Screening of medicinal plants against *A. flavus*

Nur Amalin Amni bt Mahmud (72506)

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Screening of medicinal plants against *A. flavus*

Nur Amalin Amni bt Mahmud

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SUPERVISOR: DR ROSMAWATI BT SAAT

Programme of Resource Biotechnology
Faculty of Resource Science and Technology
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Resource Biotechnology Programme
Faculty of Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Infestation of *Aspergillus flavus* has been a threat to the important crops and human's health. The usage of synthetic fungicides have resulted into environmental and health issues hence making natural fungicides a more attractive option. This study aims to investigate the effectiveness of *Cympbogon citratus* and *Nigella sativa* against toxigenic *Aspergillus flavus* growth. *C.citratus* and *N. sativa* were purchased at Kota Samarahan. The leaves and seeds were dried at 60 °C for one to two days in drying oven. The dried leaves and seeds were then ground by using laboratory blender and extracted with 96% of ethanol. *A. flavus* was sub-cultured on potato dextrose agar (PDA). In this experiment, disc diffusion technique was used as antifungal assay where the extracts were diluted in DMSO and water resulting in 100%, 75%, 50% and 25% of the ethanolic extract of the plants.. Then, the plates were incubated at 72 hours at 37 °C in inverted position. The diameter of inhibition zone was measured in mm as antifungal activity. Results obtained suggest that both plants show the antifungal activity with 100%, 75, and 50% concentration of extracts. The higher the amount of extracts used, the higher the antifungal activity exhibited towards the fungi. However, at 25% concentration of extracts, neither plants show any antifungal activity. Last but not least, *C. citratus* exhibited higher antifungal activity compared to *N. sativa*.

Keywords: *Aspergillus flavus*, *Cympbogon citratus*, *Nigella sativa*, antifungal activity, disc diffusion technique

ABSTRAK

Serangan Aspergillus flavus *telah menjadi ancaman kepada tanaman penting dan kesihatan manusia. Penggunaan racun kulat sintetik telah mengakibatkan isu alam sekitar dan kesihatan justeru menjadikan racun kulat semulajadi adalah pilihan yang lebih menarik. Kajian ini bertujuan untuk mengkaji keberkesanan* Cympbogon citratus *dan* Nigella sativa *terhadap pertumbuhan* Aspergillus flavus *yang beracun.* Cympbogon citratus *dan* Nigella sativa *telah dibeli di sekitar Kota Samarahan. Biji dan daun telah dikeringkan pada suhu 60 °C sekitar satu ke dua hari di dalam ketuhar pengeringan. Biji dan daun yang telah kering telah dikisar dengan menggunakan pengisar sebelum dieskstrak dengan etanol.* A. flavus *telah dikultur pada potato dextrose agar (PDA). Dalam eksperimen ini, kaedah disc diffusion telah digunakan dimana ekstrak telah dicairkan kepada 100%, 75%, 50% dan 25% menggunakan DMSO dan air suling. Kemudian, plat disimpan di dalam incubator selama 72 jam pada suhu 37 °C. Diameter zon perencatan telah diukur dalam mm sebagai aktiviti antikulat. Keputusannya, kedua-dua tumbuhan menunjukkan aktiviti antikulat dengan 100%, 75% dan 50% ekstrak. Semakin tinggi ekstrak maka semakin tinggi aktiviti antikulat yant terhasil kepada fungi. Namun begitu, 25% ekstrak tidak menujukkan aktiviti antikulat.* C.citratus *menunjukkan akitiviti antikulat yang lebih tinggi berbanding* N. sativa.

Kata kunci : Aspergillus flavus, Cympbogon citratus, Nigella sativa, *aktiviti antikulat, teknik resapan disk*

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LIST OF ABBREVIATIONS

A. flavus	*Aspergillus flavus*
A. alternatum	*Acremonium alternatum*
AF	Aflatoxin
A. fumigatus	*Aspergillus fumigatus*
A. niger	*Aspergillus niger*
C. albicans	*Candida albicans*
C. musae	*Colletotrichum musae*
C. neoformans	*Cryptococcus neoformans*
CFU/g	Colony forming unit per gram
C. citratus	*Cymbopogon citratus*
C. flexuosus	*Cymbopogon flexuosus*
dc	diameter control
dt	diameter treated
ddH_2O	double distilled water
F. oxysporum	*Fusarium oxysporum*
F. solani	*Fusarium solani*
mg	milligram
mg/L	milligram per litre
mm	millimetre
mL	millilitre
N. sativa	*Nigella sativa*
MIC	Minimum inhibitory concentration
PDA	Potato Dextrose Agar
ppm	part per million
P. citrinum	*Penicillium citrinum*
P. digitatum	*Penicilium digitatum*
P. maneffei	*Penicillium maneffei*
R. stolonifera	*Rhizopus stolonifera*
rpm	revolution per minute
spp.	several species
µL	microliter
°C	Degree Celsius

