IN-VITRO FUNGICIDAL ACTIVITIES OF GRAPHENE OXIDE TOWARDS *CANDIDA ALBICANS*

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(D18B0046)

A RESEARCH PAPER SUBMITTED TO FACULTY OF VETERINARY MEDICINE,
UNIVERSITI MALAYSIA KELANTAN
IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF
DOCTOR OF VETERINARY MEDICINE

FEBRUARI 2023

UNIVERSITI MALAYSIA KELANTAN

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CERTIFICATION

This is to certify that we have read this study entitled "***In-Vitro*** **Fungicidal Effects of Graphene Oxide Against *Candida albicans***" by **Syuhada binti Mat Salleh.**, and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfillment of the requirement of the course DVT 55204 - Research Project.

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ACKNOWLEDGMENT

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

Dr. Siti Nor Azizah binti Mahamud

Dr. Nor Fadhilah binti Kamaruzzaman

Lab assistants of FPV UMK

Family

DVM class of 2018/2023

Thank You

DEDICATION

Praise to God upon His permission, I was able to finish my final year project. I would like to express my sincere gratitude to a number of people for their kindness and patience to give a helping hand during my final year project. Without them, I would not have succeeded in doing my research period and gained much experience and knowledge related to veterinary practices.

I would like to express my utmost gratitude to my supervisor, Dr Siti Nor Azizah binti Mahamud for guiding me to do my research project for and also for her boundless knowledge regarding the *Candida albicans* and willingness to support during the final year project research period. Also, thank you to my co-supervisor, Dr Nor Fadhilah binti Kamaruzaman for helping me and teaching me knowledge on *Candida albicans* and also for her illimitable guidance regarding the laboratory works.

Throughout this training, I am very honored to have Cik Nani Izreen binti Mohd Sani and Puan Norsyamimi Farhana binti Mat Kamir , the laboratory assistants for virology and bacteriology lab for their encouragement and guidance from time to time during the research period. Also, I am very thankful to my teammates, Muhammad Shahin and Ashyer Andderias for their supports and advice during the research period and also giving moral support for me to apply my skills and knowledge during the laboratory works.

I also would like to express my sincere thanks to my family who gave me endless support for me to finish my project successfully. It brings a lot of challenging and valuable experiences for me throughout my research period. Lastly, thank you so much to all my friends for being supportive and kindly giving me a lot of assistance and caring about me during our internship.

Finally, I would love to thank Azwan Izhar, Abid, Aishah, and the rest of DVM class of 2018/2023 for their countless moral support during the final year research project period. Thank you.

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ABSTRACT

An abstract of the research paper was presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement for the course DVT 55402 – Research Project.

Candidiasis is an opportunistic mycotic disease of the digestive tract infecting many avian species. Candidiasis is caused by fungal yeast *Candida albicans (C. albicans)* ubiquitously in the environment. Nowadays, *Candida albicans* become one of the emerging multidrug-resistant fungal pathogens which infect various avian species, resulting in stunted growth, diarrhea and multiple cutaneous scabs. The treatment for candidiasis includes administration of antifungal drugs, which has been partially successful due to development of resistance. Thus, it is important to investigate for an alternative treatment particularly for improvement of overall animal health. This research aimed to determine the fungicidal effects of graphene oxide against *Candida albicans* in vitro and to determine the toxicity effects of graphene oxide against Feline Kidney (CRFK) cells *in-vitro*. The study's demonstrated that graphene oxide at maximum concentration of 400 mg/mL did not exhibit antifungal activities against *Candida albicans*. Also, the compound also did not show any toxicity effect towards mammalian cells. In conclusion, graphene oxide was not effective at the concentration tested and may not be useful as antifungal against *Candida albicans*.

Keywords: Graphene oxide, *Candida albicans*, Candidiasis, CRFK cells

ABSTRAK

Abstrak daripada kertas penyelidikan dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan untuk memenuhi sebahagian daripada keperluan kursus DVT 55402 – Projek Penyelidikan.

Kandidiasis adalah jangkitan oportunistik di bahagian saluran pencernaan yang menjangkiti pelbagai spesies burung. Kandidiasis adalah disebabkan oleh yis fungi iaitu *Candida albicans*, yang ada di mana-mana persekitaran. Pada masa kini, *Candida albicans* menjadi salah satu patogen kulat yang tahan dengan pelbagai ubat dan boleh menjangkiti pelbagai spesies burung dan boleh mengakibatkan pertumbuhan terbantut, cirit-birit dan mengakibatkan kudis-kudis kulit. Rawatan bagi penyakit kandidiasis adalah dengan pemberian ubat antikulat yang kurang berkesan disebabkan oleh perkembangan rintangan antikulat Oleh itu, adalah penting untuk mengkaji rawatan alternatif terutamanya untuk haiwan. Kajian ini bertujuan untuk menentukan kesan fungisida graphene oksida terhadap *Candida albicans* dalam vitro dan untuk menentukan kesan ketoksikan graphene oksida terhadap sel buah pinggang kucing (CRFK) dalam vitro. Dalam kajian ini, graphene oksida dengan kepekatan maksimum 400 mg/ml tidak menunjukkan aktiviti antikulat terhadap *Candida albicans.* Selain itu, graphene oksida juga tidak menunjukkan sebarang kesan toksik terhadap sel mammal. Kesimpulannya, graphene oksida adalah tidak efektif menggunakan kepekatan yang diaplikasikan dan mungkin tidak berkesan terhadap *Candida albicans.*

