ANTIMICROBIAL PROPERTIES OF *ANACARDIUM OCCIDENTALE* (CASHEW) LEAF EXTRACT AGAINST *STAPHYLOCOCCUS AUREUS* AND *ESCHERICHIA COLI*

NOR AIN NABIHAH MOHD ALI JINAH, (CORRESPONDING AUTHOR)
DEPARTMENT OF NUTRITION SCIENCES, KULLIYYAH OF ALLIED HEALTH SCIENCES, INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA, INDERA MAHKOTA, 25200 KUANTAN, PAHANG, MALAYSIA.
anabihah2611@gmail.com

HANANI AHMAD YUSOF @ HANAFI, PhD
DEPARTMENT OF BIOMEDICAL SCIENCES, KULLIYYAH OF ALLIED HEALTH SCIENCES, INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA, INDERA MAHKOTA, 25200 KUANTAN, PAHANG, MALAYSIA.
hanani@iium.edu.my

HANAPI MAT JUSOH, PhD
DEPARTMENT OF NUTRITION SCIENCES, KULLIYYAH OF ALLIED HEALTH SCIENCES, INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA, INDERA MAHKOTA, 25200 KUANTAN, PAHANG, MALAYSIA.
hanapi@iium.edu.my

ABSTRACT

Introduction: *Anacardium occidentale* (cashew) leaf has been recognized as a potential source of natural antibacterial and antimicrobial compounds. **Aims:** This study investigated the antimicrobial properties of *Anacardium occidentale* (cashew) leaves extract against the most common human pathogenic bacteria specifically *Staphylococcus aureus* and *Escherichia coli*. **Materials and methods:** *Anacardium occidentale* leaves were extracted using 70% methanol. The antimicrobial activities of the extracts were evaluated by determining the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) value. **Results:** The results showed that both bacteria were sensitive towards the leaves extract. However, *E. coli* was more sensitive towards the leaves extract than *S. aureus* which showed the lowest inhibitory concentration at 0.353 mg/ml and 0.705 mg/ml respectively. In addition, there was no significant different between the minimal concentrations of leaves extract that can kill both bacteria. The result also showed that no colony present in agar plates for both bacteria at concentration 2.821 mg/ml. **Conclusion:** Therefore, it can be concluded that *Anacardium occidentale* (cashew) leaves extract are effective as antimicrobial agent against *S. aureus* and *E. coli* bacteria.

Keywords: Antimicrobial, *Anacardium occidentale*, MIC, MBC, *Staphylococcus aureus*, *Escherichia coli*

INTRODUCTION

Recently, the interest of public in using phytomedicine for the treatment of diseases is increasing. Natural products such as plants are believed to cure diseases and with no adverse effects. It has been well documented that natural products play critical roles in the development of drug with anti-inflammatory and antibacterial properties (Varghese et al, 2013). There is a need to shift from using synthetic drugs and antibiotics to alternative treatment using plants products because the safety and efficacy of the drugs remain questionable. Synthetic drugs are man-made medicines that use chemical ingredients to treat, cure, prevent diseases and promote well-being. However, the excessive use of drugs may bring negative effects to health such as hallucination, addiction, loss of self-control and the worst is death (National Institute Drug Abuse, 2017).

Furthermore, synthetic antibiotics are associated with microbial resistance over the time and thus resist the effects of drugs. Therefore, the use of plants products could be a promising alternative (Turing et al., 2019). Plants contain many phytochemicals including alkaloids, flavonoids, phenolic acids, saponins, terpenes and tannins. These phytochemicals have pharmacological properties such as antimicrobial, anticarcinogenic, analgesic, antifungal, antitumor, antioxidant, antimalarial and anti-inflammation (Yadav & Agarwala, 2011). Various species and parts of plants including leaf, bark, seeds, roots, flower and fruits have different bioactive compounds which may have different medicinal properties.

