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RESEARCH ARTICLE

Cytotoxicity and Apoptotic DNA Fragmentation Evaluation of Ethanolic Extract of *Phyllanthus niruri* L. in Vero and Primary Feline Testicular Cells

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Abstract *Phyllanthus niruri* or Dukung Anak is native to Southeast Asia, particularly in Malaysia and has been used as traditional preparations and as health supplements. However, previous studies have found that *P. niruri* can impair the male reproductive functions. With booming population of stray animals within the community, a new controlling method that is more affordable and safer must be developed. This study was aimed to explore the safety of *P. niruri* as a male herbal contraceptive to be used in veterinary medicine as one of the non-surgical sterilisation methods. For the preliminary part of this ongoing research, we have investigated the cytotoxicity activity of *P. niruri* ethanolic extract on Vero and primary feline testicular cells (FTC) and DNA apoptotic activity in FTC. *Phyllanthus niruri* extract was prepared using cold maceration method in 50% ethanol. The FTC were prepared from feline testes obtained from post-routine castration from local private veterinary clinics, while Vero cells were obtained from the archived cells of Virology Laboratory, Faculty of Veterinary Medicine Universiti Malaysia Kelantan (FPV UMK). The MTT assay was performed to evaluate the cytotoxicity of these plant extracts on FTC and Vero cells. Apoptosis was assessed using DNA laddering assay on extracted DNA of the treated FTC cells and observed the DNA patterns in gel electrophoresis. The 50% cytotoxic concentration (CC_{50}) of the ethanolic extract of *P. niruri* was a dose dependent. The DNA laddering assay revealed that the plant extract does not induce apoptosis in FTC. Therefore, the study outcomes indicated that the ethanolic extract of *P. niruri* is potentially safe for animal use. However, in vivo studies are required to confirm these findings.

Keywords: Apoptosis, cytotoxicity, feline testicular cells, herbal contraceptive, *Phyllanthus niruri*.

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Introduction

The overpopulation of stray animals, mainly cats and dogs, remains a concerning issue in the community. Unowned dogs comprise approximately 75% of the dog population worldwide [22]. In addition, intact male cats tend to be involved in fights or aggressive encounters thus become predisposed to more severe complications due to fight-related wounds [19, 29]. As one of the initiatives to manage this issue, Trap-Neuter-Release (TNR) method was introduced as a humane way to control the overpopulation of stray animals by combining surgical sterilisation and natural mortality [24]. However, according to a study by Lohr *et al.* [8], TNR is considered beyond economical than trapping and euthanising. Therefore, in this study, we intend to apply a more cost-effective and affordable method to control a mass population of stray animals that can be widely used in underdeveloped and developing countries.

Herbal contraceptives are seen as one of the effective methods of controlling the fertility of animals and humans [1]. They are widely used in rural areas as an affordable way to manage human overpopulation as compared to chemical-based contraceptives [25]. Different parts of certain medicinal plants, including flowers and seeds, can be utilized for the development of herbal contraceptives for both males and females [18]. Furthermore, herbal contraceptives generally received good cultural acceptance by the local community with effective antifertility property and minimal adverse reactions [6, 40].

As the intention to provide a more affordable method for controlling stray animals' overpopulation, we have chosen *Phyllanthus niruri* (*P. niruri*) to be developed into herbal contraceptives as it possesses the antifertility effect and non-toxic [42]. *P. niruri*, locally known in Malaysia as "Dukung Anak", is one of the largest genera that is indigenous to Southeast Asian countries including Malaysia, Philippines, and Thailand [29, 55]. Physical appearances of many *Phyllanthus* species are nearly identical, hence, difficult to differentiate between the species [10]. Tuhin *et al*. [33] documented that the variations of the epidermal shape and circumference can be used to distinguish *P. amarus*, *P. niruri* and *P. debilis*. Additionally, *P. niruri* is usually around 30-60 cm in height with distichous elliptical leaves and branching stem at the base of the plant near the root region, as can be seen in Figure 1 [35, 42]. The leaves carry the fruits of *P. niruri* that are smooth capsules with globose shape and approximately 2-3 mm in diameter [28].

