PRELIMINARY STUDY OF *Phyllanthus niruri* AS A NATURAL CONTRACEPTIVE FOR ANIMALS

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CERTIFICATION

This is to certify that we have read this research paper entitled 'Preliminary Study of *Phyllanthus niruri* as a Natural Contraceptive for Animals' by Amin Mustaqim Bin Mohamad Fauzi and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course DVT 55204 - Research Project.

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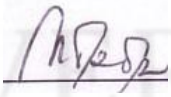

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Thank You

DEDICATION

I would like to dedicate and thank those who have given their support, advice, guidance, and recommendations throughout the completion of this project. A loving gratitude to my loving parent, Nor Aizan, who has been my pillar of strength consistently throughout my life and who consistently gives words of encouragement. My sister and brother, Zulaikha and Adam who always support me.

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ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement on the course DVT 55204 - Research Project.

Ongoing increases in the population of stray animals contributes to significant socioeconomic costs, environmental damage and zoonotic disease burden worldwide. The most common stray animals in Malaysia are domesticated pet animals such as cats and dogs, with current population control measures determined to be largely inadequate in controlling population growth. This is a preliminary research study to investigate the potential contraceptive properties of the common indigenous Malaysian Stonebreaker plant, *Phyllanthus niruri* and determine its feasibility as an alternative population control method for stray animals. Therefore, crude extracts of *P. niruri* were prepared and tested for cytotoxicity via MTT and DNA apoptosis assays on feline testicular cells. Results suggest that *P. niruri* extracts possess antifertility properties *in-vitro*. In contrast, *P. niruri* extracts are relatively non-toxic with little to no evidence of DNA fragmentation occurring at doses several magnitudes higher than predicted clinical usage doses. Further study is recommended to explore the potential of *P. niruri* in both animal models and real-world conditions.

Keywords: *Phyllantus niruri*, Feline Testicular Cells, Cytotoxicity, MTT assay, DNA apoptosis assay

ABSTRAK

Abstrak daripada kertas penyelidikan dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan untuk memenuhi sebahagian daripada kursus DVT 55204 - Projek Penyelidikan.

Kenaikan jumlah haiwan-haiwan terbiar telah menyumbang kepada kerosakan alam sekitar dari segi flora dan fauna, beban penyakit zoonotik dan aspek sosio-ekonomik di keseluruhan dunia. Haiwan-haiwan terbiar yang paling biasa ditemui di kawasan tanah air Malaysia adalah haiwan peliharaan domestik seperti anjing dan kucing rumah, dengan keadaan kawalan populasi dianggarkan sebagai tidak mencukupi untuk menjaga keseimbangan populasi haiwan-haiwan berikut. Kajian peringkat awal ini bertujuan untuk menyelidiki potensi anti-pembiakan pohon *Phyllanthus niruri*, suatu tumbuhan yang natif dan kerap ditemui di persekitaran Malaysia serta menguji kesesuaiannya sebagai kaedah kawalan populasi haiwan-haiwan liar di negara ini. Oleh itu, beberapa ekstrak ringkas *P. niruri* telah dihasilkan dan diuji untuk kerosakan pada sel (sitotoksisitas) melalui asai MTT dan asai apoptosis DNA pada sel testis kucing. Keputusan menunjukkan bahawa ekstrak *P. niruri* mempunyai potensi sebagai anti-pembiakan pada *in-vitro*. Selain daripada itu, ekstrak *P. niruri* mempunyai kadar toksisitas yang rendah kerana tiada atau kurang bukti kerosakan DNA dalam sel yang diuji walaupun pada tahap dos tinggi yang jauh melebihi penggunaan dos klinikal digunakan. Penyelidikan selanjutnya diperlukan untuk menerokai lagi potensi *P. niruri* menggunakan model haiwan serta kondisi-kondisi alam nyata.

Kata kunci: *Phyllantus niruri*, Sel Testis Kucing, Sitotoksisitas, asai MTT, asai apoptosis DNA

1.0 Introduction

Continued growth of stray animal populations represents a statistically significant problem of increasing severity affecting countries worldwide, including Malaysia. A large majority of these strays are popular domesticated pet animals such as cats and dogs, with free-roaming dogs making up around 75% of the global canine population (Hughes et al., 2013). The presence of a stray population causes a variety of problems such as acting as disease vectors between animals, biological reservoirs for zoonotic diseases such as rabies, and environmental damage due to their interactions with the local ecosystem (e.g., predation of native fauna). In order to address this problem, methods such as Trap-Neuter-Release (TNR) were developed as a more humane alternative to the traditional trap-and-euthanize approach (Levy et al., 2003). Despite this, TNR was deemed to be less cost efficient compared to trap-and-euthanize according to a research study conducted by Lohr et al. (2013).

