GRAPHENE OXIDE ANTIFUNGAL ACTIVITIES AGAINST *ASPERGILLUS VERSICOLOR*

ASHYER ANDDERIAS

(D18A0004)

A RESEARCH PAPER SUBMITTED TO THE

FACULTY OF VETERINARY MEDICINE,

UNIVERSITI MALAYSIA KELANTAN

IN PARTIAL FULFILMENT OF THE REQUIREMENT

FOR DEGREE OF DOCTOR OF VETERINARY MEDICINE

2022

UNIVERSITI MALAYSIA KELANTAN

CERTIFICATION

This is to certify that we have read this research paper entitled **'Graphene Oxide Antifungal Activities Against *Aspergillus Versicolor*'** by Ashyer Andderias, 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.

Dr. Nor Fadhilah Binti Kamaruzzaman

BSc in Biochemistry (UKM), PhD (RVC)

Senior Lecturer

Faculty of Veterinary Medicine

University Malaysia Kelantan

(Supervisor)

Dr. Mohammad Sabri Bin Abdul Rahman

DVM (UPM), MSc (UPM)

Senior Lecturer

Faculty of Veterinary Medicine

University Malaysia Kelantan

(Co-supervisor)

ACKNOWLEDGEMENT

Special thanks for those who have given their support, guidance, advice, and aid for the completion of this project paper:

Dr. Nor Fadhilah Binti Kamaruzzaman

Dr. Mohammad Sabri Bin Abdul Rahman

Lab assistants of FPV UMK

Family

DVM 5 2022/2023

Ashley Joyce Poon

Thank You

DEDICATIONS

I humbly dedicate this thesis to my beloved family, friends and members of Faculty of Veterinary Medicine, University Malaysia Kelantan for their words of affirmation and never-ending support. Especially my parents who have been there for me since day one of my journey.

I also dedicate this to lecturers and lab assistants for their knowledge and assistant throughout my research project. I am blessed for having them in my life which make my journey bearable, especially Dr. Nor Fadhilah, Dr. Mohammad Sabri, Prof Madya Dr.Maizan, Dr. Rasydan, Dr. Brenda, Puan Nani, Puan Salmah, Puan Mimi, and Encik Abid.

Table of Content

LIST OF FIGURES

LIST OF TABLES

ABBREVIATION

GO	-	Graphene Oxide
MDCK	-	Mardin-Darby Canine Kidney Cells
SDA	-	Sabouraud Dextrose Agar
µg/mL	-	Micrograms per milimeter
nm	-	Nanometer
rpm	-	Revolution per minute
DMSO	-	Dimethyl sulfoxide
CO_2	-	Carbon dioxide
NT	-	no treatment
µM	-	Micromolar
cm	-	centimeter

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.

Aspergillosis is a common fungal disease in animals and humans. The existing treatment of conventional antifungal may have disadvantages as it can cause liver damage, severe allergic and increasing resistance towards the compound makes it partially effective. Therefore, it is important to find an alternative therapy for the problem. Recently, the application of carbon-based nanoparticles such as graphene oxide (GO) has received attention for biomedical application. GO has been tested for its antibacterial properties and showed some promising outcomes. However, it is unknown if the GO can also demonstrate antifungal properties. In this research, we aimed to determine GO fungicidal activity against *Aspergillus versicolor* and the toxicity in mammalian cells. Based on the results, GO have fungicidal activity against *Aspergillus versicolor* as we can observe inhibition zone of GO on the agar plate streaked with *Aspergillus versicolor*. Also, GO is less toxic towards mammalian cells as it can induce cell proliferation of Mardin-Darby Canine Kidney (MDCK) cells.

***Keywords**: Graphene Oxide, Mardin-Darby Kidney cells, fungicidal activity, antifungal, toxicity cell growth, Aspergillosis*

ABSTRAK

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

Aspergillosis adalah penyakit kulat yang biasa terjadi pada haiwan dan manusia. Rawatan antijamur konvensional yang ada mungkin mempunyai kekurangan kerana boleh menyebabkan kerosakan hati, alergi teruk dan peningkatan daya tahan terhadap sebatian tersebut menjadikannya sebahagiannya berkesan. Oleh itu, penting untuk mencari terapi alternatif untuk masalah tersebut. Baru-baru ini, penggunaan nanopartikel berasaskan karbon seperti graphene oxide (GO) telah mendapat perhatian untuk aplikasi bioperubatan. GO telah diuji sifat antibakteria dan menunjukkan hasil yang menjanjikan. Namun, tidak diketahui apakah GO juga dapat menunjukkan sifat antijamur. Dalam penyelidikan ini, kami bertujuan untuk menentukan aktiviti racun kulat GO terhadap *Aspergillus versicolor* dan ketoksikan pada sel mamalia. Berdasarkan hasilnya, GO melakukan aktiviti fungisida terhadap *Aspergillus versicolor* kerana kita dapat melihat zon penghambatan GO pada plat agar yang dililit dengan *Aspergillus versicolor*. Juga, GO kurang toksik terhadap sel mamalia kerana boleh menyebabkan percambahan sel Mardin-Darby Canine Kidney (MDCK) sel.

