125 ppm, 62.5 ppm, 31.25 ppm, 15.6 ppm and 7.8 ppm.

Cell cultures and cytotoxicity assay

Human lung adenocarcinoma cell line (A549) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were incubated in 5% CO_2 humidified at 37^{o}C.

Viable A549 cells (5 x 10^3 cells/well) was sub cultured in a 96-well plate and kept for 24 hours for attachment. The next day, the various formula extract (100 µL) was added and cells were allowed to grow for 48 hours. Then 10 µL MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was added in each well and incubate for 4 hours. After completing the incubation, supernatant was collected and added with 100 µl ethanol-HCl, then further measured at 595 nm using ELISA reader. Screening of formula extract was necessary to assess the cell toxicity on non-infected SRV-2 and to determine the optimum concentration which has the lowest cell inhibition (Maurya *et al*., 2010)

Quantification of SRV-2 by real time-PCR

A549 infected SRV-2 cells (1 x 10^4) was sub cultured in a 12-well plate and incubate for 24 hours in 5% CO_2 humidified at 37^{o}C for growth. Formula extract (500 µL) was added to each well and incubate for 5 days. Supernatant (500 µL) harvested on day 1, 3 and 5. Each samples were kept at temperature -80^{o}C. Viral RNA was extracted from 140 ul supernatant using *QIAamp Viral RNA Mini Kits* (Qiagen, Hilden, Germany) then reverse transcribed by *Superscript^{TM} III First-Strand Synthesis System for RT-PCR* (Life Technologies, Carlsbad, CA, USA) to generate viral cDNA. The primers used in the real time PCR were SRV-2 5737U19 and SRV-2 5943L20 (IPB-PRC collection) that will amplified gp70 envelope gene. 10 pmol/ul of each primer was added to the master mix PCR containing 10 µl of SsoFast Evagreen master mix (Bio-Rad Laboratories, Hercules, CA, USA) and 2.5 µl of cDNA template. The real time PCR amplification was carried out in iCycler Thermal cycler with iQ5 multicolor real time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) at the conditions: 95° C for 3 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 47°C for 30 s, and extension at 72°C for 30 s. The standard for viral quantification was developed based on viral copy number using SRV-2 envelope plasmid at the concentration of 10^1-10^6. The tests were systematically conducted in duplicate (Besson and Kazanji 2009).

RESULTS

Water and phytochemical compound

The result showed the water content of the *P. guajava* and *A. paniculata* leaves were 8.91% and 9.10%, respectively. Yield from the leaves of *P. guajava* was 20.32% and *A. paniculata* was 11%. Qualitative phytochemical compound of both extracts contain flavonoids, saponins, tannins and steroids, but *A. paniculata* extract had hydroquinone.

The cytotoxicity of extracts

The cytotoxicity assay showed that the toxicity of various formula of extracts ranging from 8 % to 87 % (Figure 1). The lowest means of percent inhibition was the formulation of three to one parts of *P. guajava* and *A. panniculata* against non-infected A549 cells. This formulation was chosen for further antiretroviral assay.

The result of cells morphology observation of cells treated with the formula (3:1) at 125 ppm showed minimal morphology changes almost similar with control compared to 500 ppm that has extensive changes (Figure 2). For further antiviral assay, we used maximum concentration formula (3:1) at 125 ppm.

Inhibition of SRV-2 replication

Antiviral assay of the formulated extracts was conducted for 5 days. Supernatants were harvested on day 1, 3 and 5 then measured the cycle threshold (Ct) by RT-PCR. Inhibition of viral replication was determined by the copy number of SRV-2. The bigger value of copy number indicates more amount of the virus. The determination of sample's copy number values used standard curve calculation based on correlation between cycle threshold and copy number. The obtained Ct for copy number of 10^6 to 10^1 resulted from the regression equation of $y = -2.3418x + 32.713$, with value of $R= 0.9712$. According to that equation, the SRV-2 replication was determined based on copy number (Figure 3).