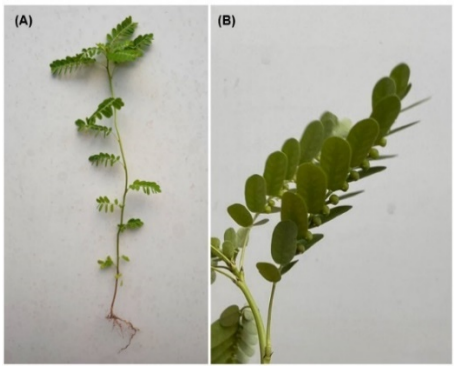

Figure 1. The morphological features of *Phyllanthus niruri* L. (A) Whole plant including roots; (B) The elliptical leaves and rounded fruits (location found: Jeli, Kelantan, Malaysia)

The phytochemical constituents in the ethanolic extract of *P. niruri* includes the presence of alkaloids, phenolics, flavonoids, terpenoids, steroids, glycosides, saponins and tannin compounds [5]. The alkaloids found in *P. niruri* is a securinega-type that includes nor-securinine, epibubbialine, nirurine and securinine [12, 36]. The quercetin and rutin are among the flavonoids found in *P. niruri* [12, 43]. One of the phenolic compounds isolated from *P. niruri* is ellagic acid and it is a common phenolic that can be found in *Phyllanthus* spp. [30]. In a previous study, it was found that the phenolic and flavonoid compounds isolated in a plant are responsible to cause antifertility [53]. Due to this fact, *P. niruri* was chosen as the best candidate for evaluation of the antifertility effect.

In societal context, *P. niruri* is used by the locals as traditional preparation for numerous diseases and as health supplements [2, 54]. The species is known to be used as antispasmodic, antihepatotoxic, antimalarial, antiviral, antibacterial, laxative, treatment for jaundice, hepatitis B and kidney-related problems [2, 54]. Besides possessing various health benefits, previous studies have reported the negative effect of consuming *P. niruri* resulting in disruption of the reproductive functions in both males and females [2, 15, 56]. The antifertility activity of *P. niruri* is related to the reduction in seminal fluid fructose level and sperm quality including sperm motility, counts and viability [56]. Following that, *P. niruri* can cause reduction in mature spermatozoa counts, degenerative effects on sperms and reduction in testosterone level [2, 15]. From histological evaluation, aqueous extract of *P. niruri* has caused shrinkage of the seminiferous tubules with testicular oedema leading to male infertility [15].

Therefore, this study aims to evaluate the testicular cellular changes in vitro using 50% ethanolic extracts of *P. niruri* as preliminary understanding for the development of a more affordable, safe, and effective male herbal contraceptive in the future. Additionally, this article also highlights the preparation of primary feline testicular cells with cytotoxicity evaluation using MTT assay and apoptosis activity assessment using DNA laddering assay onto treated cells. The findings from this study can help us to understand the safety levels of exposing the 50% ethanolic extracts of *P. niruri* in cultured cells before being applied into in vivo study.

Materials and Methods

Preparation of Plant Materials

The *P. niruri* plants were collected from local herbal farm in Jeli, Kelantan, Malaysia. Plant species authentication was conducted by Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM) Herbarium to validate the plant species prior to further analysis. The plants were authenticated as '*Phyllanthus niruri* Linn.' with voucher number, ID033/2022. Following the species identification, the whole plants including the seeds were rinsed using running water and distilled water consequently. To minimize the water content from the plants, we shade dried the rinsed whole plants for one week. Following that, the dried whole plants were cut into shorter lengths to ease grinding process using a mechanical blender available at Animal Nutrition Laboratory, FPV UMK, City Campus, Kelantan and light yellowish green fine powder was acquired.

Preparation of Plant Extracts

Cold maceration extraction method of *P. niruri* was adapted from a study by Kamarudin *et al*. [34]. In a 1000 mL borosilicate glass bottle (Schott Duran, Germany), 100 g of *P. niruri* powder was measured and added into 1000 mL of 50% ethanol and were stirred thoroughly until mixed. Following that, aluminium foil was used to cover the borosilicate glass bottle containing the mixtures and the immersion was conducted for three days at room temperature. Filtration of the mixture was performed using Whatman filter paper no. 1 to remove the solid materials from the liquid extracts. To remove the remaining solvents from the liquid extracts, the extracts were evaporated using Hei-VAP Core rotary evaporator (Heidolph, Germany) and dark greenish-brown concentrates were resulted. The concentrates were then oven dried at the temperature of 35°C for approximately three to four days to acquire the crude extracts. The crude extracts were stored in a refrigerator at 4°C prior to further analysis. To create the stock solution for cell culture study, 100 mg of viscous crude extracts were added into 1 mL of filtered 1% dimethyl sulphoxide (DMSO) and was mixed vigorously until dissolved.