***Kata Kunci**: Graphene Oxide, Mardin-Darby Kidney cells, fungicidal aktiviti, antifungal, pertumbuhan sel ketoksikan, Aspergillosis*

1.0 INTRODUCTION

Aspergillosis is an airborne fungal illness caused by the ubiquitous genus *Aspergillus* spp. This disease produces significant rates of morbidity and mortality in both people and animals (Desoubeaux & Cray, 2018). Aspergillus spores are typical components of aerosols, where they disperse over short and long distances, depending on environmental conditions. When spores come into touch with solid or liquid surfaces, the spores will deposit and germinate under optimal moisture conditions (Kanaani et al., 2008). Molds are referred to as 'ubiquitous' due to their ability to move globally on air currents and to grow practically anyplace when optimal food and water are present (Desoubeaux & Cray, 2018).

Aspergillus versicolor, a new causal agent, has been identified in a case of canine disseminated aspergillosis, which is often linked with *Aspergillus terreus* or *A. deflectus* infection (Zhang et al., 2012). The diagnosis was based on clinical symptoms, radiographic examination, and pathological results. Then, fungal culture and internal transcribed spacer ribosomal DNA sequencing were performed to confirm that *Aspergillus versicolor* was the causative agent (Zhang et al., 2012). The nonspecific clinical symptoms include sadness, fever, and cough. In contrast, a cytologic analysis of the bronchoalveolar lavage fluid frequently indicates a mixed inflammatory response characterised by an abundance of neutrophils and macrophages.

In addition to their negative consequences, fungus can be beneficial to medicine, biotechnology, and basic research because they are the primary producers of antibiotics utilised in contemporary medicine (H.N. Nguyen et al, 2018). Researchers have investigated both synthetic and natural antifungal chemicals as a means of inhibiting fungus growth.

In recent years, graphene and graphene oxide have been found to exhibit antibacterial characteristics, but nothing is known about their antifungal capabilities (H.N. Nguyen et al, 2018). A thick sheet of carbon atoms connected by sp2 hybridization and organised in a hexagonal arrangement compose graphene. Due to its unusual electrochemical features, which include high thermal conductivity, high current, density, chemical inertness, optical transparency, and very high hydrophobicity, it piques the curiosity of researchers in numerous sectors (Priyadarsini et al., 2018). It is the most straightforward type of carbon and consists of a few layers of graphite. X-ray diffraction technology reveals graphene's crystal structure to be a honeycomb-like shape. Included in the graphene family are reduced graphene oxide, graphene oxide, graphene sheets, and layered graphenes (Priyadarsini et al., 2018).

Few research has been conducted on the antifungal effects of graphene family members such as reduced graphene oxide (rGO) and graphene oxide (GO) nanoparticles on *Fusarium* sp. growth. In these tests, germination of *Fusarium* sp. was observed to be reduced by 13.70% - 77.55% relative to 10 mg L-1 and 500 mg L-1 GO concentrations, respectively (H.N. Nguyen et al., 2018). In addition, another study demonstrates a 50% decrease in *Fusarium* sp. growth on solid media with a 500 mg L-1 concentration of GO (H.N. Nguyen et al., 2018). Additionally, in a separate study utilising rGO, the ability of *Aspergillus* sp. to grow on solid substrate was examined. These studies demonstrate simply the effect of nanomaterials on fungal development; the mechanism of action in the fungi's metabolism has not yet been explained (H.N. Nguyen et al., 2018).

In this study, we examined the antifungal activity of graphene oxide against *Aspergillus versicolor*. *Aspergillus versicolor* is a slow-growing filamentous fungus that grows on food and in moist indoor conditions (V.K. Nadumane, 2016). *Aspergillus versicolor* is a mesophile with a minimum growth temperature of 9 °C, a maximum growth temperature of 39 °C, and an

optimal growth temperature of 27 °C (Pitt & Hocking, 1997). It has a pH minimum more than pH 3.1 and a pH high greater than pH 10.2 (Wheeler et al., 1991). The effects of graphene and graphene oxide on the fungus *Aspergillus flavus* and *Aspergillus niger* are being studied (H.N Nguyen et al., 2018). This experiment focuses on *Aspergillus versicolor* because no previous research has been conducted on this species.

2.0 PROBLEM STATEMENT

Aspergillosis is a common fungal disease in animals and humans. Commonly, the treatment relies on conventional antifungal. However, the treatment is limited and partly effective, meaning that the fungi can develop resistance towards this conventional antifungal. Therefore, this study aimed to investigate the efficacy of graphene oxide as an antifungal alternative against *Aspergillus versicolor in-vitro*.

