Examining wound healing properties of *Areca catechu*
Group 1-19

Abstract

Chronic wounds are extremely prone to bacterial infections, particularly those that have gained multi-drug resistance. This creates an urgent need for the research of new, medically-applicable compounds. This study investigated the antibacterial, antifungal and wound-healing properties of *Areca catechu* (AC). A colony count assay ascertained AC's anti-bacterial properties through the decreased formation of bacterial colonies, especially for DMSO-based extracts. A minimum inhibitory assay revealed that dialysed extracts demonstrated differentiated antimicrobial activity due to decreased absorbance detected by the microplate reader. AC also showed antifungal properties, showing close to 100% inhibition at 5%, but decreased inhibition at increasing concentrations due to the presence of more prominent pro-fungal growth factors. AC was also able to increase the occurrence of the mitosis late prophase and mitosis telophase, causing aberrations in the cell cycle. Finally, DMSO and water-based AC showed signs of agglutination and granulation in the crab astrocytes, suggesting immuno-stimulative properties of AC.

Introduction

Chronic wounds are susceptible to bacterial infection and cost over US$3.4 billion to care for patients with chronic wounds, 3% of the expenditure on health for the same period (Posnett & Franks, 2008*)*. Additionally, large numbers of bacteria have gained multi-resistance towards drugs and antibiotics, most notably being the methicillin-resistant *Staphylococcus aureus* (MRSA) (Nikaido, 2009). This creates an urgent need for research on new compounds and treatments of chronic wounds.

Within the clinical setting, several bacterial strains are of particular concern. *Staphylococcus epidermidis* is a Gram-positive bacteria commonly found in the human skin flora. This strain exhibits high pathogenicity and penicillin resistance, and has the highest rates of infection in indwelling medical devices, costing $2 billion in vascular catheter-related bloodstream infections in the United States alone. (Otto, 2009). *Escherichia coli* is a Gram-negative bacteria that are commonly found in the gut of humans and warm-blooded animals. Particularly worrying is Shiga toxin-producing *Escherichia coli* (STEC). It thrives in a wide range of temperatures and might cause abdominal cramps and diarrhoea in mild cases; severe disease progression results in hemorrhagic colitis and haemolytic uremic failure, characterized by acute renal failure, haemolytic anaemia and thrombocytopenia (Who.int, 2019).

Areca catechu (AC), or betel nut, is a slender, single-trunked palm that can grow to 30m, cultivated across the Arabian peninsula, tropical Asia and the central Pacific. The seed endosperm is chewed as a stimulant masticatory. (Staples & Bevacqua, 2006). AC causes many

severe diseases such as oral squamous cell carcinoma, precursors leukoplakia, submucous fibrosis and cardiovascular disease (Warnakulasuriya, Saman *et al.*, 2002), thus seldom researched on but may be a potential candidate for novel antimicrobals (need ref).

Objectives

This study aims to examine the properties of AC in antimicrobial, wound healing activity,. Also, to examine AC's effect on the cell cycle and mitosis.

Hypothesis

We believe AC demonstrates potent antimicrobial activity, facilitates wound healing properties and does not cause aberrations in the cell cycle or demonstrate changes in the rate of mitosis *in vivo.*

Materials and Methods

Extraction

Fresh AC seeds were purchased from a local market and blended with either deionised water or 1% dimethyl sulfoxide (DMSO) solution to obtain a suspension. The extract was then centrifuged under 8000 rpm for 10 minutes at 15ºC so as to separate soluble and insoluble components, and the supernatant was decanted. The supernatant was then filtered with a microfilter (pore size 0.45µm) to remove all bacteria and maintain sterility in downstream tests. The supernatant was aliquoted and was stored at 0 ºC until needed for future use.

Dialysis of extracts

Dialysis tubing with a molecular weight cut-off of 30kDA was sterilised by soaking in hydrogen peroxide solution for 1 hour. The sterile extracts were placed in the dialysis tubing and were dialysed against sterile water. The high and low molecular fractions would then be separated and stored at -4 ºC for future usage.