Kata kunci: graphene oksida, *Candida albicans*, Kandidiasis, CRFK cells

1.0 INTRODUCTION

Candida albicans is an opportunistic, yeast-like fungus that causes Candidiasis in poultry (Kabir et al., 2012). It often causes "sour crop" in which the chicken crop's unable to empty causing the feed to undergo fermentation. The crop mycosis is transmitted via spoiled feed and contaminated water (Penn State Extension, 2020). *C albicans*, which is the normal gastrointestinal microflora, will then overgrow in the favorable environment and lead to yeast infection (Smith, 2020). Several risk factors that predispose to Candidiasis and exacerbate the disease include vitamin D deficiency, malnutrition, immunosuppressive diseases, poor hygiene, and prolonged usage of antibiotics (Smith, 2020). Recent finding showed Candidiasis commonly found in flock of chickens given antibiotics for long periods, indirectly kill the normal bacteria in the gastrointestinal, causing excessive growth of *C. albicans*, leading to the yeast infection (Smith, 2020). In the consequences of the disease, affected poultry will have diarrhea, appear thin, listless and disheveled. Gauthier & Ludlow (2021) explained the susceptibility of young, old, and sick birds towards Candidiasis. *C. albicans* can also cause Candidiasis in human and causes red patches and itchiness of the skin. In severe cases, the yeast may invade the bloodstream and cause candidemia, which leads to systemic infections and causes death if not promptly treated (Revankar, 2021). Grossly, Candidiasis causes whitish, raised patches of ulceration that is known as "turkish-towel" appearance in the mucosa and pseudomembrane of the crop, esophagus and pharynx (Bauck, 1994; Schmidt et al., 2003). There are reports showing the emergence of antifungal resistance towards common antifungal agents that are used to treat Candidiasis such as fluconazole and amphotericin B (Sokół et al., 2020). Based on the reports, E-test methods are done on isolates from the poultry's' intestinal tracts to check the susceptibility of antifungal such as amphotericin B, fluconazole and itraconazole in which it proved that *C. albicans* showed lower susceptibility towards the antifungal (Sokół et al., 2020). Application of carbon nanoparticles allow potential solution as a new alternative for antifungal treatment for Candidiasis. One example of carbon nanoparticles is graphene oxide. For this study, graphene oxide is chosen as an alternative antifungal agent to treat Candidiasis.

Graphene oxide has been used in hemostasis and cell healing purposes (Lu et al., 2012). These nanoparticles also demonstrate the ability to prevent bacterial growth and also causes strong toxicity toward bacteria such as Klebsiella pneumoniae, Pseudomonas aeruginosa and Escherichia coli (Liu et al., 2011; Wu et.,al, 2017). Graphene oxide has potential to be the new alternative as antifungal agent as it has such a large surface area and good superior mechanical tenacity (He et.,al, 2012). Apart from that, graphene oxide is inexpensive and can create regeneration for the lesions caused by infectious agents, thus making it suitable as the possible alternative as an antifungal agent.

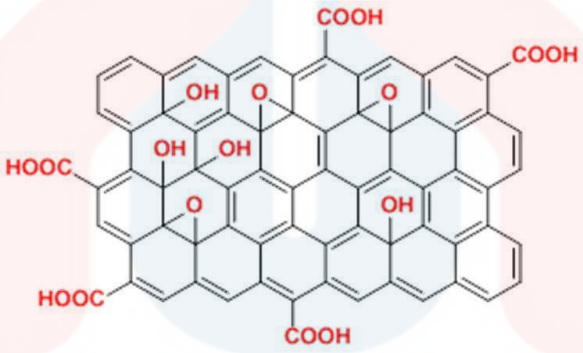


Figure 1 Graphene oxide chemical structure

2.0 RESEARCH PROBLEM

Nowadays, *C. albicans* become one of the emerging multidrug-resistant fungal pathogens which may infect hosts such as chickens, turkey, quails and even humans. The emergence of the antifungal resistance towards *C. albicans,* in part, has caused failure in treatment in infected poultry. There are reported research that show evidence that antifungal such as Fluconazole and Amphotericin B that commonly used to treat candidiasis already showed decreased activity against *C. albicans*. Therefore, it is important to find alternative to existing therapy. This study aims to determine the antifungal activities of graphene oxide, a carbon-based nanoparticles against *C. albicans in-vitro*.

3.0 RESEARCH QUESTIONS

3.1 Can graphene oxide kill or inhibit the growth of Candida albicans?

3.2 Can graphene oxide cause toxicity effect towards mammalian cells?

4.0 RESEARCH HYPOTHESIS

4.1 Graphene oxide can kill or inhibit the growth of *Candida albicans in-vitro*

4.2 Graphene oxide can demonstrate a low toxicity effect towards mammalian cells

5.0 RESEARCH OBJECTIVES

5.1 To determine the fungicidal effects of graphene oxide against *Candida albicans* in vitro.

5.2 To determine the toxicity effects of graphene oxide against mammalian cells in vitro.

6.0 LITERATURE REVIEW

6.1 *Candida albicans*

Candida albicans is the opportunistic, normal digestive microflora in poultry, which can overgrow to cause candidiasis in poultry (Kabir et al., 2012). Among all *Candida* species, *Candida albicans* is the most significant species that causes yeast infection to poultry. *Candida albicans* can be differentiated from other Candida species by the presence of germ tubes in serum-free yeast extract peptone dextrose medium (Kim et al., 2002). The transmission of *C. albicans* mainly occurs via fecal contaminated feed and water. *C. albicans* possess virulence factors such as aspartyl proteinase which causes degradation of protein in the extracellular matrix and causes phagocytosis inhibition and induces inflammatory reaction (Calderone & Fonzi, 2001). Additionally, hemolysin is also one of the virulence factors which degrades erythrocytes and releases iron to be consumed by *C. albicans* (Sumaya et al., 2020 ; Chute, 2001). The fungus also has hyphae or pseudohyphae which facilitate vascular invasion and lead to systemic infection. The yeast infection leads to formation of raised, whitish pseudo membranes and causes presence of cheesy-like materials in mainly crops, but less likely on pharynx and esophagus (Nicholds., 2021).

6.2 Candidiasis in poultry

Approximately 200 species of Candida known, which *Candida albicans* is the most isolated species from poultry. In a study, about 163 species of the genus Candida can be found in various habitats (Singh, Gurjeet & Raksha, & Urhekar, A. ; 2013). Over the past decades, the prevalence of both albicans and other species of Candida has considerably increased, which it also infects human and causes candidemia to human of various ages (Cortés & Corrales, 2018). There is also difference in susceptibility of *C. albicans* towards various species. Based on studies, turkeys are highly susceptible to infections caused by
C. albicans compared to chickens that are more resistant towards the fungi (Wyatt, R &

Hamilton, P. ,1975). Candidiasis can affects the poultry industry as this fungi causes delay in crop emptying and cause reduced appetite in the poultry, proving that it lead to reduced feed conversion ratio in poultry.