CHAPTER 1

INTRODUCTION

1.1 Research Background

According to Rudramurthy et al., (2019) *Aspergillus flavus* has been ranked 5th among the world's ten most dangerous fungus due to its pathogenicity such as mycoses in humans. In the agriculture area, this fungus has been observed to harm the seed crops, especially, the preharvest and postharvest with the carcinogenic secondary metabolite called aflatoxin (Amaike & Keller, 2011). Aflatoxin is reported to infect a wide range of agricultural seeds, although it is most significant in the field for maize, peanuts, cotton seed, and tree nuts (Kumar et al., 2017).

The complexity in innovating fungicides with optimal features, such as broad-spectrum efficiency, increased bioavailability, minimum toxicity and possible complications, has stifled the development on drugs in combating invasive fungal infections (Brauer et al., 2019). According to Masiello and co-workers, (2019) Succinate Dehydrogenase Inhibitor and fludioxonil as antifungal agents, have been shown to be successful against *A. flavus*, whereas the infestation of *A. flavus* has also been reduced by reroconazole (75%) and boscalid (56%) in a demonstration project their conducted. However, the usage of the synthetic fungicide has been controversial as it could cause environmental issues and health related effects (Brauer et al., 2019).

Hence in this study, ethanolic extraction of *Cymbopogon citratus* and *Nigella sativa* are screened against *A. flavus.* Ethanolic extraction has been found to be effective in reducing the number of *A. flavus* growth, including suppressing the production of aflatoxin (Ren et al., 2020). The ethanol extract was obtained by drying and ground the medicinal plants into powder before soaking them into ethanol. Then the antifungal activity was investigated by using disc diffusion method with different dilutions of ethanol extracts. Lastly, the

percentage of mycelial inhibition was calculated in evaluating the effectiveness of the plant extracts.

The findings of this study may provide a better knowledge of the medicinal plants' potential to prevent the growth of *A. flavus*. Subsequently, may be utilised as a main preventative and efficient strategy for controlling *A. flavus* fungal invasion.

1.2 Objectives

Therefore, this study focuses on the screening of the medicinal plants *C. citratus,* and *N. sativa against A. flavus*. Hence, the objectives of the project are as follow:

1. To identify the potential of selected medicinal plants in inhibiting the growth of *A. flavus*
2. To determine the ability of *C. citratus* and *N. sativa,* in inhibiting the growth of *A. flavus*
3. To compare the effect of different concentration of *C. citratus* and *N. sativa* ethanol extracts in inhibiting the growth of *A. flavus.*

CHAPTER 2

LITERATURE REVIEW

2.1 A*spergillus flavus*

Aspergillus flavus is a saprophytic pathogenic soil fungi belonging to the *Ascomycota* phylum (Amaike & Keller, 2011). This species is most recognised for its tendency to form aflatoxin, a severe toxin and carcinogen. This fungus originates predominantly in the soil, but with a conducive environment, it can infiltrate host plants through a variety of paths. Extenuating factors that encourage infection and aflatoxin formation in the field include heat, dryness, and pest attacks to the crops (Haschek & Voss, 2013).

According to Khan and co-workers, (2020) in their study, *A. flavus* has been observed on several cultural media in order to observe the consistency of their key morphological characteristics. As observed, the fungi exhibited a range of colours, in between olive-green, yellowish-green, and dark green colonies with a white circle that was finally encircled by conidia. Other than that, velvety or fuzzy textures with a floccose centre were commonly seen in the colonies (Khan et. al, 2020).

According to Amaike & Keller in 2011, *A. flavus* is a morphologically sophisticated species with two distinct groups depending on the size of its sclerotia, each groups have different ability to produce specific types of aflatoxins. Table 1 below shows the classifications of *A. flavus* strains

Table 1 : Classifications of *A. flavus* Strains in producing aflatoxin (Amaike & Keller, 2011).