3.0 RESEARCH QUESTIONS

i. Does Graphene Oxide demonstrate fungicidal activity against *Aspergillus versicolor in-vitro*?

ii. What is the toxicity of Graphene Oxide towards mammalian cell *in-vitro*?

4.0 RESEARCH HYPOTHESIS

i. Graphene Oxide can demonstrate fungicidal activity against *Aspergillus versicolor*

ii. Graphene Oxide is less toxic towards mammalian cell *in-vitro*

5.0 RESEARCH OBJECTIVE

i. To determine the effective concentration of graphene oxide that can demonstrate fungicidal activity against *Aspergillus versicolor in-vitro*.

ii. To determine the toxicity effect of Graphene Oxide against mammalian cell *in-vitro*.

6.0 LITERATURE REVIEW

6.1 Canine Disseminated Aspergillosis

There are three major kinds of canine aspergillosis: nasal, bronchopulmonary, and disseminated infections. In this instance, the dispersed form, aleuriospores can be identified in infected tissue (Zhang et al., 2012). Aspergillus species are capable of producing aleuriospores that can spread efficiently via hematogenous pathways. The German Shepherd is the most commonly affected breed, along with the Dalmatian, English setter, pug, Rhodesian ridgeback, springer spaniel, and whipper (Zhang et al., 2012). Clinical manifestations of disseminated aspergillosis include diskospondylitis, osteomyelitis, spinal hyperpathia, vestibular abnormalities, ataxia, paraparesis, weight loss, anorexia, uveitis, lameness, renal failure, and respiratory distress in canines (Zhang et al., 2012). The prognosis for disseminated aspergillosis is typically poor, as treatment is difficult.

6.2 Aspergillus Species

Recently, the genus Aspergillus was divided into eight separate subgenera, namely Aspergillus, Fumigati, Circumdati, Terrei, Nidulantes, Ornati, Warcupi, and Candidi. This subgenus is further subdivided into 22 sections, each containing a number of closely related species (Zhang et al., 2012). However, only a small proportion are capable of causing illnesses such as those caused by *A. fumigatus*, *A. flavus*, and *A. niger*. Sometimes, other species, such as *A. terreus* and *A. versicolor*, are isolated from clinical specimens (Zhang et al., 2012). Localized skin infection, nail infection, eye infection, and pulmonary dysfunction to invasive systemic aspergillus infection producing diskospondylitis, osteomyelitis, and pyelonephritis are the illnesses linked with aspergillosis. In this instance, the dog had advanced illness, and neither clinical nor postmortem testing confirmed portal entrance of *A. versicolor*. Most likely, the spore entered the host by inhalation or an open wound (Zhang et al., 2012). The diagnosis

of *Aspergillus versicolor* was established on the macroscopic colonial appearance of white to light tan colonies. The staining with lactophenol blue reveals brush-like and radiating conidial heads with round vesicles, biseriate phialides, and spherical conidia in short chains (Zhang et al., 2012).

6.3 Graphene and graphene oxide as nanomaterials for medicine and biology application

A thick sheet of carbon atoms connected by sp2 hybridization and organised in a hexagonal arrangement compose graphene. Due to its unique electrochemical features, which include high thermal conductivity, high current, density, chemical inertness, optical transparency, and very high hydrophobicity, it can be utilised in numerous fields (S. Priyadarsini et al., 2018). Members of the graphene family include reduced graphene oxide (rGO), graphene oxide (GO), graphene sheets and layered graphenes, including a few stacked graphenes and multilayered graphene. Unique intrinsic physical and chemical features of GO include a large surface area, oxygen-containing functionality, enhanced conductivity, and biocompatibility (S. Priyadarsini et al., 2018). In addition, graphene possesses unique catalytic, mechanical, electrical, thermal, biological, and optical properties that make it applicable for biomolecule recognition, bioassays, molecular medicine, and small molecule drug delivery. Because GO and rGO are used in osteogenic stem cells to research chondrogenesis, adipogenesis, epithelial genesis, myogenesis, cardiomyogenesis, and neurogenesis, graphene-based materials are also utilised in the field of bone repair or organ regeneration (S. Priyadarsini et al., 2018).

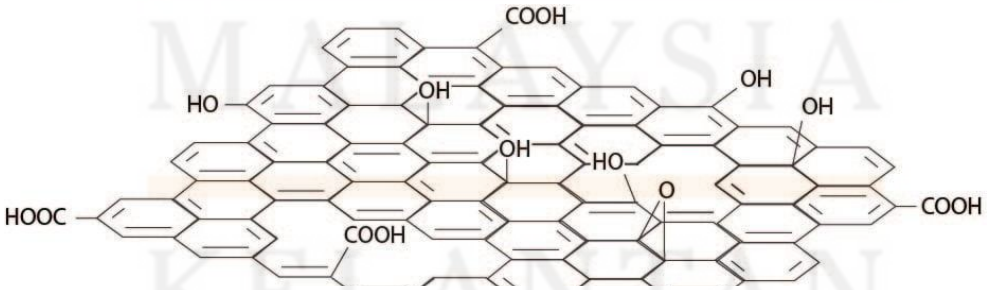


Figure 1. Graphene Oxide Structure. (n.d.-b). BiolinScientific.https://www.biolinscientific.com/blog/what-is-graphene-oxide

