Investigating the wound healing, antibacterial and antioxidant effects of *Polygonum Multiflorum* and *Areca Catechu*

Group 1-07

Abstract

In this study, the wound healing, antibacterial and antioxidant effects of *Polygonum multiflorum* and *Areca catechu* were investigated. This was to find a potential solution to the problem of a large increase in the number of bacteria which have gained multi-resistance towards drugs and antibiotics. This investigation indicates that *Areca catechu* has antibacterial effects against Gram-positive bacteria whereas *Polygonum multiflorum* exhibits no antibacterial effects at all in a water-based solvent. However, both *Areca catechu* and *Polygonum multiflorum* exhibit antibacterial effects against Gram-positive and Gram-negative bacteria in a DMSO-based solvent. Furthermore, both *Areca catechu* and *Polygonum multiflorum* exhibit strong antioxidant effects, with them being dosage-dependent. The strong antioxidative effects of *Areca catechu* also allows it to facilitate wound healing.

Introduction

Chronic wounds are susceptible to bacterial infection. The costly nature of chronic wound management is confirmed by estimates from the United Kingdom (UK), where the costs of caring for patients with chronic wounds are estimated to be at US$3.4–4.6 billion per year, representing around 3% of the total estimated out-turn expenditure on health for the same period (Posnett J, Franks PJ, 2008).

In recent years, there has also been a large number of bacteria which have gained multi-resistance towards drugs and antibiotics. One example is the methicillin-resistant Staphylococcus Aureus (MRSA) which is resistant to many antibiotics and drugs. (Nikaido, 2009). Therefore, alternative antibiotics are needed to combat this problem.

Betel nut is known to be an Indian traditional medicine. It is chewed by many people, along with piper, betel leaf, slaked lime or gambir. It is known to cause buccal cancer and is highly addictive. However, because of its notorious and infamous reputation, not much research has been done to delve deeper into its antibacterial, antioxidant and wound healing properties. Hence, this study would want to look into it's antibacterial, antioxidant and wound healing properties.

He shou wu is a herb used in Chinese Traditional Medicine to treat hair loss, back pain and cough. The biological compounds found in He Shou Wu may also positively affect blood cholesterol levels and improve conditions caused by an age-related decline of the nervous system, such as Alzheimer's disease. However, it is known to cause liver damage and hence little research on its antibacterial, antioxidant and wound healing properties have been conducted.

Objectives

This project aims to examine the efficacy of *Areca catechu* and *Polygonum multiflorum* in limiting bacterial growth, show antioxidative effects and hence facilitating wound healing.

Hypothesis

Both *Areca catechu* and *Polygonum multiflorum* will display antibacterial properties, with *areca catechu's* antibacterial properties being stronger. Both *Areca catechu* and *Polygonum multiflorum* will display antioxidant effects, with *Polygonum multiflorum's* antioxidant effects being stronger. Only *Polygonum multiflorum* will display wound-healing effects.

Materials and methods

Extraction

Areca catechu seeds were collected from a local market and placed into a blender, along with either water or 1% DMSO solution. These were blended to obtain soluble and insoluble components, and the supernatant was decanted. The supernatant was filtered with a microfilter (pore size 0.45um) to remove all bacteria and maintain sterility in downstream tests. The supernatant was aliquoted and stored at 0℃ until needed for future use.

Well Diffusion Test

Bacteria were cultured in nutrient broth at 37 degrees Celsius overnight. The resultant suspension was used for subsequent testing. Using a sterile cotton swab, the bacteria was spread evenly onto sterile agar plates. Wells was punched onto these inoculated agar plates. 100μL of either sample (*Areca catechu* or *Polygonum multiflorum)* extracts (m/v), 10% bleach (positive control) or sterile water (negative control) was introduced into each well. The set up was incubated at 37℃ overnight in an incubator and the resultant bacterial growth was observed. Any resultant zones of inhibition generated were measured.