	L strains	**S strains**
Size of sclerotia	<400 mm in diameter	>400 mm in diameter
Types of Aflatoxins	B1, B2	B1, B2, G1, G2

2.2 Antifungal agents

Antifungal agent is defined as medication that precisely eradicate fungal infections from a host while causing the least amount of harm to the host. Over the last 25 years, antifungal medicines treating widespread fungal infections have advanced dramatically. According to Miller & Wilmott, (2019) the production of antifungal drugs commences with Amphotericin B deoxycholate. This was the most effective antifungal treatment for any fungal infection. It attacks the cell by adhering to the ergosterol in the fungal cell membrane. However, because of dose-dependent renal toxicity and hypokalemia, amphotericin B lipid complex was developed, which has a lower toxicity than initial (Miller & Wilmott, 2019).

The next antifungal drug reported is azole, which, unlike Amphotericin B, blocks ergosterol production in fungal cells. Imidazole and triazole are azole derivatives. Imidazole is well-known for its direct usage. Triazole derivatives, on the other hand, have been used in a variety of mould and fungal experiments. Fluconazole has been shown to be effective towards *Cryptococcus neoformans* and a variety of *Candida* species. However, it has little effect on moulds and has inconsistent efficacy against *Candida* spp. Hence, Voriconazole was created as a result of this to combat *Candida* spp. resistance. Itraconazole has been used to combat *A. niger* and *Histoplasma capsulatum* infections. Posaconazole is famous for being the first azole exhibiting potency against mucormycosis-causing substances. However, transaminase increase and peripheral neuropathy were identified as azole detrimental effects. It was claimed that voriconazole causes vision difficulties and central neurologic damage (Miller & Wilmott, 2019).

Next, Echinocandins, which is the most recent drug works by restricting beta-1,3-d-glucan synthase, causing the fungal cell wall to be destroyed. However, since it is not expressed in mammals, this medication has a minimal complication rate. Caspofungin, micafungin, and anidulafungin are some of the other derivatives in this class. Since

echinocandins are inadequately absorbed by the digestive tract, they are only accessible in IV doses. Because of their low tissue absorption, echinocandins are not recommended for fungal infections of the central nervous system, eye, or urinary tract. Echinocandins can produce an increase in aminotransferases on rare occasions (Miller & Wilmott, 2019).

Lastly, Flucytosine, on the other hand, is a fluorinated pyrimidine analogue. It was found effective towards *Candida* spp. and *Candida neoformans*. Flucytosine is usually combined with amphotericin B or an azole since it acquires tolerance fast when it is used independently. The most common side effects of this medicine are bone marrow suppression and gastrointestinal distress (Miller & Wilmott, 2019).

2.3 Ethanol Extract

In recent research by Ren et al., (2020) ethanol was reported to have the ability to inhibit not only the growth of *A. flavus* but also to suppress the synthesis of AFB_1 by increasing the fungal oxidative stress response in a dose-dependent manner. A comparative transcriptomic analysis revealed that 3.5% ethanol could supress and inhibit most of the aflatoxin gene.

Hence, many ethanolic extraction of plants have been actively studied against *A. flavus* growth including rosemary, thyme, kaffir lime, bitter cucumber, tobacco, licorice, doum, banana peel, clove, betel and so much more (Centeno et al., 2010; Thanaboripat et al., 2006; Youssef et al., 2021; Boonpawa et al., 2019). For example, an experiment done by Boonpawa and co-workers (2019) in comparing the antifungal activity of aqueous and ethanol extract of clove bud and betel leaf against *A. flavus* in Potato Dextrose Agar (PDA) using the poisoned food technique. Other than that, they also tested the potency of the ethanol extraction in inhibiting *A. flavus* directly by soaking the dried chili pod into the ethanolic plant extract during chili storage.

This study showed the growth of *A. flavus* was supressed up to 2-5 times better than the aqueous extraction by the 95% ethanolic extract. Moreover, with 7500 ppm of the betel lead ethanolic extracts, the fungal mycelial growth was inhibited by 84% while being completely inhibited at 10000 ppm. However, at 10000 ppm of clove bud ethanolic extracts, the mycelial growth of *A. flavus* was inhibited by 83%. Moreover, Figure 1 below shows the number of fungal colony (log CFU/g) at day 14. Dried chili soaked in clove ethanolic extract for 30 min was observed to have the best result as it showed the lowest number of fungal colonies compared to others on day 14 (Boonpawa et al., 2019).