Preparation of Cell Culture

The primary feline testicular cells (FTC) were prepared using a method described by Mota *et al*. [38] but with modification. Feline testes were acquired immediately after aseptic routine castration performed at local private clinics. Prior to that, the feline candidates were selected from male domestic shorthair (DSH) breed with age under 1 year old at the day of castration. The testes were put into pre-warmed sterile PBS solution mixed with 1% penicillin-streptomycin (Sigma-Aldrich, USA) and 5% foetal bovine serum (FBS, Gibco, USA) to transport the testes from local private clinic to the Virology Laboratory, FPV UMK. At the laboratory, the testes were cut and minced using sterile scalpel to create smaller pieces. The attached tissues, apart from testicular structure, were removed. The minced testes were put into PBS solution containing 1% penicillin-streptomycin (Sigma-Aldrich, USA), 5% FBS (Gibco, USA) and 0.25% trypsin (Gibco, USA). A sterile magnetic bar was put into the solution to stir the cells on magnetic plate stirrer (C-MAG HS 7, IKA, Malaysia) at 37°C until cell suspensions were formed. The suspensions were then collected and centrifuged at 1,500 x g for five minutes. The process was repeated until the resulting supernatant was clear in color. To wash the cells, sterile PBS solution was added and centrifuged at 1,500 x g for five minutes. The supernatant was discarded and growth media, Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin-streptomycin (Sigma-Aldrich, USA) and 10% FBS (Gibco, USA), were added to the cell pellet. The cell pellet was mixed with the growth culture media by vigorous up-and-down pipetting technique. Following that, the cells were transferred into T75 cell culture flasks and were incubated at 37°C for 24 hours in the presence of 5% CO_2 and 95% humidity. Vero cells were acquired from Virology Laboratory, FPV UMK. The cells were revived from L_2 nitrogen tank and cultured in T75 cell culture flasks containing the growth medium. The Vero cells were passaged three times until reaching confluency. After the first 24 hours, both cultured cells were reviewed to monitor the cell growth and the condition of FTC can be seen in Figure 2. The cells were further incubated until 75% confluent and the cells were trypsinised using Trypsin-EDTA solution (Gibco, USA) and about 4 x 10^4 cells/mL of both cells were seeded in 96-wells tissue culture plate and grown at 37°C for 24 hours in the presence of 5% CO_2 and 95% humidity.

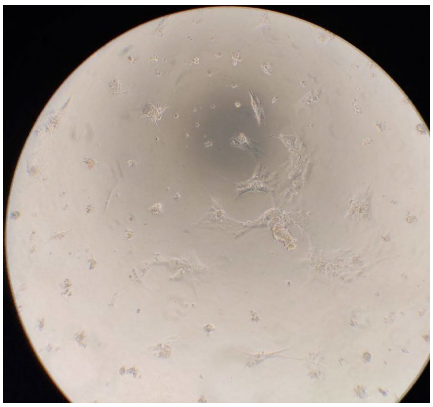

Figure 2. The condition of the adherent-type primary FTC after the first 24 hours incubation

Cell Viability Evaluation using MTT Assay

This procedure was performed to assess the effect of ethanolic extracts of *P. niruri* towards cell viability using MTT Cell Count Kit (Nacalai Tesque, Japan). The procedures were based on manufacturer's instructions. The cells were seeded into 96-well plates and incubated until 75% confluent. A total of 100 µL of 100 µg/mL *P. niruri* ethanolic extract was serially diluted and incubated at 37°C for 24 hours. After 24 hours, 10 µL of MTT solution was added into each well and the cells were incubated for three hours. Following that, the plates were observed for the formation of formazan crystal and 100 µL of solubilisation solution was added into each well and continued for incubation for another three hours. After incubation, the 96-well plates were viewed using POLARstar Omega microplate reader (BMG, Labtech, Germany) with optical density of 570 nm.