6.3 Mutli-drug resistance of *Candida albicans*

Recently, there are research papers established regarding the development of resistance of *Candida* species towards the antifungal drugs. Fluconazole and other azole antifungals are frequently chosen over other treatments for various *Candida* infections due to their low cost, low toxicity, and can be administered orally (Whaley et al., 2017). The presence of point mutations in ERG11 have been discovered as one resistance mechanism in *Candida albicans*. Previous research has revealed substitutions for amino acids that reduce fluconazole susceptibility (Marichal et al., 1999). Another research also stated that increased expression of ERG11 as a result of activating mutations in the gene encoding the zinc-cluster transcriptional regulator Upc2p is another route of fluconazole resistance in *Candida albicans* (MacPherson et al., 2005). This is the epidemiological data obtained from an article in which they showed resistance towards azole and amphotericin B for *C. albicans* isolated from feces , eggs and cloacae from laying hens (Rhimi et al., 2020).

Antifungal agents	Group A: cloacal swab		Group B: pool of faeces		Group C: eggs	
	Number of isolates (%)		Number of isolates (%)		Number of isolates (%)	
	C. albicans	*C. catenulata*	*C. albicans*	*C. catenulata*	*C. albicans*	*C. catenulate*
FLC	24/67 (35.8%)[a]	4/5 (80%)	10/13(76.9%)[a]	3/4 (75%)	3/4 (75%)	8/8 (100%)
ITZ	25/67 (37.3%)	3/5 (60%)	7/13 (53.8)	2/4 (50%)	3/4 (75%)	8/8 (100%)
VOR	18/67 (26.8%)	1/5 (20%)	4/13 (30.7)	1/4 (25%)	2/4 (50%)	1/8 (12.5%)
POS	56/67 (83.6%)	4/5 (80%)	12/13 (92.3%)	4/4 (100%)	4/4 (100%)	8/8 (100%)
MCF	4/67 (6%)[b]	0/5 (0%)	0/13 (0%)	1/4 (25%)	2/4 (50%)[b]	8/8 (100%)
ANI	0/67 (0%)	0/5 (0%)	0/13 (0%)	0/4 (0%)	0/4 (0%)	8/8 (100%)
AmB	0/67 (0%)	2/5 (40%)	0/13 (0%)	1/4 (25%)	0/4 (0%)	0/8 (0%)

Table 1 Antifungal resistance towards antifungal agents

FLC, fluconazole; ITZ, itraconazole; VOR, voriconazole; POS, posaconazole; MCF, micafungin; ANI, anidulafungin; AmB, amphotericin. Table 1 shows that *Candida albicans* and *Candida catenulata* are resistant to at least one antifungal drug tested.

6.4 Graphene oxide

Graphene oxide is a nanoparticle that is made up of carbon atoms with carboxyl, carbonyl and hydroxyl groups at the edges (Aliyev et al., 2019). It is a nano-sized carbon fiber that has excellent in vitro biocompatibility and unexcelled bio-physio-chemical properties (Sydlik et al., 2015). Research has shown that graphene oxide nanosheets have *in vitro* interaction with cell cultures and parasites such as malaria, with evidence of the nanoparticles obstructing the parasite from invading erythrocytes (Kenry et al., 2017). According to the previous report, graphene oxide shows antibacterial activity and causes toxicity towards bacteria such as *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Escherichia coli* (Wu et.,al, 2017). However, in certain concentrations, graphene oxide is proved to be highly thrombogenic and causes aggregatory response in platelets, which causes limitation in biomedical applications (Singh et al., 2012).

6.5 Mammalian cell culture

Mammalian cell culture is an approach in which cells from mammals are grown in vitro. It is a technique in which cells are grown outside of their primary tissue.(Sigma Aldrich, 2021). For this study, graphene derivatives are used as the culture medium for selected mammalian cells, which are the feline kidney cells (CRFK). In research, feline kidney cells (CRFK) have been used as a tool for development of the plaque assay for Feline calicivirus (FCV) (Bidawid et al., 2003). The plaque assay provides advantages in which the cultured cells can be stored for a long time and are more economical compared to using other mammalian cells. There are also researches in which interstitial nephritis and feline morbillivirus in cats that are inoculated with feline kidney cells (CRFK) to check for presence of any cytopathic effects of the cells toward the cell lines (Choi et al., 2020). For

instance, in this study, graphene oxide is used on feline kidney cells (CRFK) to determine the concentration of graphene oxide that causes toxicity towards the feline kidney cells and also the concentration of graphene oxide that can provide the growth of the CRFK kidney cells (CRFK).

7.0 MATERIALS AND METHODS

7.1 Candida albicans

In this study, wild strains of *Candida albicans* were obtained from the department of microbiology and parasitology, University Sains Malaysia (USM) Kubang Kerian, Kelantan, Malaysia.

7.2 Graphene oxide

Graphene oxide is obtained from GO Advanced Solutions Sdn Bhd. The material will be dried and weighed before being suspended in water to prepare graphene oxide suspension for the study. The suspension then is generated at various concentrations by dispersing the GO nanoparticles in water. The solution then is sonicated using probe sonication for one hour at a 50% strength to break down the GO to a monolayer sheet.

7.3 Feline kidney cells (CRFK)

Feline kidney cells (CRFK) are obtained from University Malaysia Kelantan (UMK) virology lab

7.4 Preparation of Sabouraud Dextrose Agar (SDA)

Briefly, 500 gram of Sabouraud Dextrose Agar (SDA) powder is weighed using a weighing scale (ML2001 Malaysia). SDA powder is then poured into a 1 litre conical flask and added with 1 litre of distilled water. The flask were then placed on a hot plate stirrer (Benchmark H4000-S1E, USA) until it boils. This process takes around 4 hours for the mixture to mix well. SDA media is left to cool down for a few minutes. Then, SDA media is poured into 20 petri dishes and stored in a chiller for further use.

