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ORIGINAL ARTICLE

Antimicrobial Activities of *Cucurbita moschata* Leaves Water Extracts Against Selected Bacteria and Their Compound Determination

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Abstract

In Malaysia, high-value cash crops such as pumpkin (*Cucurbita moschata*) are cultivated, but their production generates significant agricultural waste. Discarded fruits, stems, and leaves are waste materials with multiple potential uses such as animal feed and bioactive compounds. Therefore, this study aimed to examine the antibacterial properties and determine the types of phytochemical components available in water extracts from *C. moschata* leaves as an agricultural waste extract. Microwave-assisted extraction (MAE) is used to extract the plants' leaves due to its ease of use, quick extraction time, affordability, and increased yield. The leaves are obtained from Kampus Tembila, Universiti Sultan Zainal Abidin (UniSZA), Besut, Terengganu. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were run to visualise and determine the minimum concentration of pumpkin leave water extract that inhibits the six selected bacteria strains related to the gastrointestinal system of small ruminants which are *Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumoniae, Enterococcus faecalis, Salmonella enterica*, and *Acinetobacter baumannii.* Thin Layer Chromatography (TLC) was used for the phytochemical screening, and reagents such as Vanillin, Anisaldehyde, Dragendorff's, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) methanol solution were sprayed to identify the type of compound's presence in the aqueous extracts of both leaves. The *C. moschata* leaves show inhibition activity against all microbes tested except *B. subtilis*, and the lowest IC_{50} inhibition was 8 mg/ml against *A. baumannii* and 20 mg/ml for *E. faecalis*, respectively. In the meantime, the aqueous extracts of *C. moschata* leaves contain natural compounds such as triterpenes, alkaloids, tannins and antioxidants, as indicated by coloured spots derived from the TLC visualization method. This indicates the potential of Cucurbita moschata as an antimicrobial activity.

Keywords: agriculture waste; antibacterial activity; natural compound; pumpkin (*Cucurbita moschata*).

Introduction

Cucurbita moschata, or pumpkin, is a member of the Cucurbitaceae family, which includes 800 species and 130 genera of agricultural and ornamental plants. Cucumbers, melons, squashes, and pumpkins are members of this gourd family and are native to temperate and tropical regions of Asia, Africa, and Europe. They can be annual or perennial herbs (Ong et al., 2019). Most cucurbit species are climbing or prostrate vines with palmate leaves that alternate along the stem and have lengthy stalks.The leaves are divided or lobed, with reticulation and palmate veins. Cucurbit plants have a high concentration of bioactive substances such as triterpenes, sterol, and alkaloids and are rich in phytochemicals, carotenoids, terpenoids, and saponins (Rolnik & Olas, 2020).

As an alternative feed additive, bioactive compounds originating from plants have recently piqued the interest of certain researchers who aimed to increase animal performance and health without negatively impacting animal output (Kholif et al., 2020). Additionally, the leaves can be fed to animals, especially if they are unfit for human consumption. This application helps decrease waste and offers farmers an affordable feed alternative that may improve livestock health and productivity due to the presence of bioactive compound in the leaves (Aziz et al., 2023).

However, the results and effects of using pumpkin leaf water extract vary based on the type of bacteria present, the concentration of the sample leaves, and the type of plant residue left behind. When administering a high antibiotic concentration, the photogenic molecule may be poisonous to advantageous ruminal microbes (Nidia et al., 2017). Thus, this study aims to ascertain the antibacterial properties of *C. moschata* leaf water extract at various doses on a selected microbe and their phytochemical composition.

Materials and Methods

Culture media

Media used include Mueller-Hinton Agar (MHA), Mueller-Hinton Broth (MHB), and Nutrient Agar (NA). All the media are prepared according to the manufacturer's guide (Sigma). The agar plates and broth were kept in the chiller (4 ℃) until further used.

Microwave-assisted Extraction (MAE) Sample Water Extract Preparation

During harvest, agricultural waste, particularly green matured leaves from *C. moschata*, was gathered at Kampus Tembila, Universiti Sultan Zainal Abidin (UniSZA), Besut, Terengganu. After that, they were cleaned of any dust and grime by running tap water. The samples were left to air dry for three days at ambient temperature (20°C). An analytical balance was used to weigh the samples after they had dried. The airtight plastic bag was filled with the dried samples, which were then kept at room temperature.