Colony Count (Time Point Assay)

Bacteria were cultured overnight in nutrient broth, quantified and its numbers adjusted to a starting concentration of 10^6 CFU/ml. The bacterial suspension will then be mixed with either 100 μL of betel nut extract, 10% bleach (positive control), or sterile water (negative control). Samples of the mixtures was taken at 20-minute intervals, serially diluted and plated onto fresh agar plates. These will then be incubated at 37℃ overnight. The resultant colonies was counted and the actual bacterial numbers at various time points calculated.

DPPH assay

1.9 mL of methanol was mixed with 0.1 mL of betel nut extract and 1 ml of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution. A negative control containing 2 ml of methanol and 1ml of DPPH solution will be also prepared. The tubes were placed in the dark for 10 minutes before spectrophotometric measurements will then be taken at 517nm wavelength of light. The measurements were referenced against that obtained from the control setup. By calculating the percentage of DPPH left, we were able to calculate the antioxidant effects of the extract.

$$\left(\frac{A_{517}\text{ of control} - A_{517}\text{ of sample}}{A_{517}\text{ of control}}\right) \times 100$$

Microscopic Examination

20 µL each of extract and 20 µL of fresh bacteria culture was introduced onto a sterile glass slide. This was placed under the microscope, and any morphological changes to the bacterial were documented by photomicrographs.

Dialysis Extracts

The dialysis tubing was sterilised by putting it into hydrogen peroxide solution for 1 hour. The sterile extracts will then be placed in the dialysis tubing and dialysed against sterile water. The high and low molecular fractions will then be separated and stored in the refrigerator at -4℃ for future usage.

Wound Healing Assay

Lumbricus terrestris were washed with deionised water until no visual debris are seen. *Lumbricus terrestris* then had its last three anterior segments surgically removed with a sterile blade. Petri dishes with filter paper will then be set up, each moistened with 1ml of either 5,10 or 15% (m/v) extract or sterile water into the petri dishes. The set up was placed at room temperature and the time taken for the segments to grow back was recorded.