7.5 Preparation of liquid Yeast Extract Peptone Dextrose (YEPD) broth

Briefly, 10 gram yeast extract, 20 gram peptone powder and 20 gram sucrose are mixed together in a 1 litre conical flask. The mixture is boiled using the laboratory hot plate stirrer (Benchmark H4000-S1E, USA) and then autoclaved at 121°C for 15 minutes using the laboratory autoclave (HICLAVE HV-II, Japan).

7.6 Cultivation of *Candida albicans*

Wild strains of *Candida albicans* were inoculated on prepared SDA agar and incubated at 40°C for 48 hours with presence of whitish colony growth on the agar. Next, the colony is inoculated into a universal tube that contains 5 ml sterile yeast extract peptone dextrose (YEPD) broth and mixed well using an inoculating loop. The broth is then incubated at 30°C with continuous shaking (0·7 × g min−1) overnight.

7.7 Cultivation of mammalian cells

Feline kidney cells (CRFK) are used for this study. The cells are gently thawed by stirring the vial of cells in a 37°C water bath. The vial content is sprayed with alcohol to prevent risks of contamination. The vial content then is added into a T25 flask that contains a 5 ml medium and incubated at 37°C until the cells achieve 80-100% confluency, in which an inverted microscope is used for daily observation of the cells. For CRFK cells, the cells only can achieve 80-100% confluency in around 5 to 6 days. Thus, daily change of the culture media is done by discarding the old medium and rinsing it with phosphate buffer solution (PBS) twice. The solution is then discarded, and 6 ml of the new growth medium is added into the flask and then incubated at 37°C. By supplying the cells with new nutrients, medium changes keep the cells healthy.

Cell passage or splitting is necessary to keep the cells growing exponentially. The cell passage is done when the cells in the T25 flask achieved 80-100% confluence. First, the old medium is discarded from the flask and then rinsed with PBS twice. The solution is discarded and 1 ml trypsin is added and spread evenly to detach the adherent cells from the wall of the flask. Then, the flask is incubated at 37°C for 3 to 4 minutes. The flask is observed under inverted

microscope to examine the cells. Next, 2ml new media is added

to dilute the trypsin. In the meantime, 11ml fresh media is added into a T75 flask with another 5 ml fresh media added into the old T25 flask. Both flasks are incubated for 37°C and observed for daily cell growth. Since CRKF cells are slow growth cells, two T25 flasks and two T75 flasks are used to grow the cells.

7.8 Cell counting

7.8.1 Viable cell counts using Tryphan blue dye solution

The cells from all four flasks are combined into a sterile conical tube as the stock solution. Around 50 µl of the stock is added into a sterile centrifuge tube. Then, 50 µl of Tryphan blue dye is added into the same centrifuge tube and the mixture is gently mixed.

7.8.2 Cell counting using a Hemocytometer

A hemocytometer is used to count the cells. First, the hemocytometer is wiped using 70% alcohol. A cover slip is put on the hemocytometer chamber. After that, 50 µl of the cell mixture (cells + tryphan blue dye) is added into the chamber. In order to count the cells in each chamber, the hemocytometer is subsequently put under an inverted microscope.

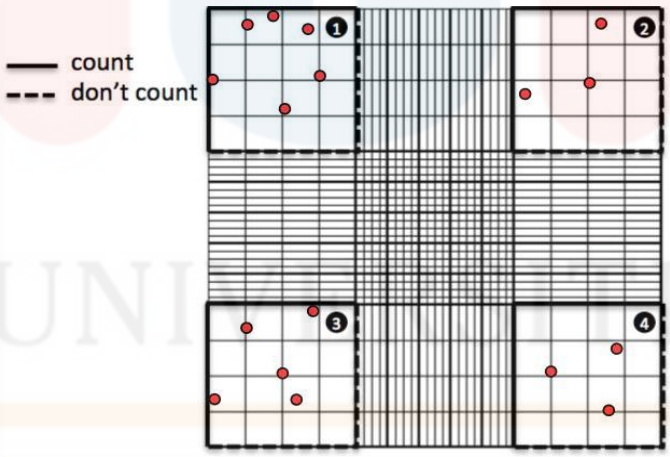


Figure 2

Number of cells on hemocytometer

Total number of cells: 6 + 5 + 3 + 3

= 17 cells

To determine the confluency of cell culture growth, this calculation is done:

a) 17 cells / 4

= 4.25 x 10^4 x 2

= 8.5 x 10^4

= 0.85 x 10^5

b) Using formula M1 x V1 = M2 x V2,

(0.85 x 10^4)(V1) = (1 x 10^5)(13 ml)

V1 = (1 x 10^5)(13 ml) / (0.85 x 10^4)

= 15 ml cell stock

7.9 Cell cytotoxicity test

7.9.1 Resazurin assay using 96-well plate

Resazurin assay is an inexpensive, rapid and sensitive test to measure the viability of cells based on the colour changes produced after a certain incubation period of time. The principle of this assay is the blue and non fluorescent resazurin is reduced to form red dye resofurin by dehydrogenase enzyme.The amount of resafurin is directly proportional to the number of living cells, thus allow the assay to detect any cytotoxic effects of cells that are treated with any drug.

Using a multi-channel micropipette, 100 µl of the cell stock are added to every well in row 1 until 9, and in column A to D only. Then, the 96 well plate is incubated at 37°C for 24 hours. For the next day, observation of the cells in the well plate is done using the inverted microscope to check the confluency of the cells. If the cells achieve 80-100% confluence, the old medium from the wells are discarded and the wells are washed with phosphate buffer solution PBS twice. After that, 100 µl new medium containing DMEM + 1% pen/strep + 10% fetal bovine serum (FBS) solution is added in each well. Using an 8- channel micropipette, the cells are treated with graphene oxide suspension of different

concentrations (6.25, 12.5, 25, 50, 100, 200, 300, 800 ug/ml). First, graphene oxide with the highest concentration; 800 ug/ml is added into the first row (A1 to D1) and mixed by gentle pipetting. Using the same tip, 100µl medium from wells of column 1 are added into column 2 (A2 through D2) to achieve graphene oxide with concentration of 400 ug/ml. The same steps are repeated down the entire column until column 8, in which the graphene oxide concentration is the lowest: 6.25 ug/ml. Column 9 (A9 through D9) are used as control and no graphene oxide added. By adding 100µl of medium to each well, the total volume of each of the wells will be 200 µl.