Minimum Inhibitory Concentration

Bacteria were cultured overnight in nutrient broth using a shaking incubator. The resultant bacterial suspension will be mixed with decreasing concentration of AC by carrying out two-fold dilutions, with sterile water as the blank. The setup was incubated at 35ºC overnight. The microplate was then tested for absorbance using a microplate reader, and the minimum inhibitory concentration was calculated.

Colony Count Assay

Test bacteria were first inoculated into 10ml of LB broth and grown overnight at 30°C in a shaking incubator. In the test setup, 0.05ml of bacterial culture was added to 0.05ml of standard

10% (w/v) plant extract and 4.9ml of LB broth. In the control setup, 0.05ml of sterile water was added in place of plant extract. Five replicates for each setup were prepared. The absorbance of the cultures at 600nm was standardised at 0.8. The mixtures were incubated in a shaking incubator at 30°C for 2h. Serial 10-fold dilutions were performed with 0.85% sodium chloride solution to the appropriate dilution factor, and 100ul of the diluted culture was pipetted and spread on LB agar. Plates were incubated at 30°C for 24h overnight. The number of colonies was subsequently counted. Lesser colonies present indicates a greater degree of inhibition.

Antifungal Assay

Aspergillus niger (Carolina) was cultured on potato dextrose agar (PDA) for a few days at room temperature. 5ml of *Areca catechu* extract was added to 95ml of PDA and left to solidify. After solidification, a small layer of *A. niger* will be cut out with a sterile scalpel and placed in the middle of both the Petri dishes containing PDA (negative control) and the Petri dishes containing the PDA which have AC extract mixed in it. The resultant diameter of fungal surface growth was monitored over a week and recorded.

Root Tip and Mitotic Zone Analysis

Beans were bought in a local market and were washed with deionised water. The beans were germinated for a few days, and submerged in the extracts, with water as the negative control. Thin slices of the root tip were made with a sterile blade. The cellular samples were placed under a light microscope. The mitotic zone of the root tip was examined to check for cells in various stages of mitosis to see if the cell had caused various aberrations of the cell division.

Immuno-stimulatory Assay

Horseshoe crabs, *Carcinoscorpius rotundicauda,* were collected from Kranji Mudflats, Singapore. These were washed to remove mud and debris and were acclimatized overnight in minimal levels of 30% seawater. Hemolymph was then obtained the next day by cardiac puncture. The carapace around the vicinity of the cardiac chamber was washed with detergent and was swabbed with 70% ethanol. The crabs were then partially bled by insertion of an 18 gauge sterile needle between the two plates of the dorsal carapace in a posterior direction, puncturing the cardiac chamber causing blood to be naturally ejected due to differences in ambient pressure and the hemolymph. This allowed an average volume of 5ml of hemolymph to be collected from each crab. The hemolymph were then centrifuged at 1000rpm for 10 mins at 10˚C. After the tubes were centrifuged, the crab astrocytes were seen at the bottom of the centrifuge tube. Using a sterile pipette, the crab astrocytes were placed on a sterile plastic film. A drop of AE was then added to the crab astrocytes to observe for granulation and agglutination

of the astrocytes. The mixture was then placed under the microscope for observation. Agglutination and granulation of the astrocytes meant that the extract of AC stimulated the astrocytes, and therefore, have immunostimulatory properties. After blood was drawn from them, the crabs were released back into Kranji Mudflats alive. ***Thin Layer Chromatography***

Pencil lines were drawn 1 cm from the top and 1 cm from the bottom of a paper-based chromatography plate, which served as the solvent front and baseline respectively. 6 evenly placed pencil marks were made on one of the lines, starting from 0.5 cm from either of the edges. The solvent, ethanol, were poured into the 250cm3 beaker to a height of 1cm to start the process. After 10 minutes, or before the solvent front reached the top of the paper, the paper was removed and a straight line was drawn over the solvent front. Thee chromatogram was dried before analysis.