6.4 The antifungal activity of graphene oxide-silver nanocomposites

Antibacterial activity based on graphene oxide (GO) nanocomposites has significant potential in modern medicine. Carbon nanoscrolls (CNSs) loaded with silver nanoparticles (AgNPs) can increase antifungal activity in this study (C. Li et al., 2013). Sonication was used to generate nanoscrolls filled with silver nanoparticles, and transmission electron microscopy revealed that AgNPs filled with nanocomposites demonstrated excellent antifungal activity against *Candida albicans* and *Candida tropicalis* (C. Li et al., 2013). Thus, carbon nanoscrolls comprised of graphene oxides and silver nanoparticles can increase and prolong antifungal activity and have a significant influence on nosocomial infections in hospitals and local antifungal therapy (C. Li et al., 2013).

6.5 Mardin-Darby Canine Kidney Cells

MDCK are generated from a normal dog kidney (McRoberts et al., 1981). This cultured cell retained many of the differentiated characteristics of kidney epithelial cells, including the asymmetric distribution of enzymes and the vectorial transport of salt and water from the apical to the basolateral sides. (K. Simsons & H. Virta, 2006). The latter results in the formation of "domes" or "blisters" in cultures, which are temporary regions where collected fluid has caused the monolayer to split from the substratum. Morphologically, the cells are cuboidal with microvilli on the apical side (K. Simsons & H. Virta, 2006). Strain I cells are derived from a low-passage MDCK cell pool, while Strain II cells are derived from a monolayer with a lower resistance of 100-200 ohm. MDCK strain I cells are preferred due of their elevated electrical resistance.

7.0 IMPORTANCE OF EXPECTED RESEARCH FINDINGS

The research finding will provide information on graphene oxide activities against *Aspergillus versicolor*. If the compound is effective, this information will be useful for future development of GO as an alternative against treatment particularly in animal.

8.0 MATERIALS AND METHODS

8.1 Graphene Oxide (GO)

Graphene oxide (GO) was purchased from a company in Malaysia known as GO Advanced Solutions Sdn. Bhd. In order to prepare the stock, GO was first dehydrated, then weighed, and then reconstituted using water. After dispersing GO nanoparticles in water, which serves as a dispersing medium, and then sonicating the suspension for one hour with probe sonication at a strength of 50% in order to break down the GO to a monolayer sheet, GO was produced at various concentrations. This was accomplished by first breaking down the GO to a monolayer sheet.

8.2 Cultivation of *Aspergillus versicolor*

The *Aspergillus versicolor* stock was obtained from the bacteriology laboratory which has preserved fungi stock in a 50mL falcon tube. The fungi stock is in agar form, and it was placed on fresh Sabouraud dextrose agar (SDA) to revive it in an incubator at 30°C for 3 to 5 days. The inoculum suspensions was prepared in a yeast extract peptone dextrose (YPD) broth. Fungi colony was taken from the SDA using inoculation loop in a biological safety cabinet and the colony was placed in a universal bottle containing 10 mL of YPD broth. The inoculum was placed in an incubator shaker at 30°C, 200rpm for 24 hours. The process gave us the target inoculum size with range of $1.0 \times 10^6 – 5.0 \times 10^6$ spores/mL.

8.3 Cultivation of mammalian cells

Mardin-Darby canine kidney (MDCK) cells were used in this experiment. A vial of cells kept at -80°C was gently stirred in a water bath heated to 37°C before use. The contents of the vial were transferred to a centrifuge tube filled with 9 mL of pre-warmed complete medium and centrifuged at 1000 pm for 5 minutes. To ensure that all traces of DMSO were completely removed, the supernatant was discarded. The cell pellet was reconstituted in 5 mL of pre-warmed complete medium before being added to a 10 mL T75 culture flask filled with pre-warmed full medium. For CO_2 incubation, cells were maintained at 37°C.

Cells were divided and passaged when they were 80–100% confluent. An inverted microscope was used to measure the cell confluency (Olympus, Tokyo, Japan). Before cell passage, the growth medium in the flask was discarded, and the cells were twice washed with sterile phosphate-buffered saline (PBS) to remove cell debris. Then, cells were placed in 4 ml of a 0.5 mg/ml trypsin-EDTA solution and incubated for 5 minutes at 37°C (Sigma-Aldrich, Cambridge, United Kingdom). By including 10 ml of growth medium, trypsin was rendered inactive. The cells continued to divide in a new flask, providing us with more cells to study.

8.4 Microscopic assessment of cell concentration

In order to count the cells, we mixed them with a trypan blue dye solution and then placed them on a hemocytometer for examination under a microscope. C equals cell concentration (cells/ml), Av equals an average number of cells counted in four different corners, and two* equals dilution factor. Cells were measured using the formula C= Av 2 x 10^4 cells ml.