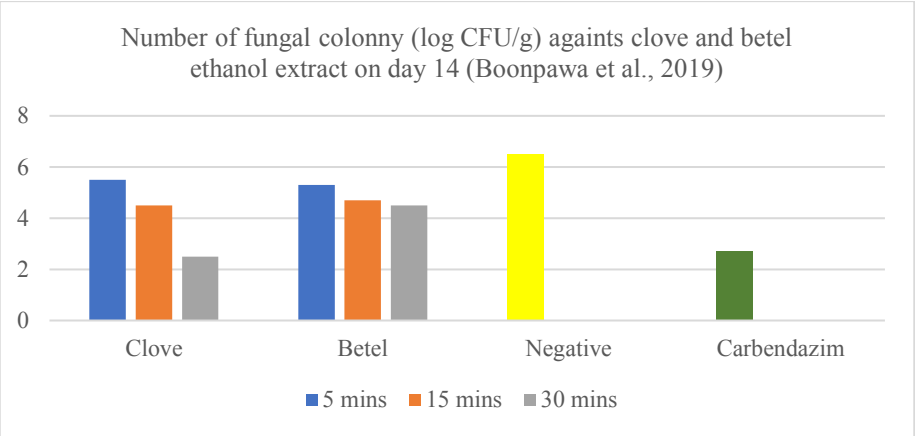

Figure 1: Number of fungal colony against clove and betel ethanolic extract on day 14 (Boonpawa et al., 2019).

2.4 Description of Medicinal Plants

2.4.1 *Cymbopogon citratus* (Lemongrass)

Cymbopogon citratus, or lemongrass from Gramineae family, has a lemony aroma due to the high concentration of citral, a cyclic manoterpene (Manvitha & Bidya, 2014). This herb is known as West-Indian lemongrass, is native to Malaysia compared to the *C. flexuosus* that is originally from India and Sri Lanka (Lawal et al., 2017). Commonly used in cooking, this herb has also been investigated for its anti-amoebic, antibacterial, antifungal, antidiarrheal, antifilarial and anti-inflammatory characteristics (Shah et al., 2011). According to Vazquez-Briones et al. (2015), citral compound, which is neral and geranial, was discovered to be the main component of this plant.

Although *C. citratus* is most studied for their essential oil's antifungal activity such as Tyagi and Malik, (2010) that studied the essential oil of *C. citratus* along with *Mentha piperita* (mint) and *Eucalyptus globulus* (eucalyptus) against *C. albicans.* The study has revealed that *C. citratus* showed the best antifungal effect compared to the other two herbs. They tested the Minimum Inhibitory concentration (MIC) of liquid phase and the vapour phase of the essential oil. As a result, after 4 hours of 288 mg/L of liquid phase and 32.7 mg/L vapour phase, the viability of the cells of *C. albicans* has completely been destroyed (Tyagi & Malik, 2010).

However, in an experiment carried out by Nyamath & Karthikeyan, (2018) *C. citratus* extracts were tested against fungal strains *Colletotrichum musae* and *A. niger.* Dried and fresh *C. citratus* were extracted with ethanol, methanol, hot and cold water before being tested by agar well diffusion method. The antifungal activity was observed and the effects were seen to be the best at 1000 ppm for each extract. For *A. niger*, the best zone of inhibition growth was carried out by dried leaves of *C. citratus* extracted by methanol (10.90 mm)

while for *C. musae,* by dried *C. citratus* leaves extracted by cold water (9.80 mm). The authors also concluded that the antifungal activity was due to the high amount of citral compound in the *C. citratus*

2.4.2 *Nigella sativa* (Black Cumin)

Nigella sativa, often known as Black Cumin or Black Seed, is a member of the Ranunculaceae family. According to Thilakarathna and co-workers, (2018) the morphological features of *N. sativa* plants include having hairy stems and bright green, tripartite leaves, as well as milky white flowers with a hint of blue or green at the end of it. While the 'black seeds' are carried in black seedpods, a single black cumin seed has a dark colour and a narrow, crescent form, as well as a powerful bitter flavour and scent. It is an annual plant native in the Iran with a wide range of medicinal characteristics (Ahmad et al., 2013). It has long been used as a folk treatment to cure a range of diseases (Shokri, 2016). D'Antuono et al., (2002) reported that *N. sativa* has benefits various diseases for having antidiabetic, antihistaminic, antihypertensive, anti-inflammatory, galactagogue and so much more. These capabilities are mostly ascribed to quinone component, thymoquinone (50%). P-cymene (40%), pinene (15%), dithymoquinone, and thymohydroquinone are some of the other compounds found in *N. sativa* (Sharma et al., 2009).