Apoptotic DNA Fragmentation Assessment using DNA Laddering Assay

The qualitative DNA laddering assay was used to assess the apoptotic DNA fragmentation of the cell lines that may be resulted from exposure to different concentration of *P. niruri* ethanolic extract. The technique was based on a study by Paul *et al*. [51]. The cultured cells were transferred into 6-well plates and were incubated to grow. After cell growth has reached 70% confluency, the prepared plant extracts were mixed with the cells using serial dilution method and the cells were incubated for 24 hours. After 24 hours, DNA extraction was performed using GF-1 Tissue DNA Extraction Kit (Vivantis, Malaysia) and the procedures were followed based on the manufacturer's instructions. The treated cells were harvested and centrifuged at 800 x g for five minutes at 4°C to obtain pellets. The cell pellets were mixed with 200 µL of PBS by pipetting. To perform cell lysis, 20 µL of Proteinase K and 2 µL of lysis enhancer were added into the samples and mixed thoroughly with 200 µL of Buffer TB and incubated at 65°C for 10 minutes. After incubation, 200 µL of absolute ethanol was added into the sample and vortexed. Following that, 650 µL of the samples were transferred into the column's mixture and centrifuged at 5,000 x g for one minute. Then, the columns were washed with 650 µL of wash buffer and centrifuged at 5,000 x g for one minute. After repeating the column washing, the column was centrifuged at 10,000 x g for one minute to remove the excess ethanol. Following that, the column was inserted into clean microcentrifuge tubes and 200 µL of preheated elution buffer and TAE buffer were added and let stand at room temperature for two minutes. The resultant column was centrifuged at 5,000 x g for one minute to elute the DNA. For DNA laddering assay, agarose gel electrophoresis (AGE) method was performed to assess any degradation of DNA following apoptosis. An amount of 10 µL of DNA for each sample was mixed with 2 µL of loading dye and loaded into wells of 2% agarose gel stained with Midori green. The electrophoresis was set for 90 minutes at 45 V. Following that, the agarose gel was examined.

Statistical Analyses

The results were analysed by One-Way ANOVA to identify significant difference between treatment and control groups using SPSS version 26.0 (IBM). Tukey's post-hoc test comparisons were performed following detection of the significant difference. For cell viability, a non-linear regression analysis was performed to plot the dose-response curves and the CC_{50} values for both FTC and Vero cells were determined using Microsoft Excel for Office 365. All data were expressed as means ± standard deviation and all significant differences were set at $p < 0.05$.

Results and Discussions

Cell Viability Evaluation using MTT Assay

The cell viability evaluation for both FTC and Vero cells against different concentrations of *P. niruri* ethanolic extracts was dose dependent. Lowest cell viability was observed at 10.0 mg/mL for both FTC (0.22 ± 0.04) and Vero cells (0.87 ± 0.04). The cell viability peaked at concentration of 0.312 mg/mL for both FTC (0.55 ± 0.03) and Vero cells (1.47 ± 0.17). These values were significantly different when compared with control group ($p < 0.05$). After 24-hour exposure, the CC_{50} for FTC was observed at higher concentration of *P. niruri* ethanolic extract in which concentration of lower than 5 mg/mL (0.29 ± 0.04) is safe and non-toxic (Figure 3). This result indicates that higher doses of *P. niruri* ethanolic extract (more than 5 mg/mL) are toxic to FTC cells. For Vero cell viability, the CC_{50} can be observed at 2.5 mg/mL (1.03 ± 0.05), at lower concentration compared to FTC (Figure 3). The result from this study indicated that *P. niruri* ethanolic extract with concentrations below 2.5 mg/mL is considered as safe and non-toxic for both cells while *P. niruri* ethanolic extract is more toxic to Vero cells compared to FTC cells.

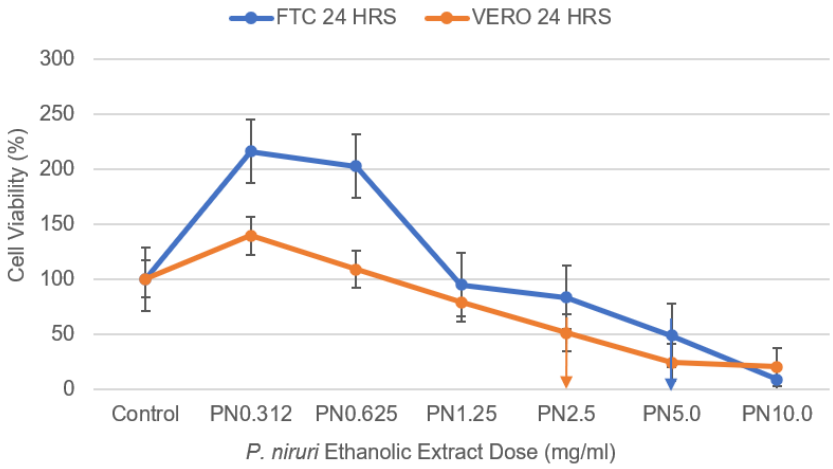

Figure 3. Cell viability (%) against different doses of ethanolic extract of *P. niruri* (mg/mL) on Vero and FTC after exposure for 24 hours. The CC_{50} of the *P. niruri* ethanolic extract is indicated by orange arrow (Vero cells) and blue arrow (FTC)