8.5 Preservation of the cell growth

They were then detached from the flask using the previously described trypsinization method to preserve the cells. 5 minutes of centrifugation at 1,000 rpm was used to transfer the

cell suspension into a tube. The pellet was resuspended in media (90% FBS, 10% DMSO) at a concentration of 5x10^6 cells/ml. In this study, DMSO was used as a cryoprotective drug to protect cells from damage caused by extreme cold. The suspensions were stored overnight in cryovials (Fisher Scientific, Loughborough, United Kingdom) marked with the date and passage number in freezers maintained at -80°C. For long-term storage, the cryovials were transported to a cryogenic freezer containing liquid nitrogen at -150°C.

8.6 Experimental Design

8.6.1 Resazurin assay

MDCK cells (1.8 x 105 cells/well) were put to a 96-well plate and cultivated for 24 hours at 37°C with decreasing concentrations of GO. As controls, only untreated cells and media were used. Resazurin sodium salt (Sigma-Aldrich, UK) was produced as a 544 µM stock solution in 50 mL PBS, and 50 µL of the stock solution was applied to each well. The plate was then incubated for a further 24 hours. The optical density was then measured at 550 nm and 630 nm using a microplate reader (BMG Labtech 96). The change in OD value (or % dye reduction) is proportional to the number of viable cells.

8.6.2 Disk Diffusion Method

SDA was prepared and four wells were made using Cork borer. The fungi inoculum was prepared into stock solution and adjusted to approximately 0.5 to 1.0 McFarland standard. Then, 100 µL of the stock solution were placed onto the centre surface of the SDA and spread evenly using sterile cotton swab. GO solution was produced at different concentrations (400µg/mL, 200µg/mL, 100µg/mL, 50µg/mL). 50µL of each concentration was placed in its respective well of the SDA. The agars were then incubated at 30°C for 24 hours. The fungal growth was observed.

8.6.1 Statistical Analysis

A descriptive statistic was used to explore the data obtained from the absorbance value for 550 nm and 630 nm wavelengths to determine the mean. The test was performed using the SPSS Version 27 software.

9.0 RESULTS

9.1 Antifungal activities of Graphene Oxide against *Aspergillus Versicolor*

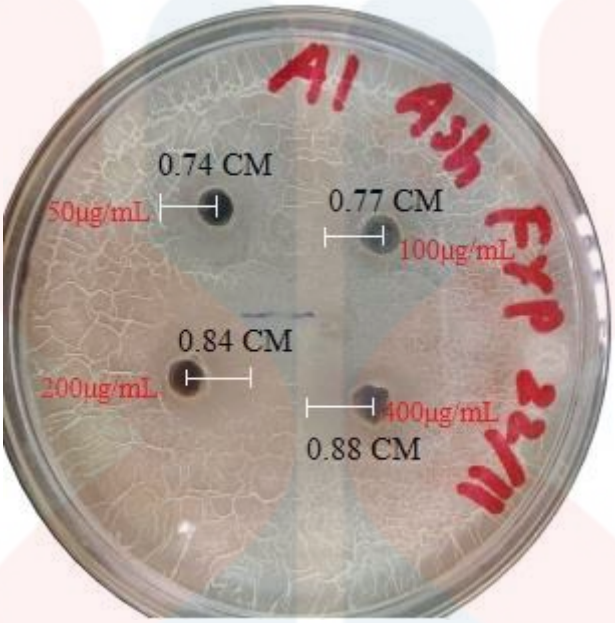


Figure 2 Zone of Inhibition *Aspergillus versicolor* on Sabouraud Dextrose Agar (SDA) treated with Graphene Oxide after 24 hours

Aspergillus versicolor was plated on SDA and increasing concentration of GO was added to the wells created on the agar. To measure the antifungal activities, diameter zone of inhibition was observed and measured using microsoft paint ruler in centimeter. There is an increase in diameter zone of inhibition as the GO concentration increase (Figure 2).

9.2 Graphene Oxide impact on the mammalian cells growth

Cells exposed to different concentration of graphene oxides between 100-400 µg/ml showed increase in the cell growth compared to the non-treated cells, suggesting the ability of compound to promote cells growth (Figure 3).