Dried leaf samples were extracted using a microwave/convection oven (Panasonic NN-CD997S) according to the procedure of Kulkarni and Rathod, 2016. In a beaker, 10 grams of the dried leaf sample were first soaked in 100 mL of distilled water (10 g/100 mL). After being exposed to a medium-low power level (350 W) of microwave radiation for 10 minutes, the leaf sample was filtered through Whatman No. 1 filter paper. This process was done thrice to extract the most compounds from the leaves. The water extracts were then freeze-dried for 72 hours until they became powdered and kept overnight at -80°C in a freezer. Following that, the water extracts were stored at -20°C in a refrigerator until further used.

Antimicrobial Assay

Bacterial strains preparation

The frozen stock of Gram-positive bacteria *Enterococcus faecalis* (ATCC 29212), *Bacillus subtilis* (ATCC 6633), and *Staphylococcus aureus* (ATCC 25923), as well as Gram-negative bacteria *Klebsiella pneumoniae* (ATCC 70063), *Acinetobacter baumannii* (ATCC 19606), and *Salmonella enterica* (ATCC 12011) were obtained from the Microbiology Laboratory of Centralised Lab Management Centre, UniSZA. After thawing, the bacteria were then added to Mueller-Hilton Broth (MHB) and incubated for an additional night at 37°C. Subsequently, a single colony was isolated by streaking the bacterial broth onto Nutrient Agar (NA).

Minimum Inhibitory Concentration (MIC) Assay

C. moschata were tested for antimicrobial activity using the disc diffusion method by Nostro et al. (2000). From the stock solution, the bacteria cultures were prepared at the concentrations of 300 mg/ml, 150 mg/ml, 75 mg/ml, 37.5 mg/ml, 18.75 mg/ml, and 9.375 mg/ml. Using a sterilized cotton swab, bacteria cultures were surface dispersed over MHA plates to initiate the inoculation process. A sterile, blank 6 mm Whatman disk was pipetted with 20 µl of prepared samples.

This method was applied to find the lowest concentration of leaf water extracts that can stop a bacterial strain from growing (Appiah et al., 2017). The optical density (OD) reading at 625 nm—which ranges from 0.08 to 1 nm—was used to adjust the bacteria to the 0.5 McFarland standard with microplate photometer (Multiskan™ FC). For twenty-four hours, the plate was incubated at 37 °C. After the incubation period, the inoculum's OD was taken at 625nm. After that, the following formula was used to determine the inhibition percentage. Using the percentage of inhibition graph, the IC_{50} value was determined. Every test was run in three duplicates. The MBC test was performed on the clear well, which showed no signs of bacterial growth.

$$\text{Percentage of inhibition: } [1- (A_{625} \text{ sample}/A_{625} \text{ negative control})]X\ 100$$

Minimum Bactericidal Concentration (MBC) Assay

Parvekar et al. (2020) state that this technique calculates the minimum bactericidal concentration (MBC) of *C. moschata* water extracts needed to eradicate a microorganism's growth by 99.9%. Using a sterile cotton swab, the sample from the clear well that showed no signs of bacterial growth during the MIC test was swabbed onto the MHA surface. For 24 hours, the plate was incubated at 37 °C. Following incubation, the growth of the bacteria was monitored, and the MBC value was determined by calculating the lowest concentration of leaf water extracts that showed no growth on the plate.

Thin Layer Chromatography (TLC)

Water extracts of *C. moschata* leaves were subjected to TLC analysis to determine the phytochemical content (Jörk et al., 1990). The mobile phase used ethyl acetate: methanol: water with a ratio of 6:3:1 (v/v/v). After that, a few drops of extract were blotted onto the silica gel plate (what brand and type), one centimetre above the plate's bottom, and allowed to dry. The saturated developing beaker held the spotted plate. The formation of the chromatogram bands was visible under UV light with short wavelength (254 nm) and long wavelength (366 nm) (model of UV light instrument). After being sprayed with vanillin, anisaldehyde, dragendorff's, and DPPH methanol solution, the plate was heated on a hot plate to observe what colour emerged. Dragendorff reagent is a general detection technique for alkaloids, nitrogen compounds, and lipids. Vanillin

and anisaldehyde are utilized to detect aldehydes, ketones, steroids, higher alcohols, phenols, and essential oils. Water extracts from leaves were tested for their antioxidant properties using DPPH in methanol solution. After that, the Rf value was determined as follows:

$$Rf\ value = \frac{\text{Distance moved by the compound}}{\text{Distance moved by the solvent front}}$$