Results

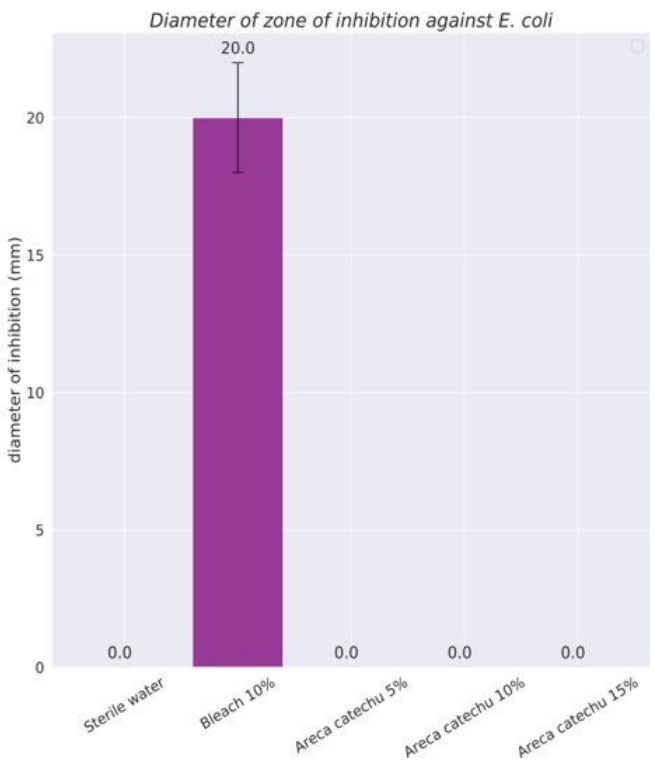


Figure 1: Well diffusion Assay results

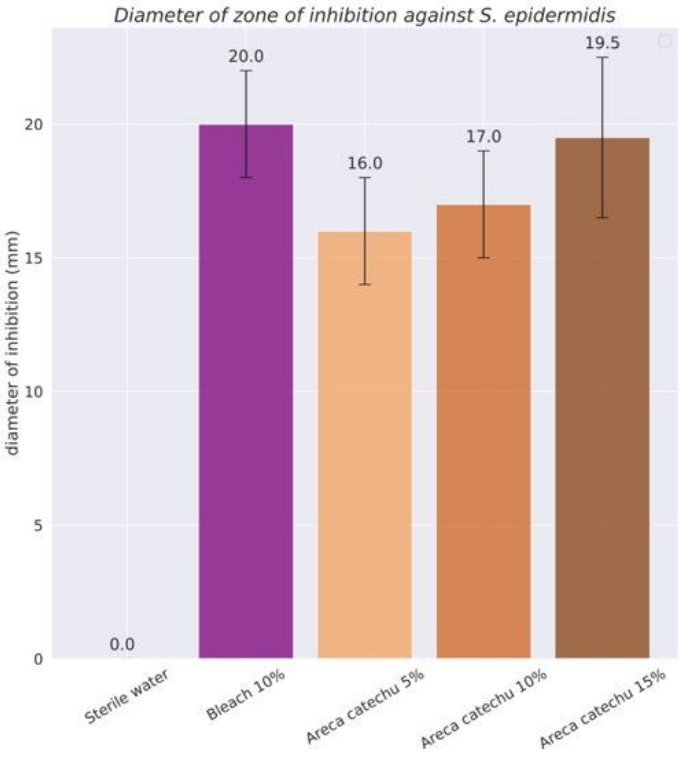


Figure 2: Well diffusion Assay results Water based *Areca catechu*

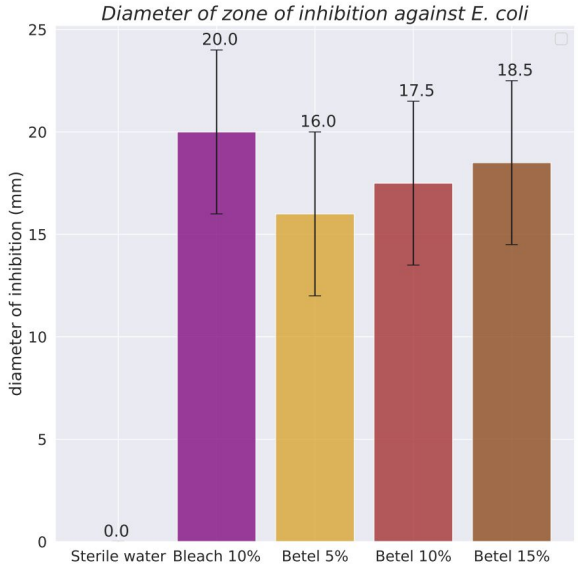


Figure 3: Well diffusion Assay results (DMSO)

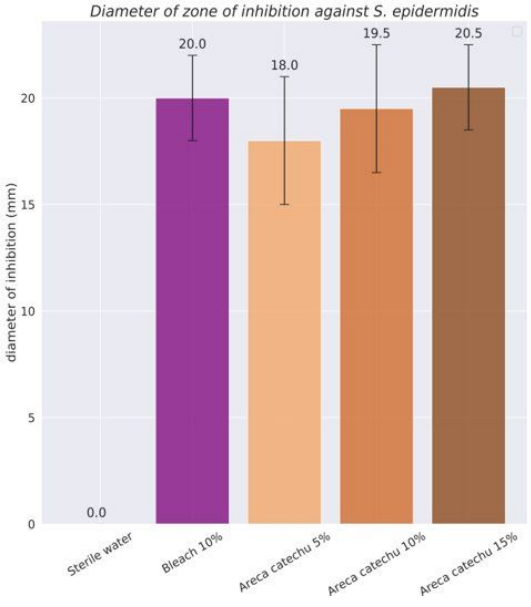


Figure 4: Well diffusion assay (DMSO)