Anacardium occidentale L. which is known as cashew plants is one of the tropical culinary herbs that provide various benefits to human health. In Southern Asia countries like Thailand, Malaysia and Indonesia, *A. occidentale* leaves are usually consumed raw (*ulam*) with white rice. *A. occidentale* is reportedly helping in digestion, urination, blood circulation and constipation (Yadav & Agarwala, 2011). Previous studies by Catherine and Anoze (2018) reported that *A. occidentale* leaves extract have antimicrobial properties against *S. aureus*, *E. coli* and *P. aeruginosa* bacteria and Chabi et al. (2014) reported that *A. occidentale* leaves extract possesses antimicrobial effect against *S. aureus*, *E. coli*, *P. aeruginosa* and *S. epidermis* bacteria.

As it is well established fact that bacteria are the leading cause of infectious disease that infects human body system. *S. aureus* bacterial infections range from mild to life threatening and the most common one is skin infection. Commonly, skin infection will cause abscesses, blisters, redness, swelling, cellulitis and skin pigmentation (Bush, 2019). *E. coli* can cause infection even if consumed in a small amount. Consuming slightly undercooked foods such as meat and unpasteurized milk that have been contaminated with *E. coli* can cause severe abdominal cramps,

food poisoning, diarrhoea and vomiting. If left untreated, it may cause life-threatening diseases like pneumonia, kidney failure and urinary tract infections (Lim, Yoon & Hovde, 2010). Therefore, this study was design to access the effect of cashew plant leaf extract against human pathogenic bacteria, *Staphylococcus aureus* and *Escherichia coli*.

MATERIALS AND METHODS

Location Study

This study was conducted in the Natural Products Laboratory, Department of Biotechnology, Kulliyyah of Science Microbiology Laboratory, Department of Biomedical Sciences & Department of Nutrition Sciences, Kulliyyah of Allied Health Sciences, IIUM Kuantan.

Sample Collection

The leaves of *A. occidentale* were collected from rural areas around Kuala Berang, Terengganu.

Extraction of the Leaves

The leaves undergone were rinsed, dried, cut, ground, soaked with 70% methanol, filtered, evaporated and freeze-dried prior using for antimicrobial process. The extracts were diluted with 70% methanol and filtered under sterile filtration process to obtain liquid form of the extract. The first leaves extract concentration was 22.570 mg/ml.

Antimicrobial Process

Agar Preparation

Nutrient agar was used for the cultivation of *S. aureus* bacteria and Eosin Methylene Blue (EMB) agar was used for the cultivation of *E. coli* bacteria. About 20g powdered nutrient agar was dissolved in 1L distilled water and was sterilized by autoclaving at 121°C for 2 hours. Then, 5ml of the medium were dispensed into each petri dish and left to solidify. Each petri dish was labelled with date, types of agar and stored in chiller at 4°C.

Broth Preparation

The function of broth is to provide a growth medium for bacteria by giving a constant amount of nutrients that allows the bacteria to reproduce quickly. Nutrient broth was used since it is a general purpose medium that can cultivate a broad variety of fastidious and non-fastidious bacteria with non-exacting nutritional requirements. Nutrient broth was used later in the glycerol stock preparation and MIC method. The 13g powdered nutrient broth was dissolved in 1L distilled water and sterilized by autoclaving at 121°C for 2 hours. Then, the nutrient broth was stored in chiller at 4°C.

Bacterial Growth

Small amount of bacteria *S. aureus* from frozen stock were streak out by using inoculation loop and transferred onto nutrient agar plate using aseptic techniques. The steps were repeated for *E. coli* bacteria which was then transferred onto EMB agar plate.

Bacterial Identification

Gram-staining method used to differentiate between two groups of bacteria. The results showed *S. aureus* is gram-positive bacteria which observed stained with violet, purple in colour and cocci in shape while *E. coli* is gram-negative bacteria that showed stained with pink colour and rod-shaped. Next, to strengthen the bacteria identification, catalase and coagulase test was done on both bacteria. *S. aureus* and *E. coli* bacteria showed presence of bubble in catalase test while clumping factor in coagulase test showed the bacteria were *S. aureus.*

Glycerol Stock Preparation

The purpose of preparing bacterial glycerol stock was to store bacteria for a longer time period to stabilizes the frozen bacteria, prevent damage of cell membrane and keeping the cell alive. Fifty (50) ml of 100% glycerol was diluted with 50ml of sterile distilled water in sterile schott bottle and autoclaved at 121°C for 2 hours. The 600µl of 50% glycerol, 400µl of nutrient broth and a small sample of *S. aureus* bacteria were transferred into microcentrifuge tube. The tube was labelled and stored at -20°C until further use. These steps were repeated for *E. coli* bacteria.