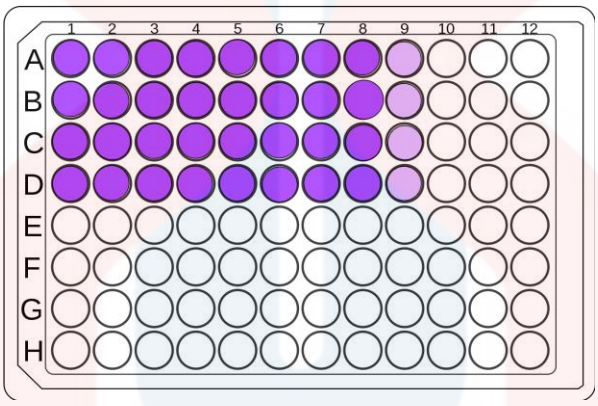


Figure 3

Serial dilutions on 96-well plates

After labeling the plate, the well-plate is incubated at 37°C for 24 hours.

The next day, the old medium from all wells are discarded one by one using the multi- channel micropipette. Then, 200 µl PBS solution is used to wash each well twice. After that, 180 µl new medium is added on each well and mixed with 20 µl resazurin dye from a mixture of 544 µl resazurin with concentration of 20.2 mg/ml + 50 ml PBS. Then, the 96 well plate is incubated for another 48 hours to observe for any colour changes. The optical density (OD) is determined using BMG LABTECH 96 microplate reader. The OD value is interpreted as the OD value is directly proportional to the number of viable cells, using wavelengths of 550 and 630 nm.

7.9.2 Antifungal sensitivity test for *Candida albicans*

Inoculum preparation: For preparation of *Candida albicans* suspension, approximately 20 µl of *Candida albicans* stock is mixed with 220 µl of YEPD broth in a sterile universal tube. The fungi inoculum suspension is adjusted according to 0.5 McFarland standard using the McFarland densitometer.

Preparation of fungal lawn on agar: For this study, Mueller-Hinton agar are used to test the antimicrobial sensitivity of *Candida albicans*. Four Muller-Hinton agar are used to obtain the average result of the experiment. The yeast suspension is inoculated on the agar using a sterile cotton swab by using the continuous streak patterns. The continuous streak pattern is done to ensure that the fungi growth is evenly distributed across the surface of the agar plate. After that, a cork borer is used to cut four holes into the agar for seeding of different concentrations of graphene oxide.

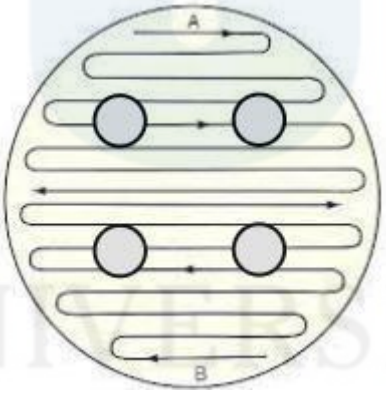


Figure 4

Inoculum preparation to test antifungal activities

Preparation of desired graphene oxide concentration: After preparing the yeast suspension, the volume of graphene oxide required to be used for antimicrobial sensitivity test is calculated using the following formula:

M1V1 = M2V2

M1 indicates concentration of graphene oxide stock V1
indicates volume taken from graphene oxide stock M2
indicates desired concentration of graphene oxide
V2 indicates final volume taken to seed graphene oxide

Based on the formula, the final volume taken to seed graphene oxide into Mueller-Hinton agar plate is 50 µl for each well. The concentration of graphene oxide used for the antimicrobial sensitivity test is 12.2 mg/ml, meanwhile the desired graphene oxide concentrations are 50, 100. 200 and 400 mg/ml. This calculation allows to determine the desired volume of graphene oxide stock and sterile distilled water for seeding on Mueller- Hinton agar plate. Finally, 50 µl of the final volume calculated to seed graphene oxide are added in every hole on the agar. After that, the agar plates are incubated at 40°C for 48 hours to observe for any presence of zones of inhibition of *Candida albicans* growth.

8.0 RESULT

8.1 Antifungal activities

Candida albicans were exposed to increasing concentrations of GO and zone of inhibition were measured. Following exposure, no zone of inhibition was measured at all concentration tested