Based on current available information, this plant has not been investigated against primary feline testicular cell line. Previous cell viability studies using MTT assay for *P. niruri* were assessed using cell lines such as Vero, HepG2 human liver cells and SH-SY5Y neuroblastoma cells [9, 17]. In our study, 10 mg/mL of *P. niruri* resulted in the lowest cell viability performance for both FTC and Vero cells. As this study is the first one to evaluate the cytotoxicity activity of FTC, we compare the result for FTC with the previous studies using various cell lines. The 50% cytotoxic concentration (CC_{50}) for FTC was recorded at 5 mg/mL. Based on a study by Dey *et al*. [9], the *P. niruri* does not cause cytotoxicity for up to 50.0 µg/mL in HepG2 cells and was discovered to possess approximately 85% cell viability when being tested on SH-SY5Y neuroblastoma cells [17]. Furthermore, the CC_{50} for Vero cells can be observed at 2.5 mg/mL in this study. This result agrees with a study by Faral-Tello *et al*. [39] where, the CC_{50} of *P. niruri* ethanolic extracts on Vero cells was 2.542 mg/mL. However, the CC_{50} value in this study is higher compared to *P. niruri* aqueous extract on Vero cells where the CC_{50} is 0.5 mg/mL [56]. The CC_{50} values of the 50% ethanolic extract of *P. niruri* for both FTC and Vero are lower compared to other medicinal plants with antifertility property such as *P. indica* (20.66 mg/mL) and *R. communis* (16.5 mg/mL) [3, 18]. According to Njeru *et al*. [48], CC_{50} above 500 µg/mL is considered as acceptable toxicity limit. These findings indicated that both cell lines' cytotoxicity activity occurred after exposing the cells at certain doses of *P. niruri* 50% ethanolic extract. Hence, we can summarize that the cytotoxic effect of *P. niruri* 50% ethanolic extract is dose-dependent. Due to the antiproliferative effect observed on FTC, our finding may also suggest that the 50% ethanolic extract of *P. niruri* possesses the antifertility property and can be further studied in animal model. For the development of male herbal contraceptive, understanding the antifertility effect on the male androgen synthesis, spermatogenesis and the evaluation of the male reproductive organs using animal model is crucial to be studied [50].

Additionally, our finding on the dose-dependency cytotoxic effect of the 50% ethanolic extracts of *P. niruri* in cell viability is in accordance with a previous study by Ooi *et al*. [26]. According to a study using HepG2 cell line, the cumulative cytotoxic response of *P. niruri* at lower dose is already sufficient to reduced 50% cell growth due to its antiproliferative potential. This is associated with phyllanthin, an active compound of *P. niruri*, where at lower concentrations, it can exert hepatoprotective property but becomes cytotoxic when higher concentrations were used [26, 37]. The finding on the dose-dependency is further supported by a study by Paul *et al*. [51] where, the MTT assay result of *P. niruri* extracts on HeLa, SiHa and C33A cell lines was found to be dose-dependent as compared to the untreated control.

Apoptotic DNA Fragmentation using DNA Laddering Assay

As DNA fragmentation activity is the hallmark for apoptosis, DNA laddering assay was performed. The DNA fragmentation assessment revealed no DNA laddering activity when different concentrations of *P. niruri* ethanolic extract were used in FTC after 24 hours post-exposure (Figure 4). For FTC cells exposed to 1% DMSO and 1% ethanol, slight laddering pattern can be observed. This result indicates that DNA apoptotic activity has occurred in both 1% DMSO and 1% ethanol treated cells.