Assessment of Antimicrobial Properties

i. Minimum Inhibitory Concentration (MIC)

The first concentration of leaf extract was 22.57 mg/ml. The next 2 to 12 concentration of leaves extract was calculated using formula below:

$$M1V1 = M2V2$$
$$22.570 \text{ mg/ml } (100\mu l) = M2\ (200\mu l)$$
$$M2 = (22.570 \text{ mg/ml } (100\mu l))/(200\ \mu l)$$
$$M2 = 11.285 \text{ mg/ml}$$

The concentration of *S. aureus* and *E. coli* bacteria were measured using ELISA spectrophotometer to obtain standardized amount of bacteria at optical density, OD 600nm wavelength ±0.010. The MIC method was done in 96-well plate. For negative control, 200µl of nutrient broth and 100µl of *A. occidentale* leaves extract were transferred into the first 3 columns in first row of 96-well plate. For positive control, 100µl of nutrient broth, tetracycline hydrochloride antibiotics and *S. aureus* bacteria were transferred into the second 3 columns in first row of 96-well plate.

Then, 100µl of nutrient broth were transferred into each 12 columns in the second row respectively. Next, 100µl of *A. occidentale* leaves extract were transferred into the first columns in second row of the plate. Serial dilution was done by transferring 100µl of leaves extract from first column to second columns and continuously in sequence until the 12th column. 100µl of *S. aureus* bacteria was transferred into each column in second row of the plate respectively. These steps were repeated for 2nd and 3rd replicate in the 3rd and 4th row of the plate. Besides, same steps were repeated for *E. coli* bacteria in the 5th, 6th and 7th row of the plate. Each columns and row were labelled with concentration and types of bacteria accordingly. The 96-well plate then was incubated for 24 hours in 37℃. After incubated, the plate was measured by VersaMax Tunable Microplate Reader and the results were recorded in Table 1.

ii. Minimum Bactericidal Concentration (MBC)

MBC is the continuity of MIC method. About 10µl of the first, second, third, fourth and fifth columns of antimicrobial *S. aureus* and *E. coli* that considered as MIC values of *A. occidentale* leaves extract were transferred into nutrient and EMB agar plate respectively. The plates were labelled and incubated for 24 hours at 37℃. These steps were repeated for the 2nd and 3rd replicate of *S. aureus* and *E. coli* bacteria respectively. The MBC results were recorded in Table 2.

Statistical Analysis

The data was statistically analysed using Mann-Whitney U test. The difference was recorded as significant at p-value ($p<0.05$).

RESULTS

Minimum Inhibitory Concentration (MIC)

The MIC results of *A. occidentale* leaves extract towards both bacteria are shown in the Table 1. The results showed that the extracts inhibit the growth of *S. aureus* bacteria at 1st until 4th concentration while *E. coli* bacteria at 1st until 5th concentration.

Table 1: MIC result

Leaves Extract Concentration, (mg/ml)	Bacteria					
	S. aureus			*E. coli*		
	1st replicate	2nd replicate	3rd replicate	1st replicate	2nd replicate	3rd replicate
5.642	(-)	(-)	(-)	(-)	(-)	(-)
2.821	(-)	(-)	(-)	(-)	(-)	(-)
1.411	(-)	(-)	(-)	(-)	(-)	(-)
0.705	(-)	(-)	(-)	(-)	(-)	(-)
0.353	(+)	(+)	(+)	(-)	(-)	(-)
0.176	(+)	(+)	(+)	(+)	(+)	(+)
0.088	(+)	(+)	(+)	(+)	(+)	(+)
0.044	(+)	(+)	(+)	(+)	(+)	(+)
0.022	(+)	(+)	(+)	(+)	(+)	(+)
0.011	(+)	(+)	(+)	(+)	(+)	(+)
0.006	(+)	(+)	(+)	(+)	(+)	(+)
0.003	(+)	(+)	(+)	(+)	(+)	(+)

Note: (+) presence growth of bacteria; (-) absence growth of bacteria

Minimum Bactericidal Concentration (MBC)

MBC results are shown in Table 2. The results showed that there is no growth of both bacteria in nutrient and EMB agar plates at 1st and 2nd concentration.