The purpose of this study is to find an alternative method that is both more effective and economical in the mass control of stray animal populations which can also be extensively utilised by poor and developing nations that lack resources to implement traditional strategies. With this in mind, we have located a plant native to Malaysia, *Phyllanthus niruri*, with potential to be transformed into herbal contraceptive due to its relative nontoxicity and fertility-inhibiting properties (Dasiman and Bahari, 2021).

It is thought that herbal contraceptives represent one of the more successful methods in regulating fertility for both humans and animals (Sitasiwi et al., 2018). Rural regions show a marked preference for herbal contraceptives as they were cheaper than commercially available pharmaceutical contraceptives employed for the same purpose of controlling human fertility (Bala et al., 2014). We have chosen to focus primarily on the

effects of herbal extracts of medical plants on the male reproductive system due to the higher efficacy of targeting males compared to females from a contraceptive perspective, with interception of a single male potentially preventing fertilisation of multiple females in a population. Our ultimate goal is to come up with a male herbal contraceptive that is both safe and highly efficient. We expect that based on the findings obtained from this preliminary research study, we will be able to employ medical herbs as male contraceptives in the future to bring the population of stray animals under control.

1.1 RESEARCH PROBLEM

Stray animal overpopulation remains a communal problem despite extensive efforts in current stray animal management protocols. Pharmaceutical contraceptives used at local veterinary clinics, although effective, are costly and too expensive to be used in stray animal management. In addition to this, pet owner's doubts regarding surgical sterilisation may lead them to abandon their animals once they proliferate. Despite this, few, if any studies have been conducted to investigate the usefulness of herbal contraceptives as an alternative strategy even though herbal contraceptives are commonly used in rural areas as a method of fertility control. In this study, *in-vitro* evaluation of a crude extract prepared from *P. niruri* will be conducted to ensure a safe and effective in-vivo assessment.

1.2 RESEARCH QUESTION

1. Does *P. niruri* plant extract induce cellular damage on feline testicular cells?

1.3 RESEARCH HYPOTHESIS

P. niruri plant extract is able to induce cellular damage on feline testicular cells.

1.4 RESEARCH OBJECTIVES

To determine cytotoxicity level of *P. niruri* extract on feline testicular cells

2.0 LITERATURE REVIEW

2.1 Concerns pertaining stray animals

Felis catus population has continued to proliferate rapidly through urbanisation since the domestication of wild cats occurring around 2300 BCE, which has led to the establishment of considerable stray subpopulations within the geographic range of the domestic cat (Feiyang, 2020; Serpell et al., 2013). The existence of stray animal populations can be wholly attributed to the following five primary causes: non-sterilization or delayed sterilisation; lack of registration; vigorous productivity/fecundity; a general lack of conscience; and ultimately, an overpopulation problem in rural areas (Feiyang, 2020), where inconsistent waste management and street waste collection enables strays to thrive and proliferate given a constant supply of easily accessible food. The rapid growth of stray animal populations occurring in close proximity to humans poses a threat not only to humans but also to other animals as it increases the risk of transmission of zoonotic diseases, transmission of diseases towards other animals and habitability issues as wild animals are attracted to areas with a large stray population density (Seimenis and Tabbaa, 2014). Examples of zoonotic diseases with potential stray animal vectors include rabies, hydatidosis, leishmaniasis and toxoplasmosis.

According to data from the World Health Organization (WHO), free-roaming dogs are responsible for the spread of rabies to humans, which results in over 55,000 deaths each year. Of these deaths, 95 percent occur within the Asian continent (Feiyang, 2020).

2.2 Trap-Neuter-Release Method

Trap-Neuter-Release (TNR), is a preferred and humane procedure that combines surgical sterilisation with the natural mortality rate of animals in an effort to reduce the overpopulation of strays (Levy et al., 2003).