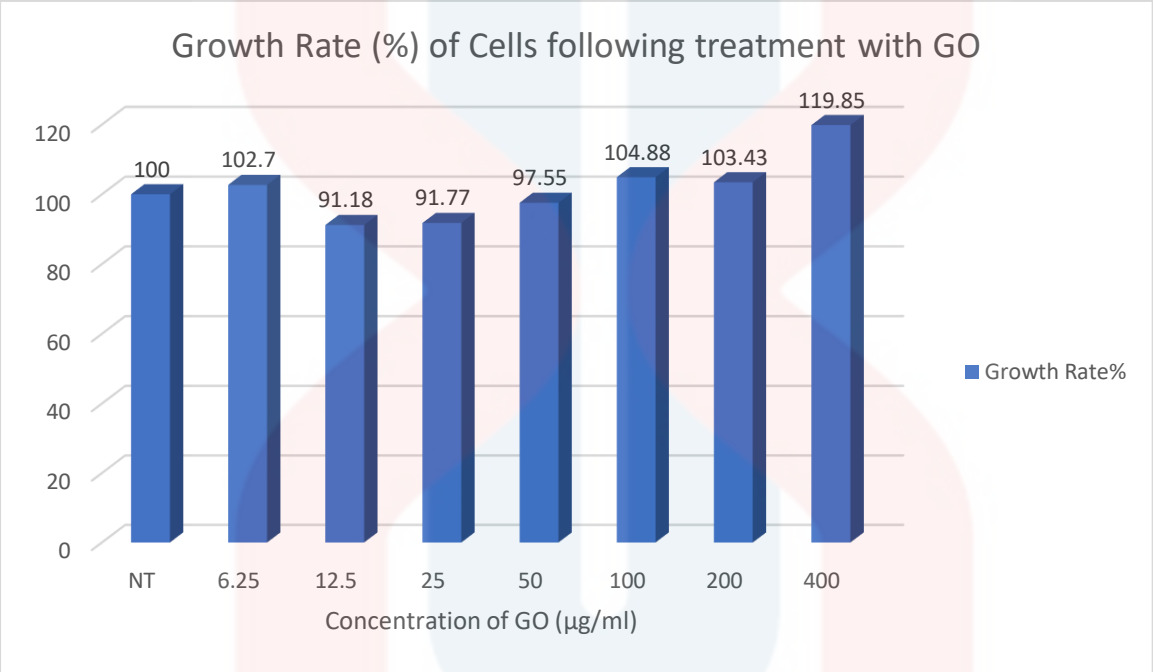


Figure 3 Growth rate of cells following treatment with Graphene Oxide

Figure 3 shows the data on growth rate of cells following treatment with GO after 48 hours. The horizontal x-axis represents different concentrations of graphene oxide in µg/ml, while the vertical y-axis represents growth rate of cells in percentage (%). The bar graph shows GO can induce cell proliferation and inhibit cell growth at different concentrations.

10.0 DISCUSSION

This study was done to investigate the antifungal properties of graphene oxide and its toxicity effect on mammalian cells. In this experiment, we used MDCK cell. The study shows that there is presence of antifungal properties of GO within concentration 400µg/mL to 50µg/mL. Based on the results, inhibition zones were observed and measured for every respective concentration. Graphene oxide at the concentration of 400µg/mL shows the biggest diameter inhibition zone at 0.88cm whereas GO at the concentration of 50µg/mL shows the smallest diameter inhibition zone at 0.74cm. Meaning that, the graphene oxide does inhibit fungal growth at 400µg/mL to 50µg/mL of concentrations.

Graphene oxide can destroy the cell membrane of the fungi because of its tremendous mechanical strength and sharp edge, Graphene may also directly damage bacteria's cell membrane by altering its osmotic pressure and causing DNA and RNA to leak out. This study proves that GO have the antifungal properties because there is presence of diameter zone of inhibition when in contact with *Aspergillus versicolor*. Moreover, the diameter of inhibition zone increased as the GO concentration increase. In this study, the zone of inhibition hardly can be measured because the GO may not be uniformly diffuse unto the agar. Thus, other method such as microbroth dilution can be used to allow the GO to be optimized. Also, we can increase the GO concentrations more than 400µg/mL to observe the zone of inhibition more prominently.

The toxicity effect of graphene oxide on mammalian cells in this experiment shows that treated cells with graphene oxide can induce increased rate in cell proliferation. Based on the results, at 400µg/mL concentrations of GO, we can observe the growth rate is 119.85% which increased 19.85% more than the non-treated cells (100%). However, at concentrations of the graphene oxide such as 12.5µg/mL, there is decrease of growth rate from 100% of non-treated

cells to 91.18%. This indicate that graphene oxide has toxic effect on mammalian cells but at certain concentrations may induce cell proliferation. Graphene oxide can induce cell death through DNA damage (Gurunathan et al., 2019). This mechanism reduced nutrition absorption, induce oxidative stress and trigger the cell to self-destruct. On the other hand, it can also induce cell proliferation because it can modify protein adsorption in the extracellular matrix, which improves cell adhesion and proliferation (K. Zhang et al., 2016). If the concentrations of the graphene oxide are used more than 400µg/mL, it may show us the accurate impact of graphene oxide on mammalian cells.

11.0 CONCLUSION

In conclusion, Graphene Oxide shows antifungal activities against *Aspergillus versicolor in-vitro*. Therefore, Graphene Oxide can be potentially used for further study its antifungal properties for treatment especially in animal. Also, Graphene Oxide is less toxic to mammalian cells as it can induce cell proliferation. However, it is dose-dependent because at some point of concentrations, GO can inhibit cell proliferation.