Table 2: MBC results

Column in plate	Concentration, (mg/ml)	Bacteria					
		S. aureus			*E. coli*		
		1st	2nd	3rd	1st	2nd	3rd
1	5.642	(-)	(-)	(-)	(-)	(-)	(-)
2	2.821	(+)	(-)	(-)	(-)	(-)	(+)
3	1.411	(+)	(+)	(+)	(+)	(+)	(+)
4	0.705	(+)	(+)	(+)	(+)	(+)	(+)
5	0.353	(+)	(+)	(+)	(+)	(+)	(+)

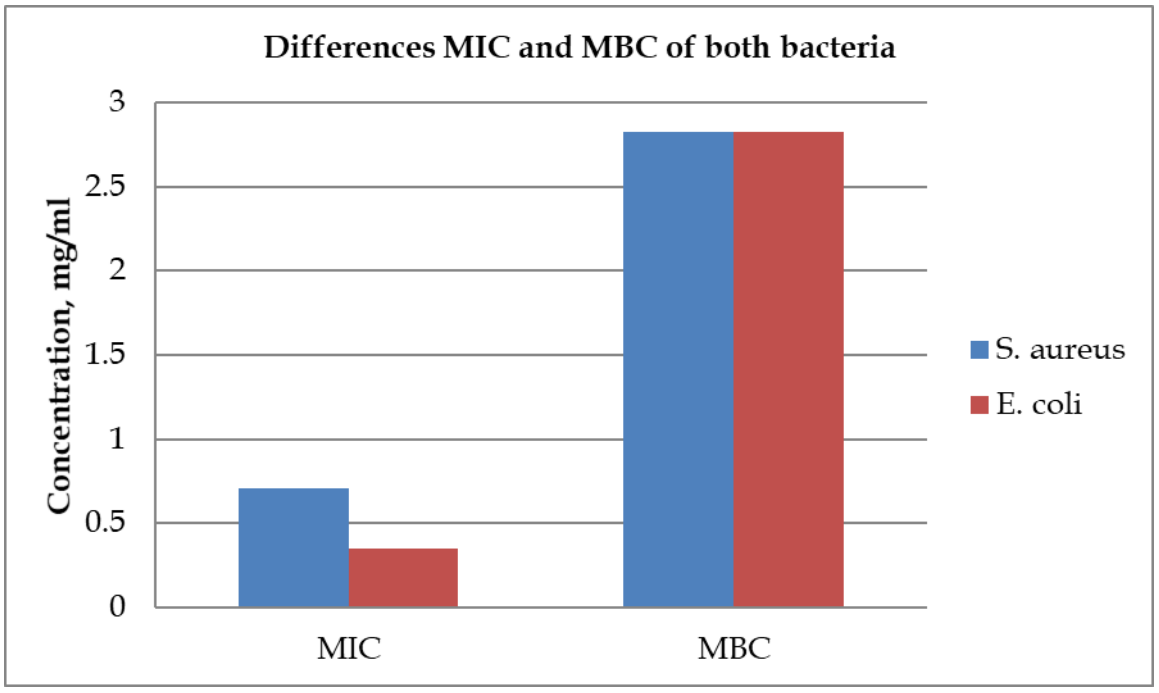
Note: (+) presence growth of bacteria; (-) absence growth of bacteria

The differences between MIC and MBC value between *S. aureus* and *E. coli* bacteria were recorded are indicated in the Table 3. The results showed that MIC value of *E. coli* bacteria was lower than *S. aureus* bacteria meanwhile MBC value was similar for both bacteria.

Table 3: Final result of MIC and MBC

Bacteria	MIC, (mg/ml)	MBC, (mg/ml)	Ratio of MIC/MBC
S. aureus	0.705	2.821	1: 4
E. coli	0.353	2.821	1: 8

Note: All results are the mean of triplicate measurements



DISCUSSION

Effectiveness of extracts between *S. aureus* and *E. coli* bacteria

In this study, *A. occidentale* leaves was extracted using methanol and tested against two types of bacteria, *S. aureus* and *E. coli*. Methanol was chosen because it is the most effective solvent for extraction. It produced the highest extraction yield as well as highest content of tannins, saponins, flavonoid, phenolic, alkaloids and terpenoids from plants (Truong et al., 2019).