4.Results and Discussions

Compounds of AC are bactericidal

Colony count assays were conducted on AC to elucidate its bactericidal properties. Water-based AE showed minimal amounts of bacterial inhibition for both *S.epidermidis and E.coli* (Fig. 1.1 and 1.2). This suggests that the compounds of AC that inflict antibacterial activity are non-polar in nature. However, it still shows great potential as a bacteriostatic agent. Notably, water-based AE showed no inhibitory effect on *E.coli*. Enhanced activity of AC against Gram-positive bacteria suggests the ability of an active compound to recognise, bind to, and cause cellular and molecular changes to result in bactericidal effects. Indeed, water-based AE causes agglutination of Gram-positive bacteria but not Gram-negative bacteria, indicating recognition and binding of specific molecules in the extract to peptidoglycan that would kick-start a cascade of molecular events resulting in bacterial death, suggesting that differential antimicrobial activity of water-based AE is dependent on recognizing specific pathogen-associated molecular patterns (PAMPS) on Gram-positive bacteria and not Gram-negative. However, DMSO based extracts of AE showed signs of bactericidal effects indicating polar compounds in AC that recognise generic structures of bacteria in general. DMSO-based extracts also show dosage-dependent efficacy, as concentration increases, the antibacterial activity increases (Fig. 1.1 and 1.2). Notably, the higher molecular weight fraction showed enhanced antibacterial activity. This study proposes that the presence of a class of proteins which are related to pentraxins that oligomerize in its inactivated state but are separated upon activation to effect enhance antibacterial activities. This would account for no enhanced activity in AE when immediately used. Dialysis, especially under non-pyrogenic conditions, activates these oligomers and causes them to fragment into individual active components and

hence causes the enhanced antibacterial activities.

Dialysed Extracts Demonstrated Differentiated Antimicrobial Activity

At decreased concentrations, the extracts were still able to show strong and positive signs of antibacterial potency. This could be seen by the decreased absorbance detected by the microplate reader. Dialysed extracts showed enhanced antibacterial activities (Fig. 2.1 and 2.2), which further suggested the presence of a class of proteins acting on the bacterium. Differential antibacterial activity was observed in extracts made up of different solvents. Suggesting specific active components are dissolved in specific solvents only, resulting in differential results. Since the higher molecular fraction is capable of effectively inhibiting bacteria at low concentrations, it has the potential to be a biological applicant, and treat wounds of many who might not have good access to healthcare.

Compounds of AC are antifungal

100% inhibition was seen in extracts with a concentration of 5 %. This suggests that *Areca catechu* is comparable to standard treatments like voriconazole but less costly. But as the concentration of extracts increases, the efficacy of inhibition decreases. This suggests the presence of both antifungal and pro-fungal growth factors. Antifungal compounds in the extract are lower in concentration and as the overall concentration increases, the effects of the latter become more obvious than the former.

4.4 Compounds of AC cause aberrations in the cell cycle

AC was able to increase the occurrence of the mitosis late prophase and mitosis telophase. This hastens the cell cycle dramatically. Whereas, the negative control, deionized water, did not cause many aberrations of the cell cycle. Suggesting active components in the extracts holding potentially carcinogenic at high concentrations. Therefore, in order for Areca catechu to be applied in the microbiology field. This calls for more tests to be done on these extracts to find a suitable concentration to prevent it from being carcinogenic and possibly even isolate the compounds that are responsible for these permutations, which could be harmful in the long-run.