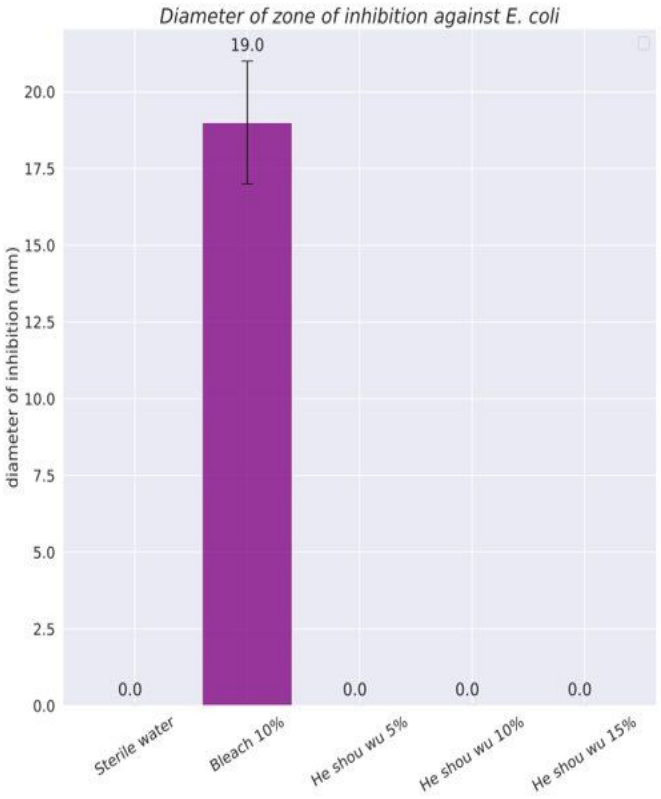


Figure 5: Well diffusion Assay

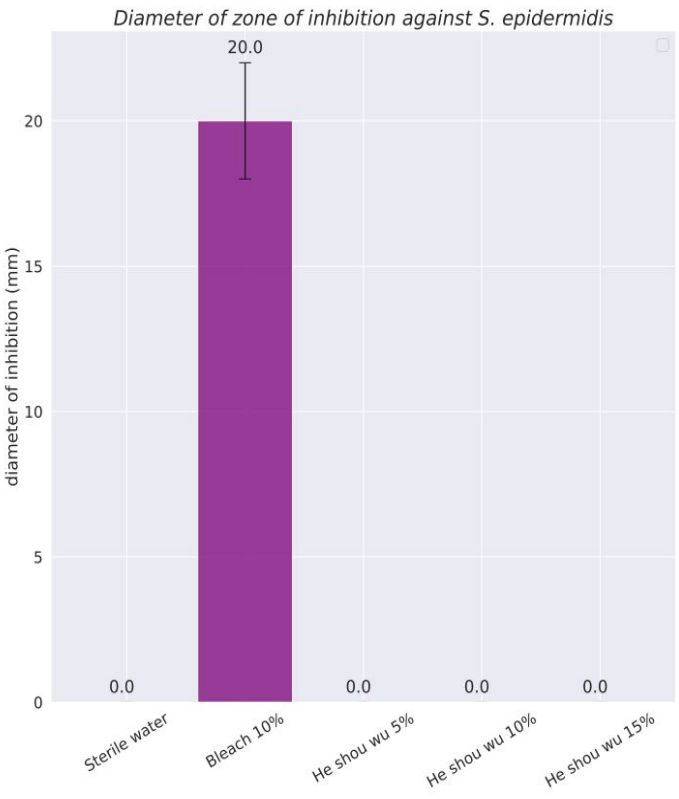
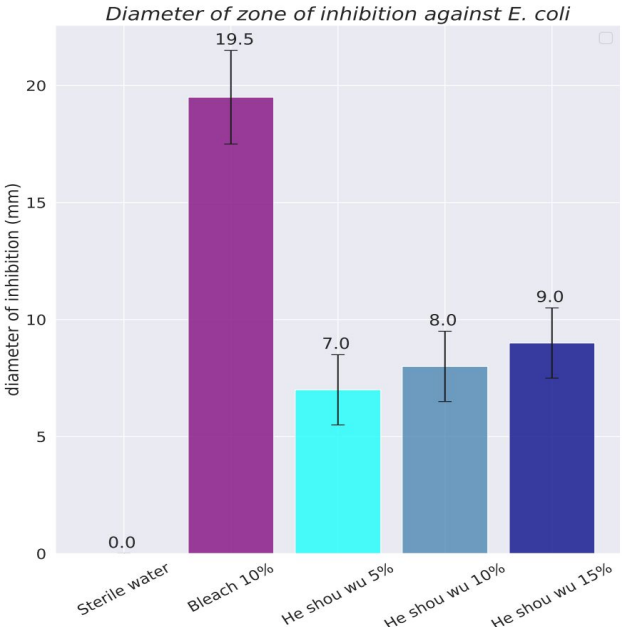