12.0 RECOMMENDATIONS AND FUTURE WORK

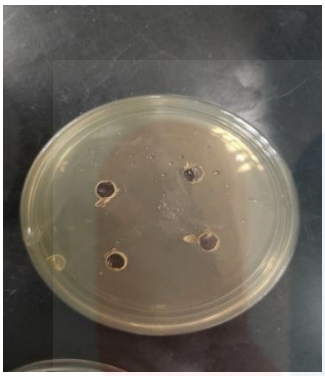
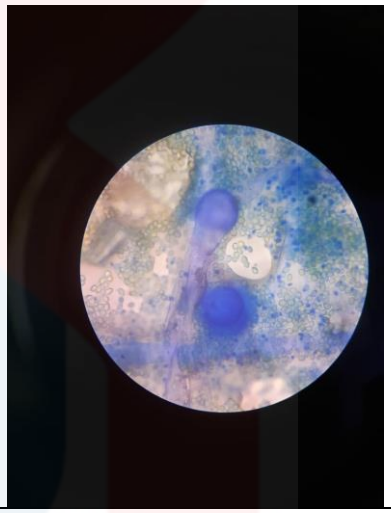
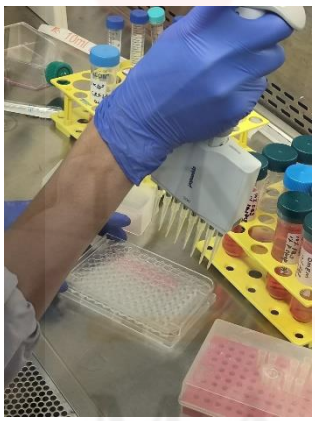
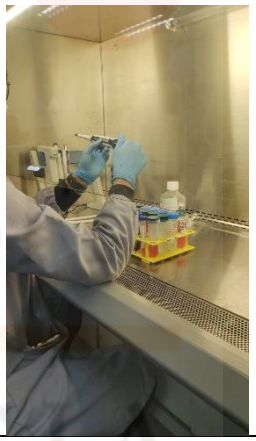
Several limitations were observed during this project. Other cell culture growth can be used to expand the knowledge of cytotoxic effect of Graphene Oxide towards other cells. The cytotoxicity test can be done to evaluate and test the cell growth, reproduction and morphological effects by medical devices. We can use larger range of Graphene Oxide concentrations to get a bigger picture of the results. This can apply the same for fungicidal effect of Graphene Oxide. Disk diffusion method may not be superior because the GO may not diffuse properly to the disk. Other method such as microbroth dilution method can increase the likelihood of the Graphene Oxide to be more effective.

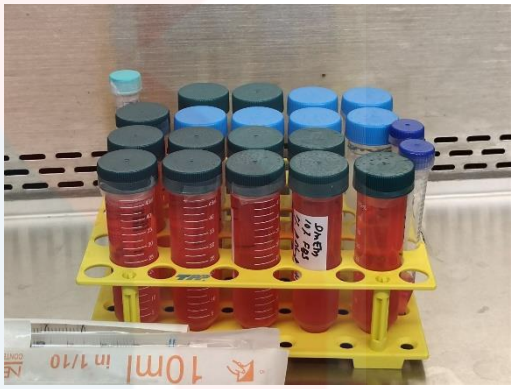
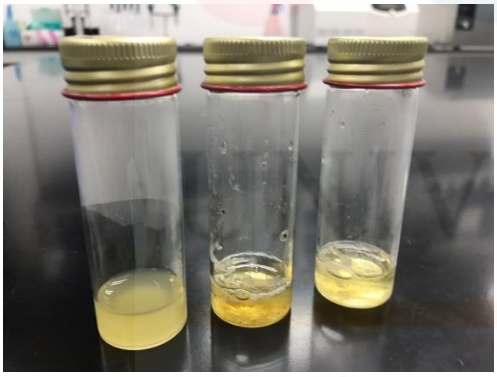
APPENDICES

Table 1 Absorbance Value of Mardin-Darby Canine Kidney cells treated with Graphene Oxide after 48 hours

Graphene Oxide (µg/ml)	Net Value (nm)	Cell Growth (%)
NT	0.408	100
400	0.489	119.85
200	0.422	103.43
100	0.427	104.66
50	0.398	97.55
25	0.374	91.77
12.5	0.372	91.18
6.25	0.419	102.70

Table 1 shows the absorbance value of mammalian cells after being treated with graphene oxide after 48 hours. The absorbance value shows the vitality of the cells. Absorbance values that are less than those of the control cells suggest a slower rate of cell proliferation. Alternatively, a greater absorbance value suggests a rise in cell proliferation.

