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The antiviral activity of *Laportea decumana* Methanolic extract against NDV virus

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ABSTRACT

The avian species virus that causes Newcastle disease is an extremely contagious illness. Avian paramyxovirus 1, or Newcastle disease virus (NDV), is a virus that brings harm to poultry's central nervous system and digestive tract. The NDV outbreak was initially documented in 1928 in Java, Indonesia. Newcastle disease does not currently require therapy. One popular and useful strategy for preventing and treating viral infections, such as Newcastle disease, is vaccination. With a variety of native medicinal plants and an abundance of biodiversity, Indonesia presents a promising area for biotechnology and pharmaceutical research. *Laportea decumana*, is a native plant in the Eastern part of Indonesia that contains alkaloids, glycosides, steroids, flavonoids, tannin, and saponin. Its cytotoxic, analgesic, antioxidant, and antibacterial properties have all been demonstrated. The antiviral properties of *L. decumana* have not been extensively researched. Thus, the purpose of this study is to examine L. decumana's antiviral activity, particularly against NDV, using a variety of techniques, including the plaque assay, cytotoxicity test, and gene expression experiment. *L. decumana* extracts at 100 μg/mL or less is a safe concentration to consider, as it still has 65% and above cell viability based on the results of the cytotoxic assay.

Keywords: NDV, *Laportea decumana*, antiviral, plaque assay

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INTRODUCTION

Newcastle disease is a highly contagious disease caused by avian viral infection. This virus is known for its harmful effects on the central nervous system and digestive tract of poultry (Butt et al., 2019). NDV is a non-segmented virus structured into six genes that encode various proteins: NP, M, HN, P, L, and F. L is considered a virulence marker of NDV. NDV is a negative-stranded RNA virus (Murulitharan et al., 2013). The NDV outbreak was first reported in Indonesia in 1928, specifically in Java (Dzogbema et al., 2021). Currently, there are no treatments for Newcastle disease (Absalón et al., 2019). Vaccination is a common and effective method to prevent and control viral infections, including Newcastle disease (Ellebedy & Ahmed, 2016).

Due to its wide range of native medicinal plants and rich biodiversity, Indonesia is an ideal area for biotechnology and pharmaceutical research. Moreover, Indonesia is known as a high-humidity country which promotes the growth of natural plants, including Laportea. One of the commonly studied species of Laportea is *Laportea decumana*. This species has been determined to contain alkaloids, steroids, tannins, saponins, flavonoids and glycosides, which have been widely used in many research areas (Thalib et al., 2021). Flavonoids, a type of phenolic compound, have been shown to work as an antiviral agent by inhibiting proteases, DNA/RNA polymerases, and viral neuraminidase (Ninfali et al., 2020). Interestingly, *L. decumana* has been tested for its antioxidant and antibacterial purposes; however, the antioxidant properties of this plant have yet to be investigated (Simaremare et al., 2018; Thalib et al., 2022). Immortalized cell line has been widely used as a host cell in much research since it is shown to provide a reproducible outcome and consistent sample (Kaur & Dufour, 2012). One of the examples of commonly used immortalized cell lines is HeLa cells. Some antiviral studies have shown the use of HeLa cells as host cells, including (Chu et al., 2018), where the influence of NDV on HeLa cells was tested through the MEK/ERK signaling activation.

Hence, the aim of this research is to investigate the antiviral activity of *L. decumana*, especially against NDV in the HeLa cells, by utilizing several methods. A plaque assay was performed to determine the amount of infectious virus in the sample. The cytotoxicity properties of *L. decumana* were then tested using an MTS assay, followed by the determination of the gene expression ability, which was conducted through qRT-PCR towards the NDV gene.

MATERIALS AND METHOD

Materials

The *Laportea decumana* used was purchased from the local market in Papua, marked by being a perennial plant with slightly woody, approximately 2 meters in height, with well-branched stems and leaves densely armed with irritant hairs with lengths of approximately 0.5 to 1 cm. The HeLa cell culture used was obtained from ATCC. NDV virus used in this experiment was a La Sota strain grown in embryonated chicken eggs (Chu et al., 2019; Masoud et al., 2022; Mast et al., 2006).