Our current study showed that, *A. occidentale* leaves extract has bacteriostatistic and bactericidal effects towards gram-positive bacteria, *S. aures* and gram-negative bacteria, *E. coli*. Based on MIC and MBC, these antimicrobial properties of *A. occidentale* leaves extract could be attributed by the presence of abundance phytochemicals found in the plant. Tannins, saponins, flavonoid, phenolic, alkaloids and terpenoids were found in the leaves extract which served as antimicrobial agents (Goncalves & Gobbo, 2012). For example, it was shown that flavonoids could destroy the cell wall of bacteria and thus affect the entire mechanism of microbial cell (Catherine & Anoze, 2018). However, it appears that the antimicrobial effects of *A. occidentale* leaves extract were more effective against gram-negative bacteria, *E. coli* than gram-positive bacteria, *S. aureus*. The fact that gram-positive bacteria devoid of outer membrane in their cell walls may suggest that the membrane may be responsible of the difference observed in the sensitivity level between gram-positive and gram-negative bacteria in presence of the extracts (Agedah, Bawo & Nyananyo, 2010).

The effects of *A. occidentale* leaves extract on MIC from other studies showed mixed results. Ajileye et al. (2015) used methanol (80%) leaves extract reported a lower MIC value (0.125mg/ml) than our study for both bacteria. Similarly, Dahake et al. (2009) used ethanol-based extraction and reported lower MIC values for *S. aureus* and *E. coli* at the concentration of 0.015mg/ml and 0.031mg/ml, respectively compared to our study. Chabi et al. (2014) also used ethanol-based extraction showed that MIC value was lower for *S. aureus* (0.313mg/ml) but higher for *E. coli* (0.625mg/ml) as compared to our study. In addition, Catherine and Anoze (2018) reported a higher MIC compared to our study when using ethanol-based extraction method. The MIC value was at concentration between 250mg/ml and 500mg/ml for both bacteria. For MBC value, the results from other studies also showed a similar trend. When compared to our study, the MBC values were higher for both *S. aureus* and *E. coli* at the concentration of 500mg/ml and 20mg/ml, respectively (Catherine

& Anoze, 2018). However, Ajileye et al. (2015) reported a lower MBC value (0.500mg/ml) for both bacteria compared to our study. In another study, the MBC value was also lower (1.25mg/ml) for *S. aureus* but higher for *E. coli* (10mg/ml) (Chabi et al., 2014).

We believe the differences observed from our study with other studies could be explained by the different methods used in the extraction resulting different yield of phytochemicals. A study by Ayepola & Ishola (2009) reported that methanol extract of the leaves was more effective than aqueous leaves extract due to the ability of methanol to extract the wider range of antimicrobial properties. Another study by Varghese et al. (2013), reported that the aqueous extract was more effective than methanol extract against periodontal pathogens like *P. gingivalis* and *P. intermedia*. The methanol extract was highly active against selected pathogens like *E. coli* and *Bacillus subtilis*. In another study, the zone of inhibition of plates inoculated with *S. aureus* was greater than *E. coli* bacteria when exposed to ethanolic extract Agedah et al. (2010) Thus, these findings may suggest that extraction methods probably have major impact on antimicrobial properties of the leaves.

Overall, this study showed the potential use of *A. occidentale* leaves extracts as antimicrobial agents against infection caused by *S. aureus* and *E. coli* bacteria. Future studies should focus more on the antimicrobial properties of *A. occidentale* leaves extracted using methanol at various concentrations and which can be tested against different types of bacteria.

CONCLUSION

The present study result showed that *A. occidentale* leaves extract have antimicrobial properties against *S. aureus* and *E. coli* bacteria through MIC and MBC method. *E. coli* bacteria required a lower concentration of leaves extract to inhibit their growth compared to *S. aureus* bacteria. It can be suggested that *A. occidentale* leaves extract contains antimicrobial properties against both types of pathogenic bacteria.