Compounds of AC are immune-stimulative

DMSO and water-based AE showed signs of agglutination and granulation in the crab astrocytes, with the latter being more extensive (Fig 4). Crab astrocytes represent

undifferentiated white blood cells, unlike the human immune system which is differentiated with many different types of immune cells (Marggraf *et al*., 2018). The crab astrocytes agglutinated and granulated in contact with AE. This indicates that AE contains active components that are associated with the activation of several immune cell lines and suggests a possibility of increased cytokine production and hence increased cell proliferation which possibly promotes wound healing. However, care must be taken to optimise dosage; if taken in large amounts, it may result in clinical response to a nonspecific insult of either infectious or noninfectious origin defined as systemic inflammatory response syndrome (SIRS).

Compounds of AC can be Broadly Divided into 2 Groups

Two bands of compounds were separated from 99% ethanol. The compounds were found in the acetone-based and water based-extracts, and not the DMSO-based extracts. Further testing of the compounds is required to check for its antimicrobial, carcinogenic properties.

5. Limitations and Conclusions

Compounds of AC have the potential to be developed into a potent wound-healing agent

Four main conclusions could be drawn from this study.

Firstly, *Areca catechu* extracts (AE) is an effective bactericidal agent. DMSO-based extracts and the dialysed extracts showed potent antibacterial properties on both Gram negative and positive bacteria. This suggests that the DMSO-based extracts and dialysed extracts had compounds that recognised generic structures of bacteria, to effect antibacterial activity. However, water-based extracts showed differential antimicrobial efficacy on Gram negative bacteria. This suggests that polar compounds dissolved in water is dependent on recognizing specific pathogen associated molecular patterns (PAMPS) on Gram positive bacteria and not Gram negative bacteria

Secondly, AE is antifungal. It had both profungal and antifungal properties. At low concentrations, most notably at 5% (m/v), showed enhanced antifungal properties. For the 5% DMSO-based AE, even showed 100% inhibition for all 7 days.

Thirdly, AE is immune stimulative. This suggests that it could activate several immune cell lines, hence causing the release of pro-inflammatory cytokines and chemokines. The release of these molecules modulates the balance between humoral and cell-based immune response. They also regulate maturation of epithelial cells, hence in turn increasing cell proliferation, and

hence promoting wound healing

Fourthly, AE is carcinogenic, This suggests that AE is causing aberrations in the cell cycle.

Discussions

This study postulates that there is a class of proteins which are related to pentraxins that oligomerize in its inactivated state but are separated upon activation to effect antibacterial activities. These pentraxins function as opsonins which coat the microbial surface and increase the number and type of binding sites on microbial surfaces. *In vivo*, binding to the Fc gamma receptors activates the complement pathway and the phosphoinositide 3-kinase pathway (PI3K), promoting phagocytosis (Clos & W., 2013). PI3K is also critical for wound healing, as it activates a G-protein, Rac GTPase, which in turn activates AKT, causing downregulating of apoptosis and upregulating the cell cycle which in turns increases cell proliferation (Castilho, Squarize, & Gutkind, 2013). In this way, we believe that the same compounds of betel nut counter bacterial infections while promoting healing. Betel nut also activates several immune cell lines, which releases pro-inflammatory cytokines and chemokines and activates the adaptive immune response. In appropriate doses, compounds of betel nut might further stimulate the immune system, helping mount robust defensive mechanisms against multiple pathogens. After establishing the presence of pentraxin like-proteins. We could also establish that compounds of betel nut are immune-stimulative. The immune stimulatory assay showed that the astrocytes were activated and the immune system of the crab is potentially deregulated causing the cell cycle to dysregulate via the activation of the complement pathway. Verification of the activation would depend on testing the extracts on higher organisms with separate innate and adaptive immune systems such as mice. Which is currently unavailable in the lab, because of BSL restrictions.

Limitations

Despite the use of 1% DMSO solution as a solvent, compounds not soluble in it might be lost during the filtration process, causing betel nut to lose some of its antimicrobial potency. Only a small range of bacterial agents were used for the tests, which might cause undiscovery of hidden potentials betel nut might have to offer. The lack of human tissues used in the experimental process (physical constraints of the lab) might cause uncertainty for the practical usage of betel nut on humans.