According to Al-Ameedy and co-workers, (2019) their study proved that *N. sativa* ethanolic extract has the potential in inhibiting the growth of strain fungal *Fusarium Solani.* Other than that, they also stated that 96% ethanol was the best solvent to extract the active materials from black seeds. Moreover, the ethanol and hexane fraction of the *N. sativa* extract was found to be the most potent antifungal agent against *F. solani.*

In another study by Shah et al., (2018) *N. sativa* seeds extracts in ethanol, methanol and water were investigated for its antifungal activity against *A. niger, A. fumigatus, Rhizopus stolonifera, Penicilium digitatum* and *Acremonium alternatum*. The antifungal activity was measured by calculating the zone of inhibition (ZI) showed by different strains towards each extract. As a result, *N. sativa* seeds methanol extract exhibited the best growth inhibition against the fungal strain. Other than that, ethanol extract showed optimum antifungal activity whilst aqueous extract however showed the lowest antifungal activity compared to the other two. Figure 2 below shows the diameter or inhibition zone (mm) against *N. sativa* seed extracts (Shah et al., 2018)

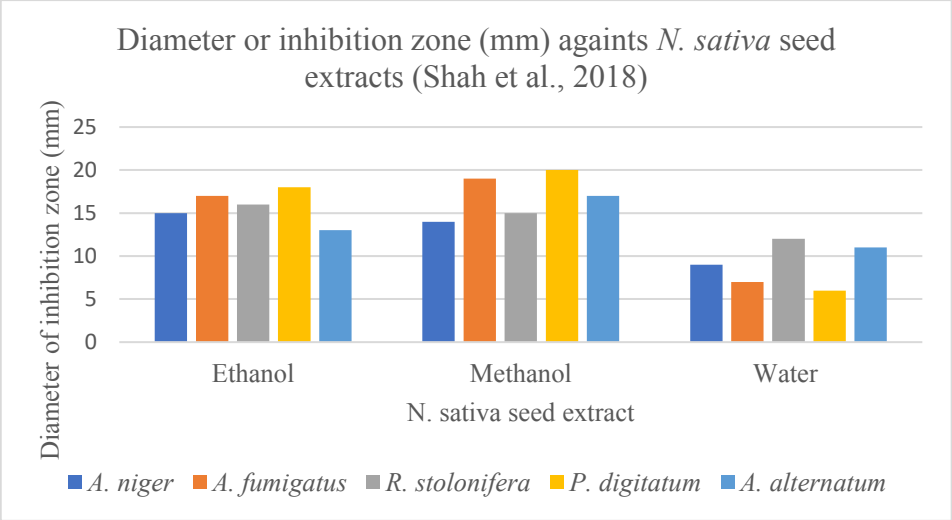

Figure 2: Diameter or inhibition zone (mm) against *N. sativa* seed extracts (Shah et al., 2018).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Laboratory blender, conical flasks, filter funnels, Whatman filter papers, parafilm, aluminium foil, electronic balance, rotary evaporator, Erlenmeyer flasks, round bottomed flasks, water bath, universal bottles, falcon tubes, micropipette, micropipette tips, petri dishes, test tube racks, forceps, schott bottles, measuring cylinder, ruler, *Aspergillus flavus*, *N. sativa* seeds and *C. Citratus*.

3.2 Chemicals

Ethanol, distilled water, 50 mg/ml ampicillin, potato dextrose agar (PDA), solvent dimethyl sulfoxide (DMSO)

3.3 Collection of plant materials

C. citratus and *N. sativa* seeds were purchased from the supermarket at Aiman Mall Kota Samarahan, 94300 Kota Samarahan, Sarawak.