Figure 3 showed that formulation (3:1) at 7.8 ppm and 15.6 ppm did not inhibit the SRV-2, while concentration of 31.25 ppm, 62.5 ppm and 125 ppm on day-5 showed 0 copy number of virus. Therefore, percent inhibition of viral replication was almost 100%, which was twice higher compared to lamivudine (44.65%).

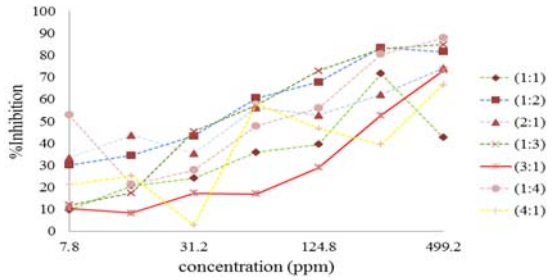

Fig. 1: Inhibition of various formula of combined extract of *P. guajava* and *A. paniculata*

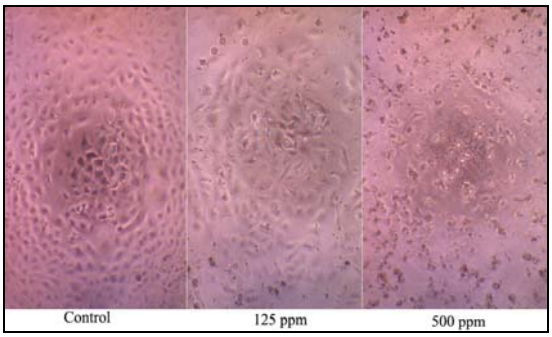

Fig. 2: Morphological A549 uninfected cells treated with 3:1 extract formulation at 125 ppm and 500 ppm compared to control (80x microscope)

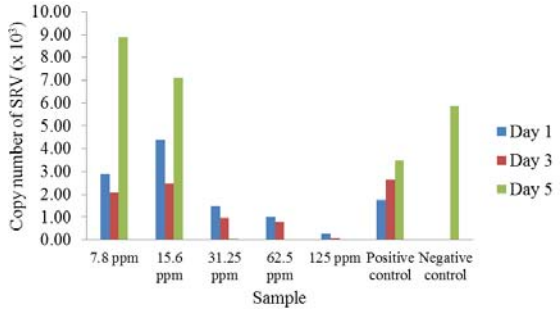

Fig. 3: The value of SRV-2 copy number (positive control: Lamivudine, analyzed only on day-5).

DISCUSSION

Extracts of both medicinal plants with formulation of 3 parts *P. guajava* and 1 part of *A. paniculata* at concentration 31.25 ppm, 62.5 ppm and 125 ppm showed inhibition to the replication of SRV-2 on the fifth day of incubation. This result was higher in potency compared to the single HIV drug lamivudine tested in this study. However, in practice the standard HIV therapy given as combined regimen, even though it has evolved to single-tablet regimen due to drug resistant and toxicity (Astuti and Maggiolo 2014). The increase inhibition of viral replication by the combined extracts was believed due to the synergistic effect. The suggested active compound responsible in the antiviral activities was flavonoid (Olivero-Verbel and Leonardo 2002). This phytochemical were found in both ethanol crude extracts. Flavonoid, bioactive compound of *P. guajava* was reported the one responsible in inhibiting the reverse transcriptase enzyme of RNA virus (Metwally *et al*., 2011). Bioactivity flavonoid compound of *A. paniculata* also been reported capable to inhibit the protease enzyme on process of cutting the protein chains to be used as a precursors of virus genetic material (Elfahmi *et al.*, 2014). By combining the two medicinal plants, therefore there are two targets of mechanism of action produced by each plants that increased the antiretroviral activities.

Conclusion

The result of our study demonstrates the combined crude ethanol extract leaves of *P. guajava* and *A. paniculata* with certain formulation (3:1) inhibits *in vitro* replication of SRV-2. Based on this, we can recommend that the two medicinal plants can be potential source as antiretroviral that is accessible and affordable. For best practice, further *in vivo* chronic toxicity need to be performed.

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