Future work

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A swimming and swarming assay can be conducted to check if betel nut inhibits biofilms commonly found in wounds. Locomotion, thrashing and omega-bend assays can be conducted to check if betel nuts pose a threat to *C.elegans* and its survival. A genotoxicity assay can be conducted to ascertain if betel nut is genotoxic, exploring the mechanism of how betel nut inhibits bacterial growth. A scratch wound healing assay can also be conducted to ascertain if betel nuts have wound healing effects on human tissues. Finally, compounds with carcinogenic properties can be isolated to find the concentration of which betel nut could perform its antimicrobial and wound healing properties without hurting the user.

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Appendix

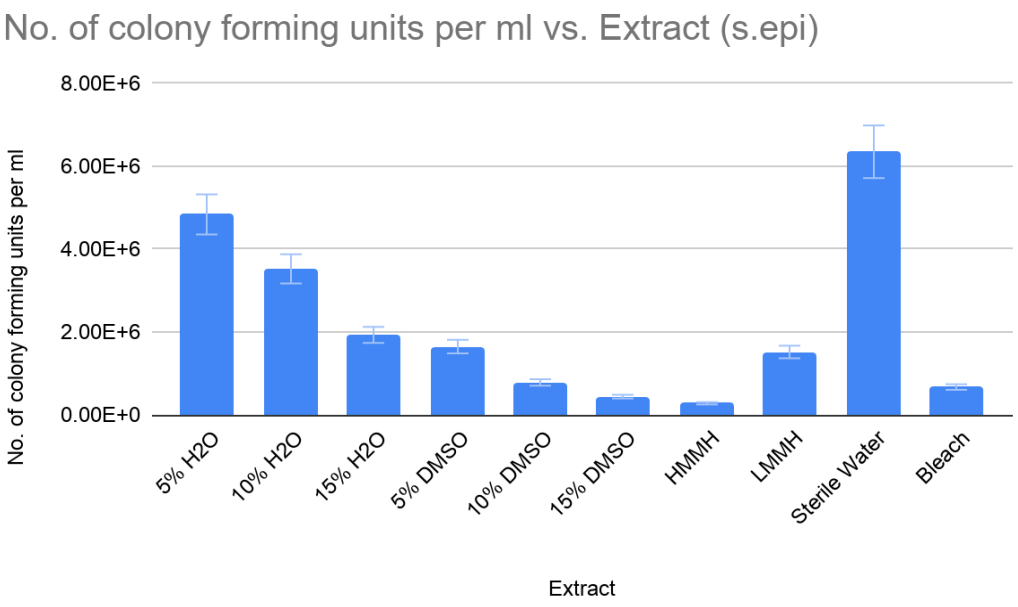

Fig. 1.1: Colony count assay of number of colony forming units/ml vs extract, for *Staphylococcus epidermidis*.