Methods

Generation of L. decumana methanolic extract

Two hundred grams of *L. decumana* powder were put in two Erlenmeyer, one hundred grams each. The powder was macerated for three days at 180 rpm after being soaked in 500 mL of methanol. A Buchner pump and Whatman filter paper (Grade 1) were used to filter the extract. There were two iterations of the maceration procedure. A Buchi rotary evaporator was employed to concentrate the filtrates at 65°C. After that, the surplus solvent was eliminated using an evaporating dish set over a 50°C water bath under a fume hood for two days or until the weight stabilized and the extract yield could be determined. The extracts were placed in the refrigerator to be used later on and covered with aluminum foil.

HeLa cell culture

HeLa cell lines were grown at 37°C in a 5% CO_2 incubator in a complete Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS), 3.7 gr/L Sodium Bicarbonate

($NaHCO_3$), and 1% Penicillin-streptomycin. The cells were passaged thrice a week in sterile conditions under a biosafety cabinet.

Cytotoxicity Assay of L. decumana extract

HeLa cell lines were seeded with complete DMEM at a density of 1 x 104cell/well in a 96-well plate and incubated in a 5% CO_2 incubator at 37°C for 24 hours. The cells were then washed and starved for 3 hours in DMEM and treated with different concentrations of *L. decumana* extracts (50, 100, 150, 200, and 250 µg/mL) and incubated for 48 hours. The cells were supplemented with 15 µL MTS reagent. The absorbance of untreated and *L. decumana* treated cells was readed using a microplate reader at 490 nm. The half-maximal inhibitory concentration (IC_{50}) was then assessed using the following equation (Bayer et al., 2023):

$$y = D + \frac{A + D}{1 + 10^{(x - \log C)B}} \quad \text{..(1)}$$

Plaque assay

The plaque assay had been conducted according to previous methods devised with modification (Chu et al., 2018, 2019). The HeLa cell line was first seeded in a 24-well plate in DMEM and incubated to a confluency of 80-90% in a 37°C incubator with 5% CO2 for 48 hours. The HeLa cells were infected with 200 µL of diluted (10^{-5}) NDV in complete DMEM, and they were incubated for 1 hour. After the incubation, the viral inoculum was taken out, and the cells were then washed with DMEM. 500 µL of semi-solid medium containing the diluted *L. decumana* extracts (20, 40, 60, 80, and 100 µg/mL) mixed with 1.5% Carboxymethylcellulose (CMC) in DMEM supplemented with 2% FBS and 1% Penicillin-Streptomycin were added to the cell monolayers. After being incubated for 72 hours, the cells were stained with 1% crystal violet (100 µL per well) without removing the CMC media and were incubated at room temperature for 15 minutes. After incubation, the dyed medium was removed, and the cells were washed once with PBS. Finally, cells were preserved with 2% formaldehyde. The plaque size measurement was performed after the removal of formaldehyde.

Gene expression analysis

HeLa cell lines were seeded in a 24-well plate and incubated for 24 hours. The cells were then infected with NDV (MOI: 1) for 1 hr before the viral inoculum was removed and treated with 40 µg/mL of *L decumana* for 24 hrs. After 24 hrs, RNA of HeLa cell lines was extracted using GeneAid RNA Extraction Kit based on the provider's protocol. The quality of the RNA was then assessed using a Nanodrop spectrophotometer in which all of the samples had a 260/280 ratio of 2.0 to 2.2 and a 260/230 ratio of 1.8 to 2.1. The cDNA was then synthesized by following the RevertAid First Strand cDNA Synthesis Kit protocol. The qRT-PCR data was generated in TOYOBO Sybr qPCR Mix based on the provider's protocol; the primers to detect NDV virus expression were 5' – AAAGTGGTGACACAGGTCGG-3' as forward and 5'- CCGATGTATTGCCGCTCAAG-3' as reverse (Mao et al., 2022). The data is examined using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Data Analysis

The quantitative data used in this experiment was analyzed using GraphPad, using two-way ANOVA for statistical significance and Student T-test for pair-to-pair comparison of data.