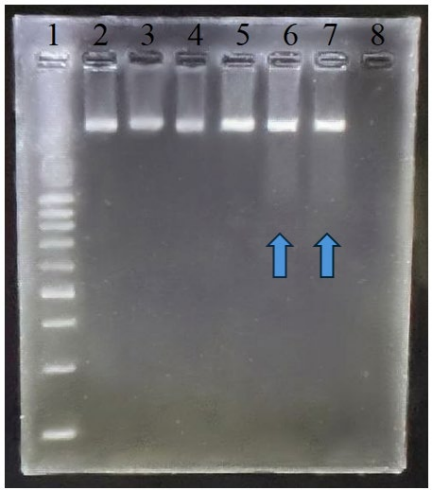

Figure 4. Gel electrophoresis result following DNA laddering assay using *P. niruri* ethanolic extracts at different concentrations on FTC. Lane 1 indicates DNA ladder, ethanolic extract of 100 mg/mL (lane 2), 50 mg/mL (lane 3), 25 mg/mL (lane 4), 12.5 mg/mL (lane 5), 1% DMSO (lane 6) and 1% ethanol (lane 7), while lane 8 is a blank. The fragmented DNA patterns in Lane 6 and 7 can be noted through observation of faint lines below the detected bands (blue arrow)

Cell apoptosis is a programmed cell death which referred to the series of orderly processes of cell death which occurred through intrinsic or extrinsic apoptosis pathways [14, 49]. Initially, apoptosis was defined as dying cells based on alterations of their morphological characteristics where the cellular plasma membrane is showing blebbing feature [20, 49]. However, there are other apoptotic characteristics that also includes DNA fragmentation [14, 31]. In the event of DNA fragmentation following cell apoptosis, the presence of DNA ladder pattern can be observed using certain DNA staining technique and can be achieved using assays such as DNA laddering assay [41]. In conjunction with this information, DNA laddering assay was conducted in our study. However, we found out that the DNA ladder pattern in FTC cells treated with different concentrations of *P. niruri* was absent. The DNA fragmentation was reported in other plants possessing antifertility potential such as *C. borivilianum*, *L. sativum*, *A. rohituka* and *C. dichotoma* [11, 44, 47, 52]. The absence of DNA fragmentation indicates that the 50% ethanolic extract of *P. niruri* does not induce genotoxicity in reproductive cells in vitro. However, the lack of DNA ladder pattern observed in treated FTC cells with declining cell viability from MTT assay could mean that the apoptosis is not the main cause of cell death. Cell death can be induced by several major types including apoptosis, necrosis, autophagy and pyroptosis following the series of cellular stress and damage [21]. With absent of DNA fragmentation in gel electropherosis, we can conclude that the apoptotic pathway is not the main cause of cell death in this study. Despite that, the absence of DNA ladder pattern using this method alone does not prove that there is no apoptosis as this method is a qualitative method to assess only the late-stage apoptosis due to internucleosomal cleavage of DNA [4, 16, 41, 46].

For the confirmation of the apoptosis in the different stages, quantitative methods such as Western blot to measure apoptotic cell-related proteins, real-time quantitative Polymerase Chain Reaction (RT-qPCR) by measuring the mRNA expression of apoptotic cells or flow cytometry by detecting the apoptotic cells stained with Annexin-V or propidine iodine (PI) can be performed [7, 13, 32, 58]. The slight laddering pattern displayed could indicate that the treated cells were still progressing to the late-stage apoptosis as the intranucleosomal cleavage of DNA occurred [7, 16, 41, 46]. The DNA laddering occurs when the DNA is being degraded by caspase-activated DNAse (CAD), a crucial process of apoptosis [46]. The DNA separation by agarose gel electrophoresis can be observed using staining such as ethidium bromide or Midori green, as used in this present study, resulting in laddering pattern [27, 46]. Hence, according to these findings, no apoptotic activity can be observed using different concentration of *P. niruri* ethanolic extracts on FTC as compared to using 1% DMSO and 1% ethanol in which, the laddering pattern was visible. However, to confirm the apoptotic activity, the cells need to be further tested using assays such as TUNEL and COMET assays [41].

Conclusion

This is a preliminary study for the development of oral male herbal contraceptive using *P. niruri* on Vero and primary FTC cells. In vitro cell viability indicates that *P. niruri* ethanolic extract is safe and non-toxic to the treated cells at concentration below 2.5 mg/mL. The DNA fragmentation assessment revealed no apoptotic activity, indicating the lack of genotoxicity effect of *P. niruri*. The data provided from this in vitro study will be further used as a reference or fundamental for in vivo study. In the future, we hope that our findings can contribute to controlling the stray animals' overpopulation thus minimizing the transmission of zoonotic and infectious diseases within the community without the need for surgical sterilization or euthanization.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgement

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