Several organisations, including the World Society for the Prevention of Cruelty to Animals (WSPA), do not condone the use of mass destruction as a method for controlling the population of stray dogs and cats, with exceptions granted in situations in which euthanasia is seen as the most humane alternative. These animals who are either terminally ill or pose a significant threat to the public are candidates for euthanasia. However, euthanasia may also be tolerated for animals that cannot be adopted and must be released back into the wild, as well as for the purpose of reducing the number of animals housed in shelters in order to prevent the welfare of the animals in those facilities from being compromised by overcrowding (Ortega-Pacheco et al., 2011). However, a study by Dorothy et al. (2019) found that Malaysians prefer the neutered stray animals to be rehomed rather than being released back into the wild as strays again due to concerns regarding the transmission of zoonotic diseases.

2.3 Current Alternatives to Animal Surgical Sterilisation

Even though surgical sterilisation is generally regarded as the most effective method for neutering companion animals, large scale application in population control efforts of stray animals is deemed impractical due to a combination of both time and financial requirements (Kutzler et al., 2006). Due to these factors, a number of alternatives to surgical sterilisation for the management of strays have been developed and are currently being evaluated for their level of efficiency and risks in small-scale pilot studies.

Alternatives to surgical sterilisation include the use of hormonal down-regulation principles, such as the administration of progestins, androgens, and gonadotropin-releasing hormone analogs (GnRH) (England, 1997; Junaidi et al., 2003; Kutzler et al., 2006; Shafik, 1994). Other methods include the injection of chemicals into the testicles, epididymis, or vas deferens, the suppression of spermatogenesis through the use of ultrasonic frequencies, and the administration of reproductive toxins such as ketoconazole (Fahim et al., 1977; Kutzler et al., 2006; Vickery et al., 1985). The use of magnetic nanoparticle hyperthermia (MNH), which applies a localised increase in temperature on testicles by converting magnetic energy into heat, has been identified as a recent advancement. These methods cause the degeneration of seminiferous tubules and gonadal atrophy, both of which are detrimental to the functions of the testicles (Jivago et al., 2021).

2.4 *Phyllanthus niruri* and its properties

According to Dasiman et al. (2021), *Phyllanthus* spp. is a species that belongs to the Phyllanthacea family and is indigenous to Southeast Asian countries such as Malaysia, the Philippines, and Thailand. It has alternate leaves that are sessile and oblong in shape. Their length ranges from 7 to 12 cm. It bears inconspicuous flowers with a bluish-greenish hue that are single, auxiliary, pedicellate, apetalous, and monoecious. *P. amarus* and *P. sellowianus* are closely related to *P. niruri* in appearance, phytochemical contents, and history, but they are found in drier regions of India and Brazil, as well as in Florida and Texas. Among the species, the *P. amarus* and the *P. niruri* are the ones that have been recognised as having an antifertility effect (Dasiman et al., 2021; Ezeonwu, 2011; Rao and Alice, 2001). In Malaysia, the species is commonly referred to by its local name, “Dukung

Anak" (Markom et al., 2007). Other common names for *P. niruri* are "gale of the wind" and "stonebreaker".

P. niruri has been attributed with antispasmodic, antihepatotoxic, antimalarial, antiviral, antibacterial and laxative properties, also has been investigated for the treatment of jaundice, hepatitis B and kidney-related issues (Manjrekar et al., 2008; Poh-Hwa et al., 2011). The antifertility properties of *P. niruri* are related to a reduction in the quantity of fructose secreted in the seminal fluid as well as inducing degenerative effects in sperm such as reduced sperm motility, sperm numbers and overall spermatozoa viability (Ezeonwu, 2011). In addition to this, *P. niruri* was also reported to be capable of reducing blood testosterone levels (Asare et al., 2013; Manjrekar et al., 2008).

On the other hand, *P. niruri* is also non-toxic and safe to use, as shown in studies that employed 5000 mg/kg of the extracts in acute oral toxicity tests. These tests were conducted to determine whether the extracts were hazardous (Asare et al., 2011). In addition, a sub chronic oral toxicity test was carried out using an ethanol extract of *P. niruri* at a dose of 300 mg/kg, and the results showed that the plant extract was not cytotoxic or genotoxic, indicating that it is generally non-toxic and safe substance (Asare et al., 2012).

2.5 IMPORTANCE OF EXPECTED RESEARCH FINDINGS

P. niruri has the potential to become one of the options for non-surgical contraception that is utilised in veterinary medicine. When we apply the concepts using animal models, having the results of the *in-vitro* evaluation of the antifertility effects using this plant extract will help us assess the safety margin and the effectiveness of the treatment. With the help of this natural contraceptive, we are hoping to make a contribution to the

community in the form of an effective and reduced risk alternative in the overpopulation of stray animals.