ACKNOWLEDGEMENTS

This research was funded by the Ministry of Higher Education's Fundamental Research Grant Scheme (FRGS19-022-0630) to which the authors would like to express their gratitude as well as the International Islamic University Malaysia.

REFERENCES

Agedah, C., Bawo, D. D. ., & Nyananyo, B. (2010). Identification of antimicrobial properties of cashew, *Anacardium occidentale L*. (Family *Anacardiaceae*). Journal of Applied Sciences and Environmental Management, 14(3). https://doi.org/10.4314/jasem.v14i3.61455

Ajileye, O. O., Obuotor, E. M., Akinkunmi, E. O., & Aderogba, M. A. (2015). Isolation and characterization of antioxidant and antimicrobial compounds from *Anacardium occidentale* L. (*Anacardiaceae*) leaf extract. Journal of King Saud University - Science, 27(3), 244–252. https://doi.org/10.1016/j.jksus.2014.12.004

Ayepola, O., & Ishola, R. (2009). Evaluation of Antimicrobial Activity of *Anacardium occidentale* (Linn.). Advances in Medical and Dental Sciences, 3(1), 1–3.

Bush, L. M. (2019, June). *Staphylococcus aureus* Infections. Retrieved from https://www.msdmanuals.com/home/infections/bacterial-infections-gram positive-bacteria/staphylococcus-aureus-infections

Catherine, B., & Anoze, A. M. (2018). Antibacterial Activity of *Anacardium occidentale* (Cashew) Leaf Extracts on *Staphylococcus aureus*, *Escherichia Coli* and, 4(1), 19–27.

Centre for Disease Control and Prevention. (2018, September 10). About Antimicrobial Resistance. Retrieved from https://www.cdc.gov/drug resistance /about.html

Chabi, S. K., Sina, H., Adoukonou-Sagbadja, H., Ahoton, L. E., Roko, G. O., Saidou, A.,Baba-Moussa, L. (2014). Antimicrobial activity of *Anacardium occidentale L*. leaves and barks extracts on pathogenic bacteria. African Journal of Microbiology Research, 8(25), 2458–2467. https://doi.org/10.5897/ajmr 2014.6859

Dahake, A. P., Joshi, V. D., & Joshi, A. B. (2009). Antimicrobial screening of different extract of *Anacardium occidentale Linn*. leaves. International Journal of ChemTech Research, 1(4), 856–858.

Gonçalves, G. M. S., & Gobbo, J. (2012). Antimicrobial effect of *anacardium occidentale* extract and cosmetic formulation development. Brazilian Archives of Biology and Technology, 55(6), 843–850. https://doi.org/10.1590/S1516-891320120 00600006

Lim, J. Y., Yoon, J., & Hovde, C. J. (2010). A brief overview of *Escherichia coli* O157:H7 and its plasmid O157. Journal of microbiology and biotechnology, 20(1), 5–14.

National Institute on Drug Abuse (NIH). (2017, March). Health Consequences of Drug Misuse. Retrieved from https://www.drugabuse.gov/related-topics/health-consequences-drug-misuse

Truong, D. H., Nguyen, D. H., Ta, N. T. A., Bui, A. V., Do, T. H., & Nguyen, H. C. (2019). Evaluation of the use of different solvents for phytochemical constituents, antioxidants, and in vitro anti-inflammatory activities of *severinia buxifolia*. Journal of Food Quality, 2019. https://doi.org/10.1155/2019/8178 294

Turing, A.M. et al., (2019). Antibiotic. Retrieved from http://www.madehow.com/Volume-4/Antibiotic.html

Varghese J., Tumkur, V.K., Ballal, V., Bhat. G.S (2013,). Antimicrobial effect of *Anacardium occidentale* leaf extract against pathogens causing periodontal disease. Advances in Bioscience and Biotechnology, 2013, 4, 15-18 ABB http://dx.doi.org/10.4236 /abb.2013.48A2003

Yadav, R., and M. Agarwala. "Phytochemical Analysis of Some Medicinal Plants". Journal of Phytology, Vol. 3, no. 12, Dec. 2011, https://updatepublishing.com/journal/index.php/jp/article/view/2737.