Figure 6: Well diffusion Assay



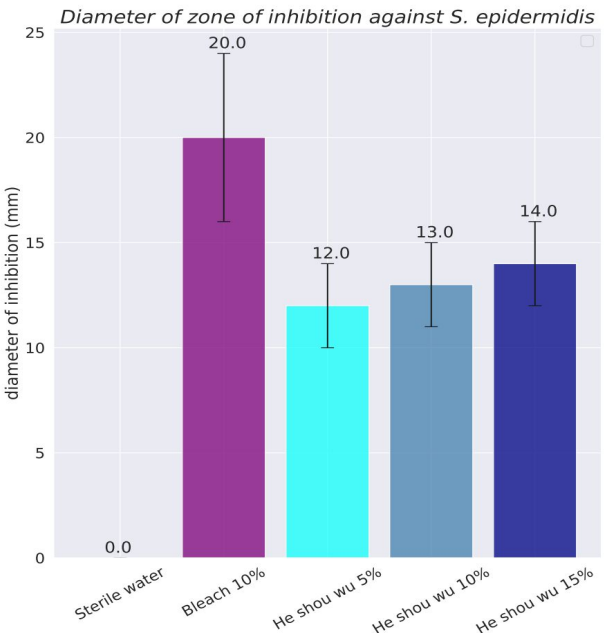
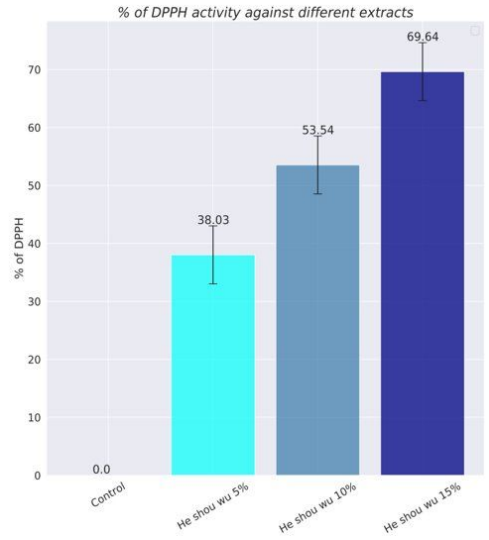


Figure7: Well Diffusion assay results

Figure 8: Well Diffusion Assay



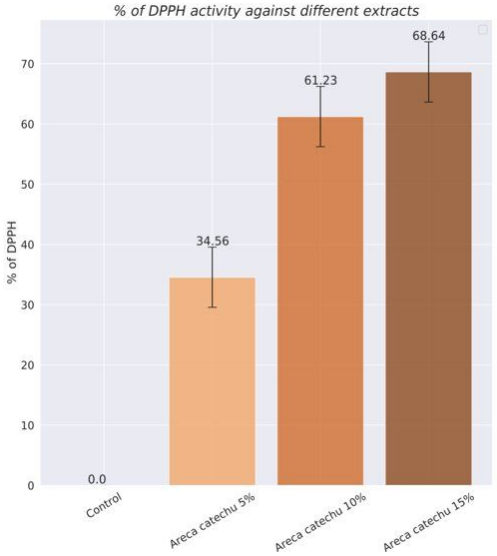
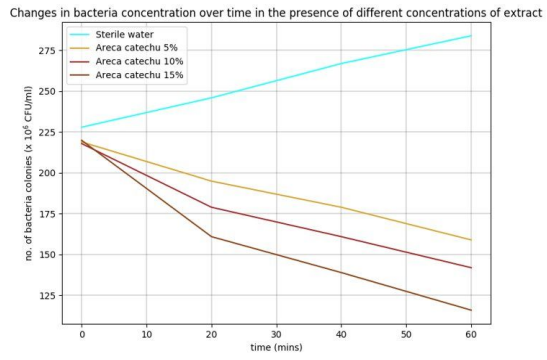