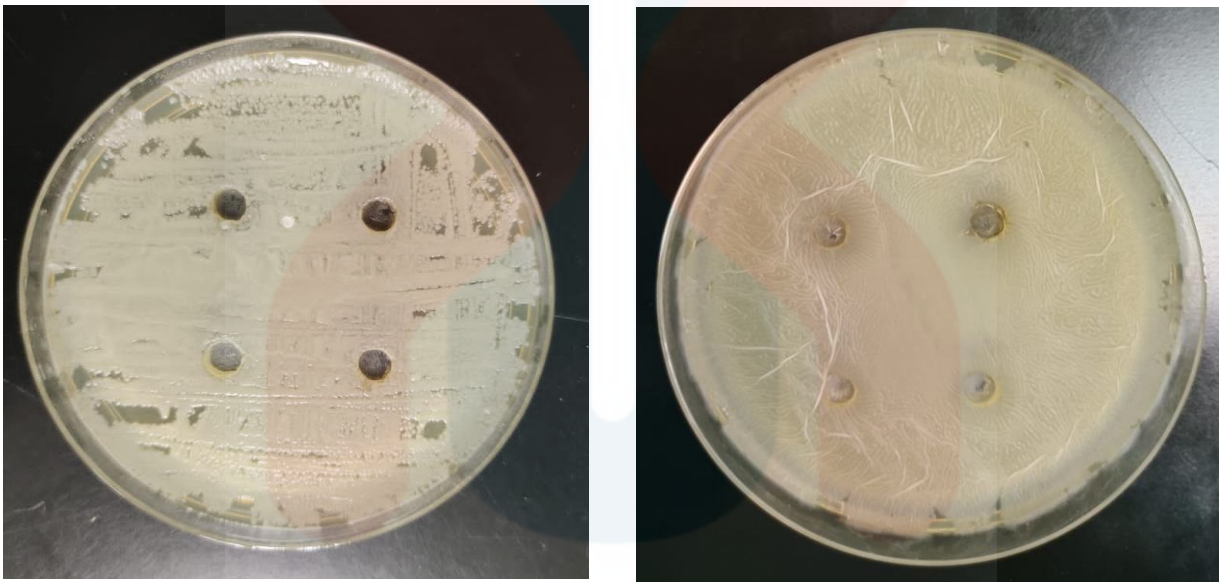


Figure 5

No presence of inhibition of *Candida albicans* growth on both Mueller-Hinton agar plates

After incubation for 48 hours, the plates are checked to see if the antimicrobial discs have created clear zones around the seeded holes which may indicate inhibition of bacterial growth. Based on the result, there is no presence of clear zone and the *Candida albicans* grow up to the rims of all four holes, indicating that *Candida albicans* is resistant to the graphene oxide.

8.2 Assessment of GO toxicity towards mammalian cells.

Cells exposed to increasing concentration of GO, showed enhancement in the cell growth (more than 100% growth) from concentration of 6.25 to 50 ug/mL compared to the untreated cells. At 400 ug/mL, GO inhibited cells growth, suggesting that the concentration causes toxic effects to the cells and unable to regrowth the cells.

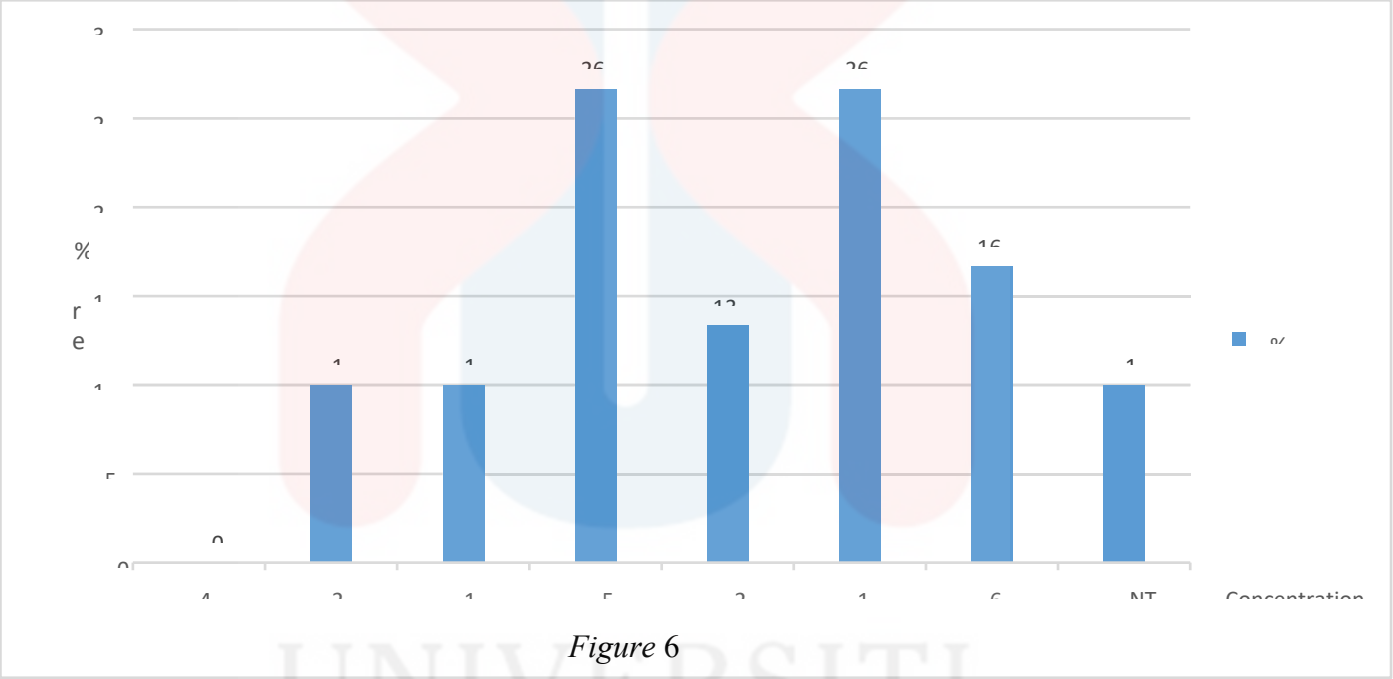


Figure 6

Growth rates of Feline Kidney cells after being treated with graphene oxide after 48 hours.

The growth rates of cells following treatment with graphene oxide. Non treated cells (NT) was normalized to 100%.

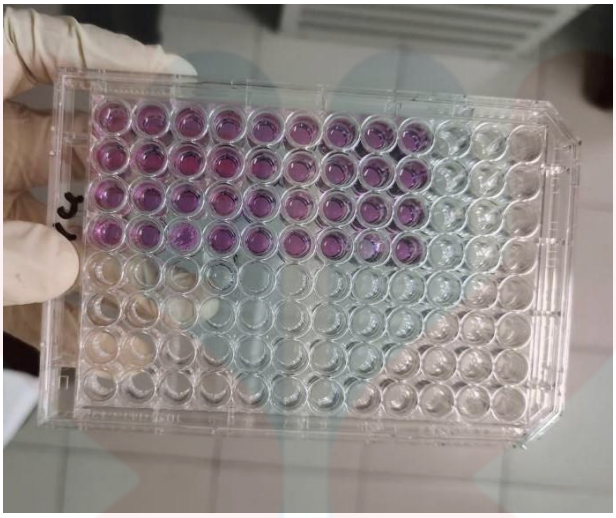


Figure 7

After incubation for 48 hours.