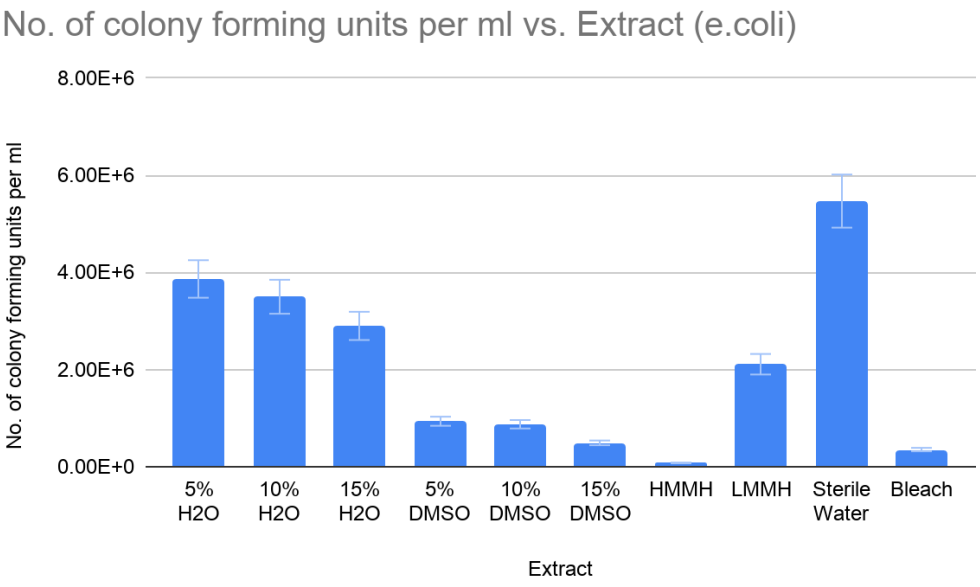

Fig. 1.2: Colony count assay of number of colony forming units/ml vs extract for *Escherichia coli*.

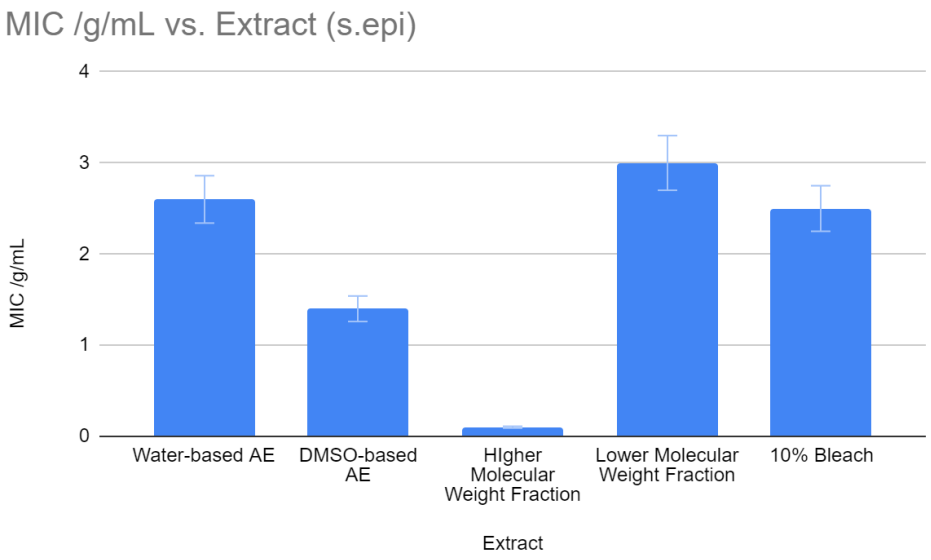

Fig. 2.1: Minimum inhibitory assay of minimum inhibitory concentration/g mL^{-1} vs extract, for *Staphylococcus epidermidis.*