Figure 9: DPPH assay (*P.multiflorum)*

Figure 10: DPPH assay (*Areca catechu)*



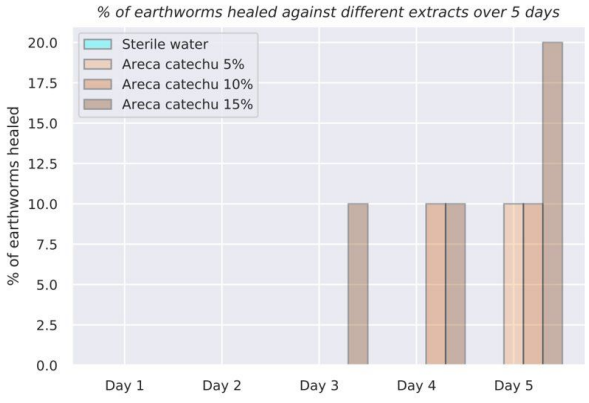


Figure 11: Timepoint Assay (*A.catechu)*water based

Figure 12: Wound healing assay

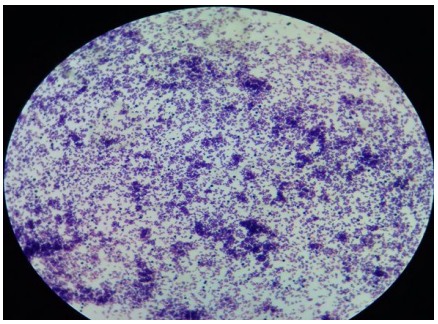


Figure 1: Effects of *Areca catechu* against *S. epidermidis*

Areca Catechu (water-based) extracts causes agglutination of Gram-positive bacteria but not Gram-negative bacteria, suggesting specific recognition and binding of specific molecules in the extract that would kick-start a cascade of molecular events resulting in bacterial death.

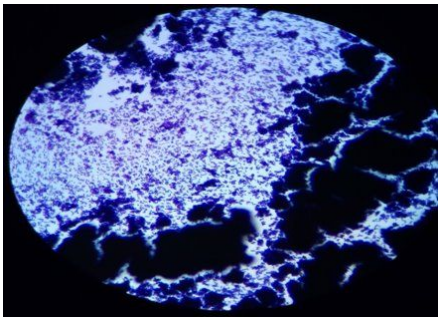


Figure 2: Effects of *Areca catechu* against *E.coli*

Areca catechu (water-based) extracts does not affect agglutination in *E.coli*, likely because it cannot recognise the specific

Discussion

In this project, we did a well diffusion assay to determine the efficacy of *Areca catechu* (water-based and DMSO-based) and *Polygonum multiflorum* (water-based and DMSO-based) in limiting bacterial growth against *S. epidermidis* and *E. coli*. *Areca catechu* (water-based) showed a zone of inhibition towards *S.epidermidis* and not *E.coli.* This suggests that *Areca catechu* shows antibacterial effects against Gram-positive bacteria and not Gram-negative bacteria. Microscopic visualization confirms that the water-based *Areca catechu* Extracts shows signs of cell agglutination on *S.epidermidis.* However, when done on *E.coli, Areca catechu* showed no signs of antibacterial effects against *E. coli.* This suggests that differential antimicrobial activity of *Areca catechu* is dependent on *Areca catechu* recognizing specific pathogen-associated molecular patterns (PAMPs) on Gram-positive bacteria. However, when *Polygonum multiflorum* was tested against *S. epidermidis* and

E.coli it showed no zone of inhibition. A DPPH Assay was also done to evaluate the antioxidant effect of both *Areca catechu* and *Polygonum multiflorum.* From the results, both *Areca catechu* and *Polygonum multiflorum* have strong antioxidant effects, with dosage-dependent effects. It is also observed that the antioxidant effect increases along with the concentration of the extracts. The strong antioxidative effects of *Areca catechu* allows it to facilitate wound healing.