REFERENCES

1. Asadi Shahi, S., Roudbar Mohammadi, S., Roudbary, M., & Delavari, H. (2019). A new formulation of graphene oxide/fluconazole compound as a promising agent against Candida albicans. Progress in Biomaterials, 8(1), 43–50. https://doi.org/10.1007/s40204-019-0109-6

2. Desoubeaux, G., & Cray, C. (2018). Animal Models of Aspergillosis. Comparative medicine, 68(2), 109–123.

3. Enoch, D. A., Yang, H., Aliyu, S. H., & Micallef, C. (2016). The Changing Epidemiology of Invasive Fungal Infections. Methods in Molecular Biology, 17– 65. https://doi.org/10.1007/978-1-4939-6515-1_2

4. Gurunathan, S., Arsalan Iqbal, M., Qasim, M., Park, C. H., Yoo, H., Hwang, J. H., Uhm, S. J., Song, H., Park, C., Do, J. T., Choi, Y., Kim, J. H., & Hong, K. (2019). Evaluation of Graphene Oxide Induced Cellular Toxicity and Transcriptome Analysis in Human Embryonic Kidney Cells. *Nanomaterials*, *9*(7), 969. https://doi.org/10.3390/nano9070969

5. Hussein, K. H., Abdelhamid, H. N., Zou, X., & Woo, H. M. (2019). Ultrasonicated graphene oxide enhances bone and skin wound regeneration. *Materials Science and Engineering: C*, *94*, 484–492. https://doi.org/10.1016/j.msec.2018.09.051

6. Li, C., Wang, X., Chen, F., Zhang, C., Zhi, X., Wang, K., & Cui, D. (2013). The antifungal activity of graphene oxide–silver nanocomposites. Biomaterials, 34(15), 3882–3890. https://doi.org/10.1016/j.biomaterials.2013.02.001

7. Nguyen, H. N., Chaves-Lopez, C., Oliveira, R. C., Paparella, A., & Rodrigues, D. F. (2019a). Cellular and metabolic approaches to investigate the effects of graphene and graphene oxide in the fungi *Aspergillus flavus* and *Aspergillus niger*. Carbon, 143, 419–429. https://doi.org/10.1016/j.carbon.2018.10.099

8. Priyadarsini, S., Mohanty, S., Mukherjee, S., Basu, S., & Mishra, M. (2018). Graphene and graphene oxide as nanomaterials for medicine and biology application. Journal of Nanostructure in Chemistry, 8(2), 123–137. https://doi.org/10.1007/s40097-018-0265-6

9. Rodriguez-Tudela, J., Arendrup, M., Barchiesi, F., Bille, J., Chryssanthou, E., Cuenca-Estrella, M., Dannaoui, E., Denning, D., Donnelly, J., Dromer, F., Fegeler, W., Lass-Flörl, C., Moore, C., Richardson, M., Sandven, P., Velegraki, A., & Verweij, P. (2008). EUCAST Definitive Document EDef 7.1: method for the determination of broth dilution MICs of antifungal agents for fermentative yeasts. Clinical Microbiology and Infection, 14(4), 398–405. https://doi.org/10.1111/j.1469-0691.2007.01935.x

10. Shakhatreh, M. A., Al-Smadi, M. L., Khabour, O. F., Shuaibu, F. A., Hussein, E. I., & Alzoubi, K. H. (2016). Study of the antibacterial and antifungal activities of synthetic benzyl bromides, ketones, and corresponding chalcone derivatives. *Drug Design, Development and Therapy*, *Volume 10*, 3653–3660. https://doi.org/10.2147/dddt.s116312

11. SIMONS, K., & VIRTA, H. (2006). Growing Madin-Darby Canine Kidney Cells for Studying Epithelial Cell Biology. Cell Biology, 127–131. https://doi.org/10.1016/b978-012164730-8/50016-2

12. Viegas, C., Dias, M., Carolino, E., & Sabino, R. (2020). Culture Media and Sampling Collection Method for Aspergillus spp. Assessment: Tackling the Gap between Recommendations and the Scientific Evidence. Atmosphere, 12(1), 23. https://doi.org/10.3390/atmos12010023

13. Wang, X., Peng, F., Cheng, C., Chen, L., Shi, X., Gao, X., & Li, J. (2021). Synergistic Antifungal Activity of Graphene Oxide and Fungicides against Fusarium Head Blight In Vitro and In Vivo. Nanomaterials, 11(9), 2393. https://doi.org/10.3390/nano11092393 12

14. Zhang, K., Zheng, H., Liang, S., & Gao, C. (2016). Aligned PLLA nanofibrous scaffolds coated with graphene oxide for promoting neural cell growth. *Acta Biomaterialia*, *37*, 131–142. https://doi.org/10.1016/j.actbio.2016.04.008

15. Zhang, S., Corapi, W., Quist, E., Griffin, S., & Zhang, M. (2012b). Aspergillus versicolor, a New Causative Agent of Canine Disseminated Aspergillosis. *Journal of Clinical Microbiology*, *50*(1), 187–191. https://doi.org/10.1128/jcm.05388-11