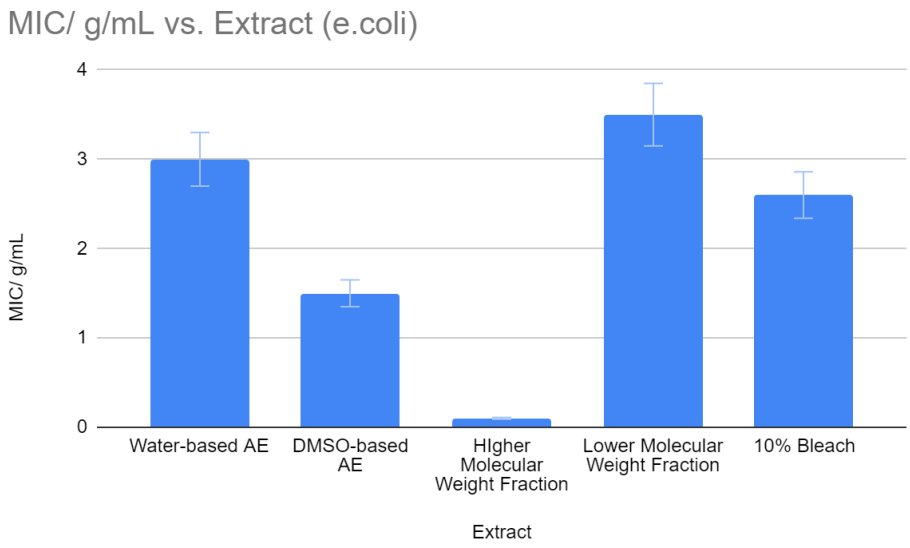

Fig. 2.2: Minimum inhibitory assay of minimum inhibitory concentration/g mL^{-1} vs extract, for *Escherichia coli*.

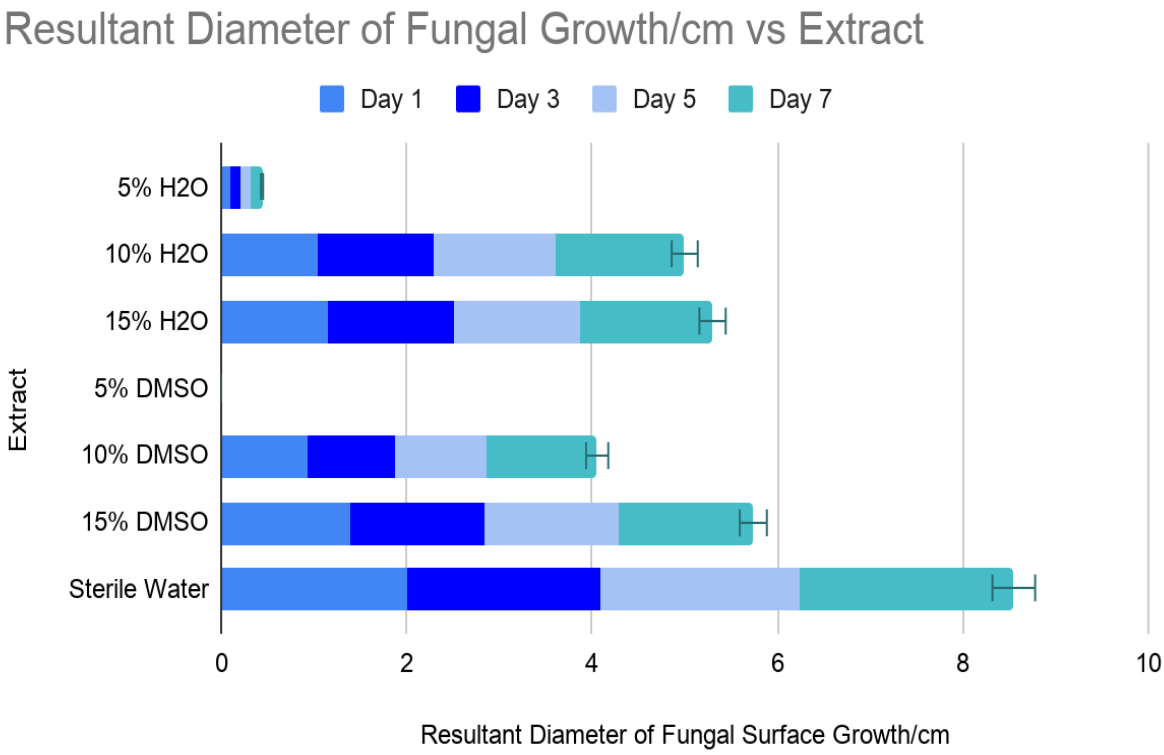

Fig. 3.1: Antifungal assay of resultant diameter of fungal growth/cm vs extract.