9.0 DISCUSSION

The purpose of this experiment is done to investigate the *in vitro* fungicidal effects of graphene oxide towards *Candida albicans* and the cytotoxicity effects of graphene oxide on the Feline kidney cell (CRFK). In this study we found that GO did not exhibit antifungal effect at concentration tested and promoted mammalian cell growth at low concentration. GO is chosen as possible alternative for antifungal drugs as it showed antimicrobial activities towards bacterial species such as *Staphylococcus aureus*. This can be attributed by the structure of GO that has sharp edges which can causes physical damage to the cell membrane of the organisms and lead to leakage of electrolytes and intracellular materials, eventually causes cell lysis. GO also has great in vitro biocompatibility that allows it to cause apoptosis to various type of cells, including fungi cells.

Antifungal susceptibility testing technique is used to identify antifungal resistance and choose the most effective course of action for *Candida albicans*. As demonstrated in the results, there is no presence of inhibition zones around the holes treated with graphene oxide for all agar plates. Thus, it is proved that graphene oxide with concentration of 50, 100, 200 and 400 mg/ml did not inhibit the growth of the fungi which indicates that this specific concentration of graphene oxide did not demonstrate fungicidal activity against *Candida albicans in vitro*. However, the clinical interpretation of this experiment is that *Candida albicans* tested can be inhibited by graphene oxide of higher concentrations. However, there might be few factors that lead to the negative result for this experiment. This can be due to insufficient time for treatment. Based on comparison of a study that treated GO with *B. sorokiniana* (Zhang et al., 2022), the fungi was treated with GO for seven days to observe the growth curve of the fungi. Thus, the duration of treatment may be one of the reasons on why GO did not show fungi activity post two days treatment on Mueller-Hinton agar.

One of the aim of the antifungal sensitivity test is to find out the Minimum inhibitory Concentration (MIC). Minimum inhibitory Concentration (MIC) is the lowest concentration of drug that can prevent the fungi growth. Thus, the resistance result of Candida albicans

towards graphene oxide can be due to the concentration of the graphene oxide (50, 100, 200, 400 mg/ml) did not achieve Minimum inhibitory Concentration (MIC).

Another alternative that can be done to test the fungicidal effects of graphene oxide against *Candida albicans* is by initiating a broth microdilution method based on CLSI standard. Experiments based on CLSI documentation may provide more accurate result as their interpretative data is based on pharmacokinetic properties of drug, clinical studies results and the microbiological data of microorganisms (Reller et al., 2009).

The efficacy of different concentrations of graphene oxide towards C. albicans in vitro are evaluated based on Clinical and Laboratory Standards Institute (CLSI) guidelines. CLSI recommends Minimum Inhibitory Concentration (MIC) assay which is commonly used to determine the antifungal susceptibility towards fungi . For this study, results are generated to produce reliable MIC breakpoints and interpretive categories that can be the guidelines for the efficacy of graphene oxide towards the fungi. For the broth microdilution procedure, the medium used is RPMI 1640 that contains 0.2% glucose and requires 96- well microdilution plates of polystyrene for the tests. According to Berkow et al., 2020 , the amount of drug powder used for the broth solution can be done using the following formula:

$$\text{weight (mg)} = \frac{\text{volume (ml)} \cdot \text{ concentration } (\mu\text{g/ml})}{\text{assay potency } (\mu\text{g/mg})}$$

$$\text{volume (ml)} = \frac{\text{weight (mg)} \cdot \text{assay potency } (\mu\text{g/mg})}{\text{concentration } (\mu\text{g/ml})}$$

For the preparation of the *C. albicans* stock solution, the colonies of *Candida albicans* are suspended in sterile saline. Then, the solution is adjusted using a spectrophotometer that

is equal to 0.5 McFarland standard at a wavelength of 530 nm. After that, the stock solution can be used as working dilution by doing 1:100 dilution and followed by 1:20 dilution using RPMI 1640 culture medium. The final inoculum should be around 0.5 × 103 to 2.5 × 103 cells per ml. Following this, graphene oxide can be inserted into the solution and incubated at 35°C for 24 hours. After incubation, the growth in the well plates are observed for any absence or presence of growth. To score the growth, the wells with graphene oxide need to be compared with the control wells. The MIC value is determined by visual inspection at 50% growth inhibition based on different concentrations of graphene oxide.

For the cell viability using resazurin assays on 96--well plate, the resazurin colour changes from blue to pink. This is because resazurin is reduced to a fluorescent pinkish resofurin, which indicates that there is presence of active microbial metabolism that represents the cells treated are metabolically active and viable. This indicates that the graphene oxide did not kill the treated cells and exerts low cytotoxicity effects towards Feline kidney (CRFK) cells. This result also implies that graphene oxide is a good biocompatible that can be non-toxic and can be accepted by the mammal's body.

10.0 CONCLUSION

This study revealed the graphene oxide do not have fungicidal activity against *Candida albicans* in vitro thus making it incapable of being the alternative antifungal drug to treat Candidiasis in poultry. Collectively, graphene oxide also did not show any cytotoxic effects against Feline kidney (CRFK) cells, indicating that it is a safe agent against feline cells. There is no evidence of graphene oxide suitable as an alternative antifungal agent against *Candida albicans*. However, further therapeutic strategies using graphene oxide can be done in the future due to its low cytotoxic potential against feline cells.