RESULT AND DISCUSSION

Cytotoxic Activity of L. decumana extract towards HeLa cells using MTS assay

L. decumana exerts a cytotoxic effect on 150 µg/mL, 200 µg/mL, and 250 µg/mL (cell viability of 32.2%, 29.6 %, and 26.5%). At a concentration of 100 µg/mL or lower, L. decumana increased the viability of Hela cells by 65% (Figure 1.) The IC_{50} (50% cytotoxic concentration) for *L. decumana* obtained was at 131 µg/mL.

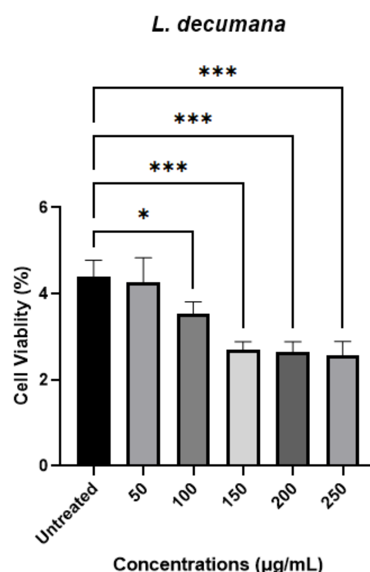

Figure 1. Cytotoxicity result of HeLa cells treated with *L. decumana*. The data presented as mean ± SD (*) $P \leq 0.05$ & (***) $P \leq 0.001$

In this study, *L. decumana* proved high toxicity at 150 µg/mL and higher concentrations. The IC_{50} of *L. decumana* was approximately 131 µg/mL, which killed 50% of the HeLa cells. The safe concentration to be considered is *L. decumana* at 100 µg/mL or lower since it still has 65% and above cell viability. This *in vitro* study should be investigated further with an *in-vivo* model since the results of the *in vitro* assay do not always match with the results of the *in-vivo* analysis (Di Nunzio et al., 2017).

Assessment of antiviral activity of L. decumana extracts against NDV by plaque assay

The Hela cells infected with NDV under different conditions were observed under the microscope to examine the cell morphology and proliferation. As seen in Figure 2A. and Figure 2B., NDV-infected Hela cells had thinner cell morphology and lower cell density compared to the healthy ones. When being treated with *L. decumana* with a concentration of 20 µg/mL, the infected HeLa cells looked healthier, resulting in similar cell morphology to the negative control but slightly lower in cell density (Figure 2C). Figure 2D indicated a significant improvement in cell morphology and cell proliferation as well as very high cell confluency with monolayer formation by treating the infected cells with 40 µg/mL of *L. decumana* leaf extracts.

HeLa has been reported to be a preferred target by NDV due to its rapidly replicating nature (Chu et al., 2019). Hence, the infection and cytopathic effect (CPE) was observed. After removing the stain and fixing the cells following the plaque-forming assay protocol, the virus growth was measured through the diameter of the plaque (Goh et al., 2016). The plaque sizes were visibly reduced in the 20 µg/ml and 40 µg/mL *L. decumana* extract treated wells compared to the untreated one. By increasing the *L. decumana* extract concentration to 60, 80, and 100 µg/mL, no plaques could be observed anymore. However, it should be noted that there were less visibly stained cells when they were treated with higher concentrations. A higher concentration of *L. decumana* extract could exert growth inhibition of the HeLa cells, marked by a relatively lighter density of stained HeLa cells (Figure 2 B, C, D). Cytotoxicity of plant extracts towards HeLa cells has been demonstrated from various plants as well (Amna et al., 2019; Ismail et al., 2020; Mavrikou et al., 2020).

In this study, to explore whether the treatment had an impact on NDV replication, qPCR was performed with 40 µg/mL of *L. decumana*-treated HeLa cells, and the untreated group served as the control. However, the qPCR results of NDV in HeLa cells were not significant when being treated with 40 µg/mL of *L. decumana* (Figure 3). The non-treated, infected cells still showed CPE, and the *L.*

decumana-treated cells showed less CPE and cell death. This indicates that *L. decumana* extract did not affect the replication activity of NDV. The outcome most likely indicates that the active compound is not a replication-inhibiting agent for viruses. The viral entry, assembly, and release are the most likely targets for the compound, as has been demonstrated on *Polyalthia longifolia*. It is shown that it inhibits viral entry and budding but not attachment and viral RNA replication (Yadav et al., 2020).