3.4 Preparation of plant materials

C. citratus and *N. sativa* were washed with distilled water before being oven dried at 60 ºC for one day. Dried leaves and seeds were ground to powder form using a laboratoroy blender and stored tightly in a sealed container until extraction at room temperature. Figure 3 below show A*) N. sativa* in powder form and B) *C. citratus* in powder form in differently labelled container.

A) B)

Figure 3: A*) N. sativa* in powder form and B) *C. citratus* in powder form

3.5 Ethanol extraction

Ground materials of 100 g were soaked in of 1000 ml of 96% of ethanol in the ratio of 1:10 (plants and ethanol). Then, they were shaken for 3 days at room temperature in a rotary shaker at 140 rpm. Then, the mixture was filtered using Whatman Filter paper. The liquid obtained was concentrated using rotary evaporator. The thick substances obtained was kept in a universal bottle and sealed with aluminium foil and kept at 4 ºC in the refrigerator. Figure 4 below shows the sealed universal bottle to be kept in the refrigerator.

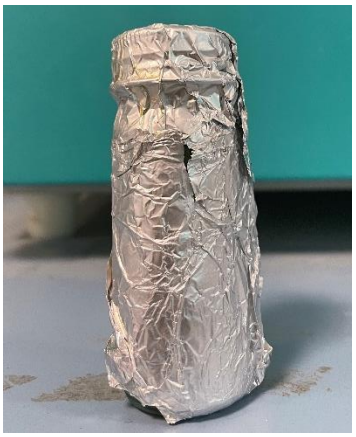

Figure 4 : Extract was kept in universal bottle and sealed with aluminium foil

Each crude extract was then diluted into 100%, 75%, 50% and 25% of the concentrations by dissolving the crude extract in organic solvent solvent dimethyl sulfoxide (DMSO) and water, then, vortex was performed for 30 minutes. Figure 5 below show the different concentrations of *N. sativa* and *C. citratus* extract.

A) B)

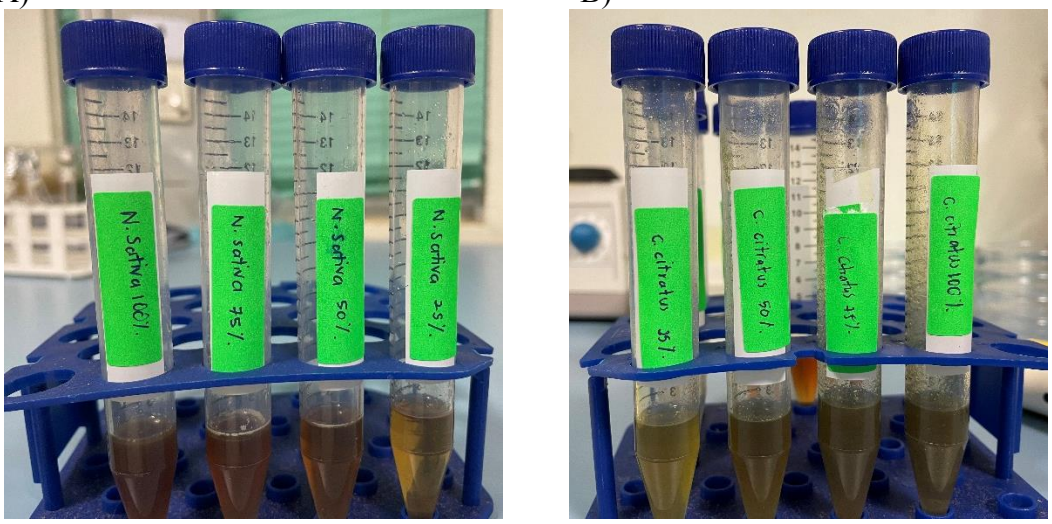

Figure 5: A) *N. sativa* extract in 100%, 75%, 50% and 25% concentrations and B) *C. citratus* in 100%, 75%, 50% and 25% concentrations

3.6 Media preparation

Potato dextrose agar (PDA) was prepared in a sterilized Schott bottle before being sent into autoclave machine for sterilization. The sterile PDA were then cooled under running water before 50 mg/ml ampicillin was added. The PDA were then poured into petri dish and allowed to be solidified at room temperature.

3.7 Fungus preparation

A. flavus stock was obtained from the fungal collection of Molecular Biology Lab, Faculty of Resources Science and Technology, UNIMAS. The fungus was sub-cultured onto PDA under aseptic condition. Then, the culture was incubated at room temperature until the