3.0 METHODOLOGY

3.1 Sample Preparation

3.1.1 Preparation of Plant Extract

Fresh, whole plants of *P. niruri* were acquired from a Kelantanese herbal farm. Species authentication procedures were then conducted at Universiti Kebangsaan Malaysia. The plants were rinsed with running water before being cleansed with distilled water and left for a week to dry in the shade. The dried plants were cut into shorter, more manageable lengths and pulverised into a fine powder using a mechanical blender available at the Animal Nutrition Laboratory, Faculty of Veterinary Medicine (FPV), UMK City Campus, Kelantan. After that, an ethanolic extract of *P. niruri* was prepared by combining 100 g of dry powdered material to 200 ml of ethanol for 24 hours. Using No. 1 Whatman filter paper, the extract was filtered to remove its solid residues from its liquid form. Utilising a rotary evaporator, the extract was evaporated to dryness under decreased pressure and dissolved in distilled water before being refrigerated for future use.

3.1.2 Preparation of Cell Culture

Feline testicles were obtained from Klinik Haiwan Kubang Kerian. The feline testicular cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS). In the culture medium, 1% penicillin/streptomycin (Sigma-Aldrich) antibiotics were added to inhibit microbial contamination. The culture medium ($4x10^4$ cells/well) was incubated at 37°C for 24 hours

with 5% CO_2 and 95% air. In accordance with the manufacturer's instructions, trypsin solution was employed to pass the culture medium.

3.2 Sample Analysis

3.2.1 Cell Viability Evaluation using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) Assay

The MTT assay was performed to determine the effect of *P. niruri* ethanolic extract on the viability of feline testicular cells in a 96-well plate. 10mcl of MTT solution was placed into each well containing 50ul of *P. niruri* and incubated for 3 hours at 37°C, then another 100mcl of solubilization solution was added in each well and incubated for another 3 hrs at 37°C. The plate was then shaken on an orbital shaker for 15 minutes. At 570 nm, the optical density was measured with a POLARstar Omega microplate reader (BMG, Labtech, Germany).

3.2.2 Genotoxicity Evaluation using DNA Fragmentation Assay using Gel Electrophoresis

The DNA fragmentation assay was performed to determine the genotoxicity of *P. niruri* on feline testicular cells incubated for 24 hours. 24 hours were spent incubating the cell culture in 6 well plates with 100 mcg of *P. niruri* and was serially diluted into 50 mcg, 25 mcg, 12.5mcg while DMSO was added for positive controls and untreated cells were used for negative controls. Incubated cells were pelleted in a clean microcentrifuge tube by centrifugation at 800 xg for 5 minutes at 4°C. A total of 200 ul of PBS was decanted and added into the solution as a supernatant agent before the cells were resuspended via pipetting. Cells in each sample were lysed with the addition of 20 ul of Proteinase K and 2

ul of lysis enhancer, mixed immediately with 200 ul of Buffer TB via pulse-vortexing. The samples were then allowed to incubate at 65°C for 10 minutes.

Following this procedure, 200 ul of glacial ethanol was added and the samples went immediate pulse-vortexing in order for a homogenous solution to be obtained. Vigorous mixing was employed to prevent the uneven precipitation of nucleic acid from high local ethanol concentrations.

A total of 650 ul of each sample was then transferred and loaded into columns assembled in clean collection tubes and centrifuged at 5000 xg for 1 minute. Residual flow through was discarded. Each loaded column was then washed with 650 ul of ethanol Wash Buffer and centrifuged at 5000 xg for 1 minute, with excess flow through discarded. The process was then repeated.

All traces of ethanol from the column were then removed through high-speed centrifugation at 10000 xg for 1 minute. Each column was then placed into clean microcentrifuge tubes and 200 ul of preheated Elution buffer was added into the column membrane. The columns were then allowed to stand at room temperature for 2 minutes.

DNA was eluted from the samples via high-speed centrifugation at 5000 xg for 1 minute and the obtained DNA was stored at -20°C prior processing step. The DNA samples were subject to gel electrophoresis on 2% agarose gel for 90 minutes at 40 amps and 100 v before data obtained was viewed and recorded.