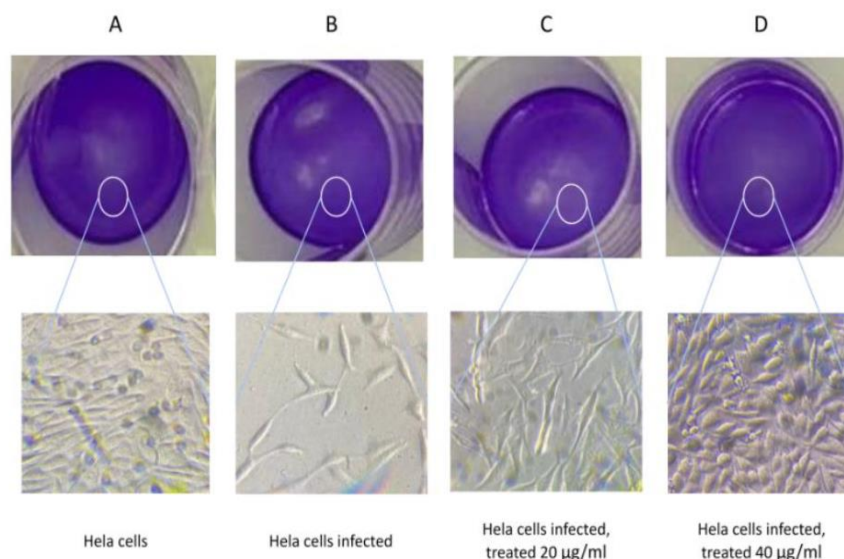

Figure 2. Plaque forming assay result of HeLa cells against NDV (A) HeLa cells without NDV infection and *L. decumana* treatment, (B) infected with NDV and not treated with *L. decumana*, (C) infected with NDV and treated with 20 µg/mL of *L. decumana*, and (D) infected with NDV and treated with 40 µg/mL of *L. decumana*

Viral gene expression analysis

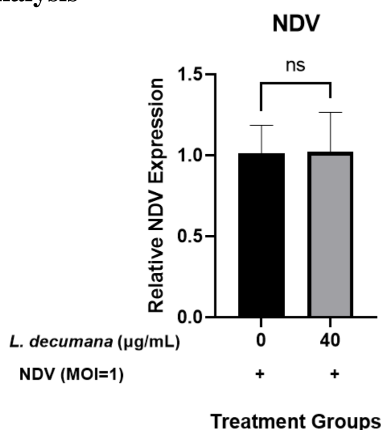

Figure 3. The relative expression of NDV in HeLa cells treated with *L. decumana*.

CONCLUSION

The experimental results show that *L. decumana* extract inhibits NDV production at a dose of 20 µg/mL, with no evidence of viral gene downregulation. It is suggested that the inhibition of *L. decumana* extract occurs at the protein level, where it could produce non-infectious viral particles. Further investigation is necessary to confirm its mechanism.

At a concentrarion of 100 µg/mL, the methanolic extract of *L. decumana* produced above 65% cell viability in HeLa cells. At a dose of 20 µg/mL, the NDV virus plaque assay showed inhibition,

indicating the antiviral activity. The gene expression research revealed no downregulation of viral gene expression, suggesting that other mechanisms, such as entrance, assembly, or budding, are being inhibited rather than viral replication. Mechanistic studies on *L. decumana* methanolic extract can also be done to further elucidate the antiviral activity.

In conclusion, *L. decumana* has proved to produce a high toxicity at 150 μg/mL and higher concentrations where its IC_{50} lies at 131 ug/mL. *L. decumana* also shows an inhibitory effect at the dose of 20 μg/mL, which indicates antiviral activity. However, the gene expression analysis result shows that *L. decumana* could not downregulate the viral gene expression, suggesting that other mechanisms, such as entrance, assembly, or budding, are being inhibited rather than viral replication. Mechanistic studies on *L. decumana* methanolic extract can also be done to further elucidate the antiviral activity.

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