Dialysis of water based *Areca catechu* against sterile water was done for 24 hours under non sterile conditions. A well diffusion assay was conducted for the extracts collected. After the test, the average diameter zone of inhibition increased of *Areca catechu* against *S.epidermidis* and *E.coli* exponentially. We propose that in *Areca catechu* . There is a class of proteins which are related to pentraxins that oligomerise in its inactivated state but are separated upon activation to effect antibacterial activities. The reason why there was no enhanced activity in *Areca catechu* extracts it was because it was immediately used or was frozen. Dialysis, especially under non sterile conditions, activated the oligomers and caused these to fragment into individual active components and hence causes the enhanced antibacterial activities.

Conclusion

Areca catechu has antibacterial effects against Gram-positive bacteria whereas *Polygonum multiflorum* has no antibacterial effects at all in a water-based solvent. Differential antimicrobial activity of *Areca catechu* is dependent on *Areca catechu* recognizing specific pathogen-associated molecular patterns (PAMPs) on Gram-positive bacteria. Both *Areca catechu* and *Polygonum multiflorum* have strong antioxidant effects, while *Areca catechu* has wound-healing effects.

References

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Appendix

Table 1: Diameter of zone of inhibition effected by *Areca catechu* water-based extracts (mm)

	Areca catechu 5%	*Areca catechu* 10%	*Areca catechu* 15%	Bleach 10%
E. coli	No	No	No	19.0±1.0
S. epidermidis	16.0±1.3	18.5±1.4	19.5±2.1	20.0±1.0

Table 2: Diameter of zone of inhibition effected by *Areca catechu* DMSO based extracts (mm)

	Areca catechu 5%	*Areca catechu* 10%	*Areca catechu* 15%	Bleach 10%
E. coli	16.0±1.0	17.0±1.0	18.0±1.0	19.0±1.0
S. epidermidis	17.0±2.0	18.5±2.0	20.0±1.5	20.0±1.0

Table 3: Diameter of zone of inhibition effected by *Polygonum multiflorum* water-based extracts (mm)

	Polygonum multiflorum 5%	*Polygonum multiflorum* 10%	*Polygonum multiflorum* 15%	Bleach 10%
E. coli	No	No	No	19.0±1.0
S. epidermidis	No	No	No	20.0±1.0

Table 4: Diameter of zone of inhibition effected by *Polygonum multiflorum* DMSO based extracts (mm)

	Polygonum multiflorum 5%	*Polygonum multiflorum* 10%	*Polygonum multiflorum* 15%	Bleach 10%
E. coli	No	No	No	19.0±1.0
S. epidermidis	7.0±0.5	8.0±0.75	9.0±0.5	20.0±1.0

Table 5: Concentration of *S. epidermidis* against time points for *Areca catechu* water based extracts ($\times 10^6$)

	Areca catechu 5%	*Areca catechu* 10%	*Areca catechu* 15%	Sterile water
0th minute	219	218	220	228
20th minute	195	179	161	246
40th minute	179	161	139	267

60th minute	159	142	116	287

Table 6: DPPH Assay (% of DPPH activity remaining)

Areca catechu 5%	38.03
Areca catechu 10%	53.54
Areca catechu 15%	69.64
Polygonum multiflorum 5%	36.15
Polygonum multiflorum 10%	61.34
Polygonum multiflorum 15%	67.97
Vitamin C	71.0
Control setup	0.0

Table 7: Wound healing assay for *Areca catechu* water based extracts (% of healing)

	Areca catechu 5%	*Areca catechu* 10%	*Areca catechu* 15%	Sterile water
1st Day	0	0	0	0
2nd Day	0	0	0	0
3rd Day	0	0	0	0
4th Day	0	11	11	0
5th Day	0	11	22	0