4.0 Results

4.1 Cell Viability Evaluation using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) Assay

Figure 1 shows the horizontal X-axis that represents the concentration of ethanolic extract of *Phyllantus niruri* (mg/ml) against the vertical y-axis, which represents the cell viability (%) for 24 hours and 48 hours of incubation period. Both the 24 and 48 hours of incubation period suggests that the higher the concentration of ethanolic extract of *P. nirui*, the lower the percent of cell viability, which suggests a lower cellular metabolic activity. Inversely, the lower the concentration of ethanolic extract of *P. niruri*, the higher the percent of cell viability suggesting higher cellular metabolic activity.

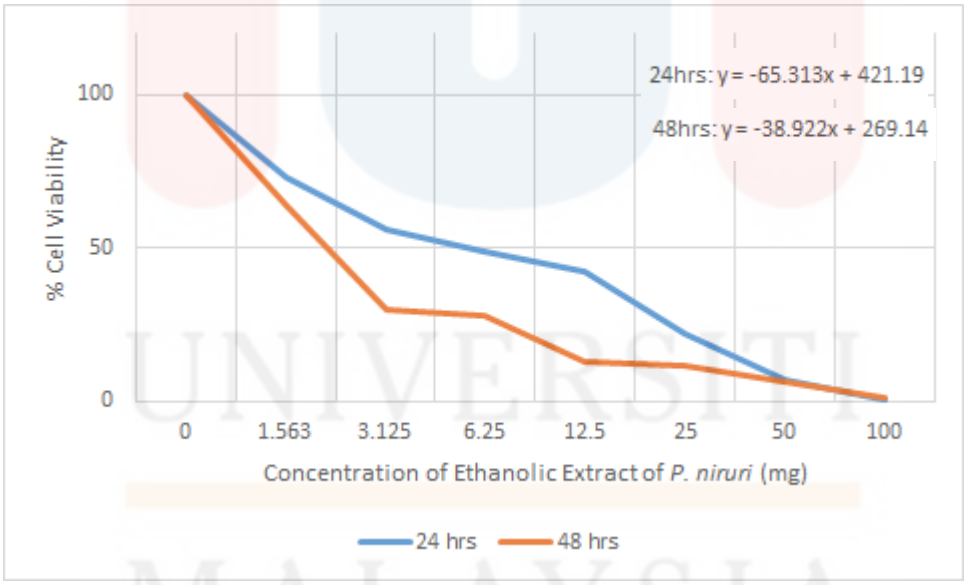

Figure 1: The percentage of cell viability against concentration of ethanolic extract of *P. niruri* (mg/ml) for 24 hours and 48 hours

4.2 Genotoxicity Evaluation using DNA fragmentation assay using gel electrophoresis

Figure 2 shows the result of DNA fragmentation assay using gel electrophoresis. From the results of DNA fragmentation assay using gel electrophoresis, there is no laddering of the bands suggesting that there is no or minimal fragmentation of DNA that occurs. This indicates that the *P. niruri* extract which despite the high concentrations used, still does not cause genotoxicity to the Leydig and Sertoli cell DNA.