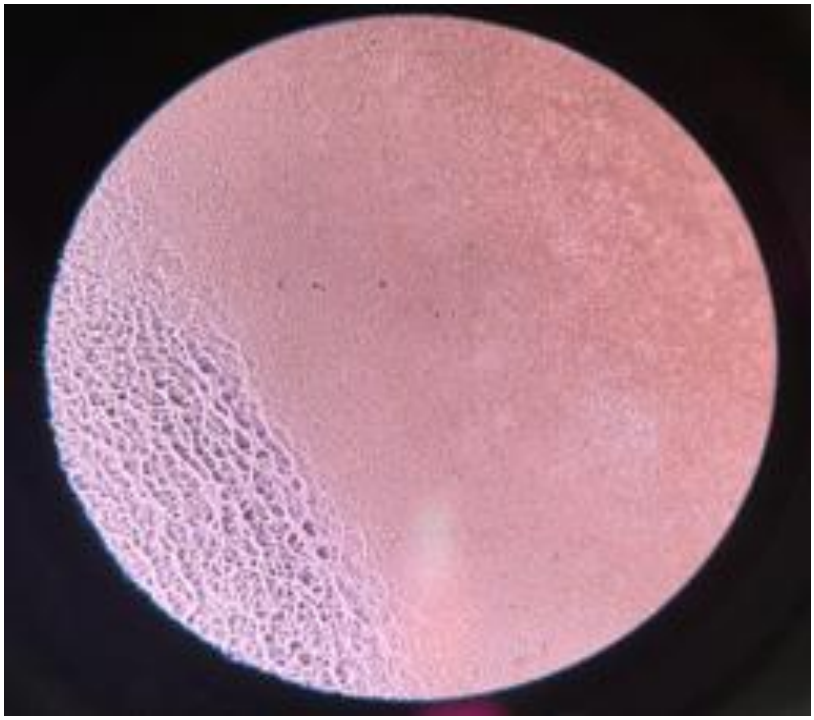

Fig. 4.1 Immuno-stimulatory assay of DMSO-based AE against astrocytes

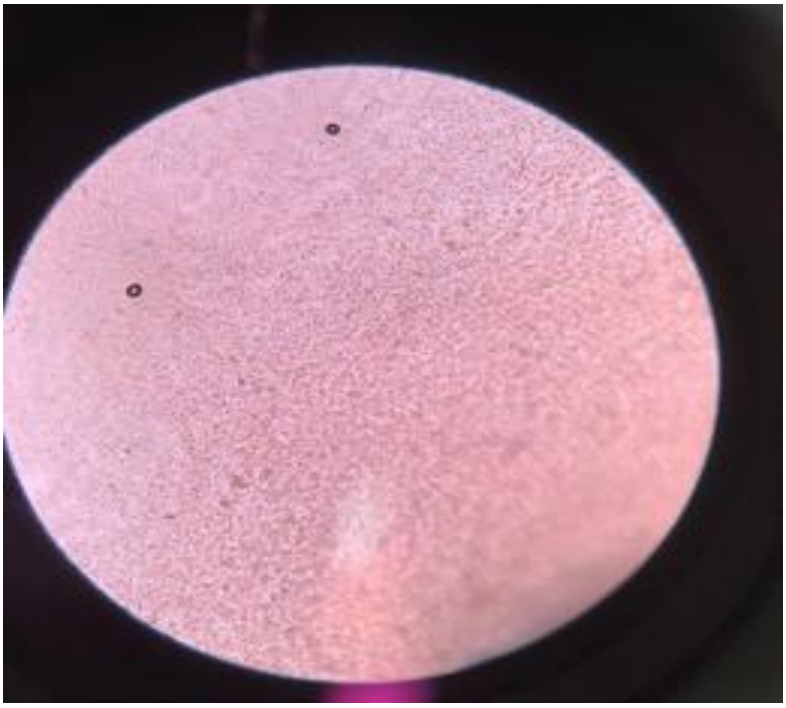

Fig. 4.2 Immuno-stimulatory assay of water-based AE against astrocytes