11.0 RECOMMENDATIONS AND FUTURE WORK

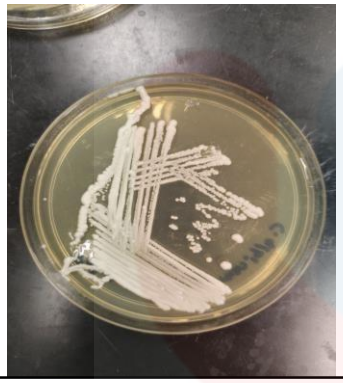
Graphene oxide has good physicochemical properties and is a good biocompatibility agent which exerts bactericidal activity towards few bacterial species. There is also a research study regarding the formulation of graphene oxide with fluconazole compound that exerted synergistic effects against *Candida albican*s through DNA fragmentation assay (Shahi et al., 2018). Thus, I suggest combining graphene oxide with other antifungal drugs such as amphotericin B as the new therapeutic strategies to combat Candidiasis based on the CLSI standard and guidelines.

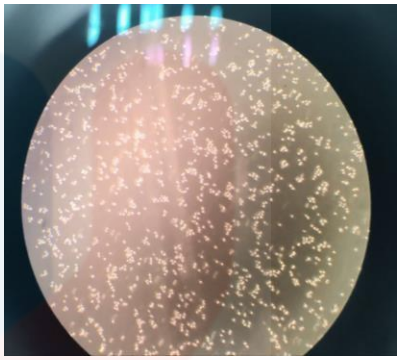
12.0 APPENDICES

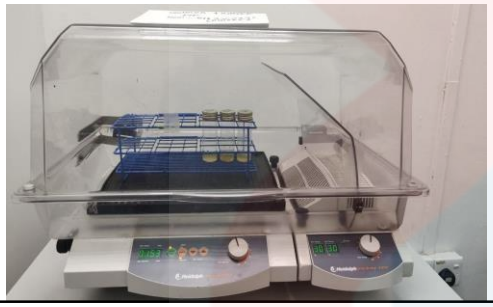
Table 2: Absorbance value of Feline kidney cells after being treated with GO

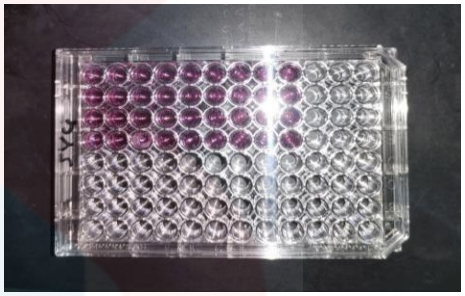
Graphene oxide (µg/ml)	Absorbance 550 nm	Absorbance 630 mm	550-630	% growth
NT	0.041	0.04025	0.00075	100
400	0.03875	0.03875	0	0
200	0.038	0.03725	0.00075	100
100	0.0395	0.03875	0.00075	100
50	0.03825	0.03625	0.002	266.67
25	0.03575	0.03475	0.001	133.34
12.5	0.037	0.035	0.002	266.67
6.25	0.0365	0.03525	0.00125	166.67

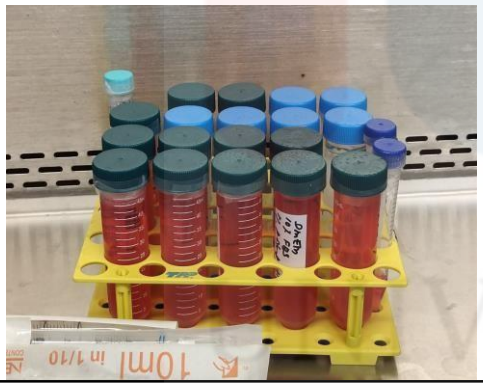
Table 2 shows the absorbance value of mammalian cells after being treated with graphene oxide after 48 hours. The absorbance value shows the vitality of the cells. Absorbance values that are less than those of the control cells suggest a slower rate of cell proliferation. Alternatively, a greater absorbance value suggests a rise in cell proliferation.

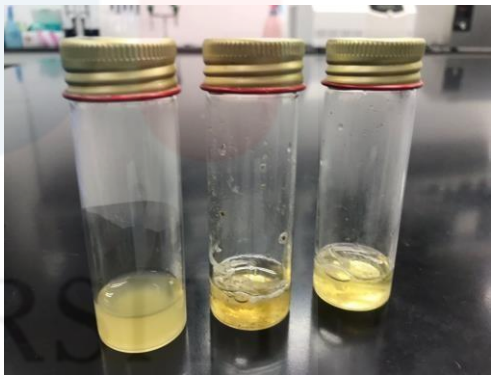


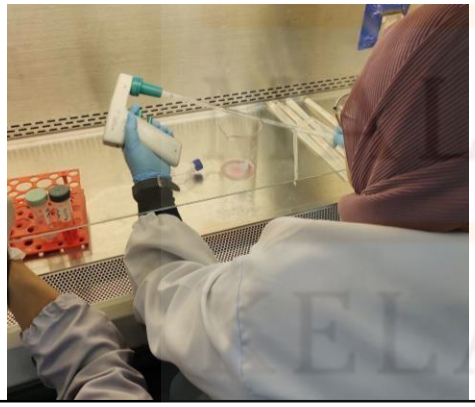


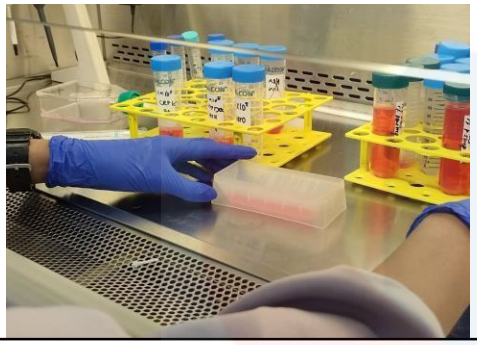


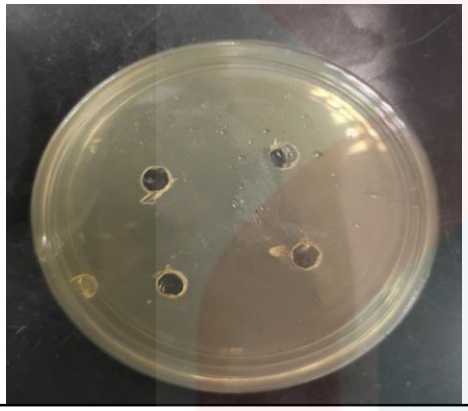












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