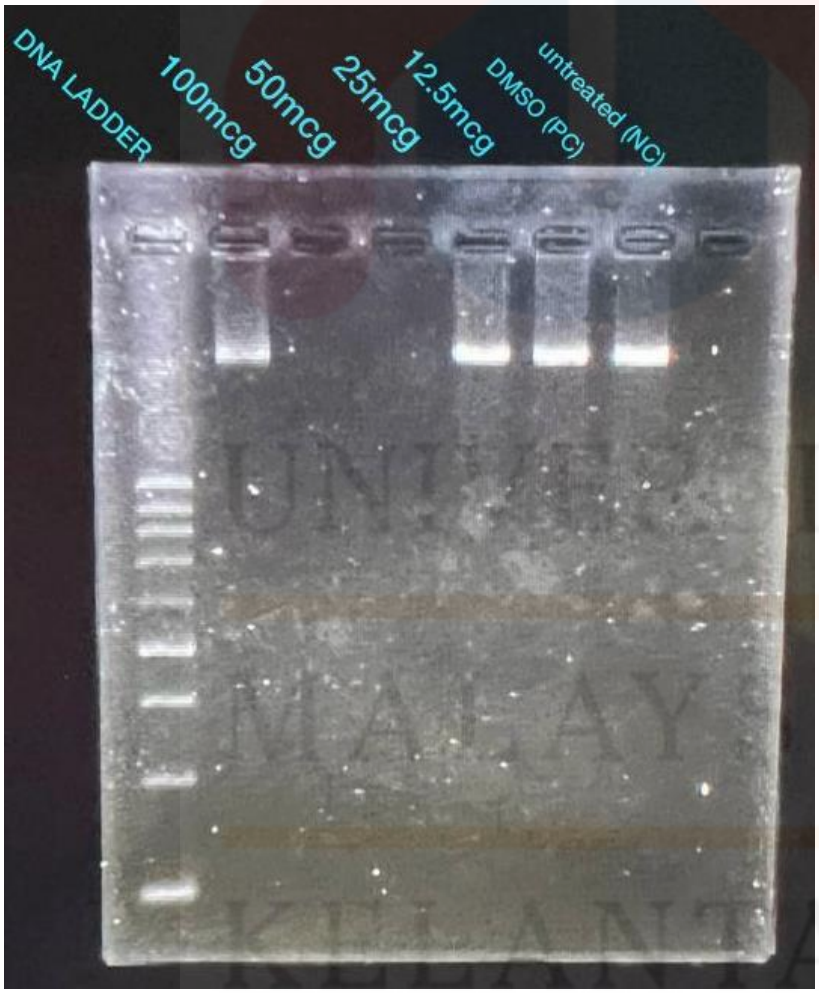

Figure 2: DNA fragmentation assay results

5.0 Discussion

The results indicated that *P. niruri* possess significant antifertility and contraceptive properties based on their capability to downregulate the functions of Sertoli and Leydig cell functions in in- vitro model. P. niruri extract demonstrated a dose-dependent correlative relationship with the degree of cellular metabolic inhibition being almost perfectly linear to the concentration of the extract in solution at lower doses, with an exponential increase in cell inhibition (and cellular mortality) at higher doses as observed in the large differences in 100 - 12.5 concentration respectively. The 50% cytotoxic concentration (CC50) of this crude extract was calculated to be roughly 5.68 mg/ml for 24 hours and 5.63 mg/ml for 48 hours, suggesting that relatively large concentrations of the extract are required to exert inhibitory effects on cell function and concentration above that is cytotoxic and below that is safe to be used (Atanasov et al., 2021).

We believe that this is due to the presence of large amounts of non-active pharmacological compounds in the extract, and propose further investigation, isolation and refinement of chemically active compounds as Active Pharmaceutical Ingredients for future studies. Despite this, little to no evidence of DNA damage was detected via the DNA fragmentation assay as we observed negligible band laddering at even high concentrations far exceeding clinically appropriate doses, suggesting that *P. niruri* exerts its effects through non DNA-damaging mechanisms. Possible mechanisms include the inhibition of cellular tyrosine kinases, modification of protein synthesis or interference through some sort of downstream signalling mechanism without causing DNA damage (Lam 2007).

The research outcome suggests that the compound has minimal genotoxic effects and possesses little potential for causing mutagenic damage if treatment is discontinued. Suggested further research efforts should focus primarily on determining and isolating active pharmaceutical compounds from the crude distillate, conducting adsorption,

distribution, metabolism, excretion and toxicity (ADMET) tests and perhaps X-ray crystallographic studies of ligand-receptor interactions to further develop the compound via the application of medicinal chemistry (Atanasov et al. 2021).

6.0 Conclusion

In conclusion, *P. niruri* extract is able to inhibit feline Sertoli and Leydig cell activity at *in-vitro* level, in a dose-dependent relationship with minimal genotoxic and teratogenic effects, through an unknown mechanism of action. We believe that the compound has demonstrated its potential for future research studies and further action should be taken to refine the compound further.

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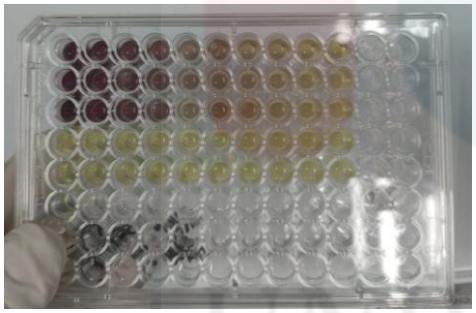
8.0 Appendices

Appendix A: Cell growth media

Appendix B: Trypsin

Appendix C: MTT assay



Appendix D: Pink coloured media indicating enriched nutrient within media

Appendix E: Biosafety chamber with MTT assay	Appendix F: Feline testicular cells