Data Analysis

An independent t-test using SPSS software was used to determine the statistical significance ($\rho<0.05$). Each result was given as mean ± standard deviation.

Results and Discussion

Antimicrobial Assay

Minimum inhibitory concentration (MIC) test

In this study, *C. moschata* leaves water extract demonstrated antimicrobial activity towards *E. faecalis, S. aureus, S. enterica, A. baumannii*, and *K. pneumonia* bacteria with IC_{50} values 20 mg/mL ± 2.98, 180 mg/mL ± 3.39, 82.38 mg/mL ± 2.92, 8 mg/mL ± 1.39, and 240 mg/mL ± 2.73, respectively as in Figure 1. Previous studies have demonstrated that pumpkin extract is effective against various microorganisms. According to Mohammed et al. (2018), *Cucurbit pepo*, another leaf in the Cucurbit family, has the ability to inhibit both *S. aureus* and *K. pneumonia*. Additionally, it was demonstrated by Del Castillo et al. (2019) that the pumpkin leaf extract exhibited antibacterial activity against *E. coli, K. pneumonia, and S. aureus*. Furthermore, the development of *B. subtilis, E. coli, P. aeruginosa, and S. aureus* was efficiently suppressed by the pumpkin fruit extract (Rolnik & Olas, 2020).

At the maximum concentration used in this investigation, 300 mg/mL of *C. moschata* leaves water extract is resistant to *B. subtilis* a Gram-positive bacteria. The ability of *B. subtilis* to produce endospores, which comprise over 70 distinct proteins and function as a multilayered protective structure, has been shown to influence the resistance to water extract from pumpkin leaves (McKenney et al., 2013). Contrary to the outcome, the methanol extract technique of *C. moschata* leaves demonstrated antibacterial activity against *B. subtilis* (Jayasundara et al., 2018).

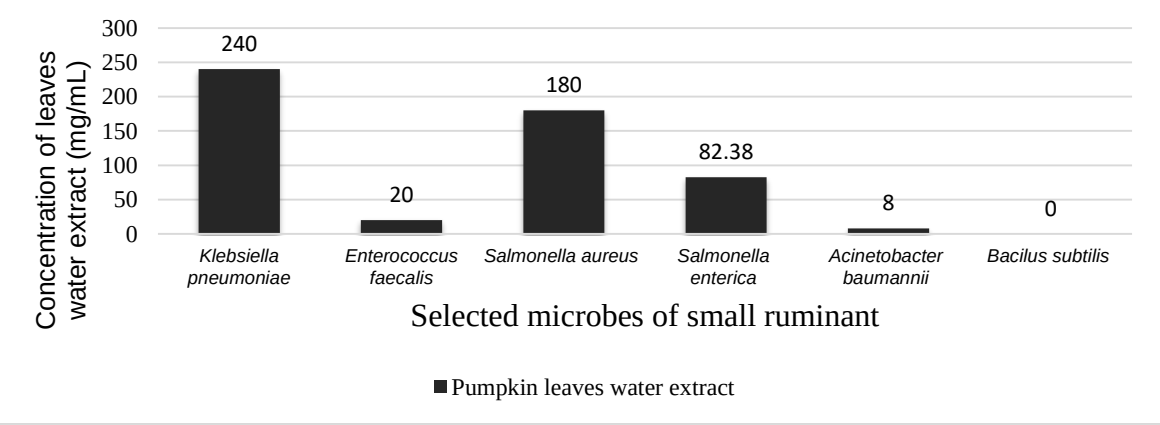

Figure 1. IC_{50} of *C. moschata* leaves water extract against selected microbes from small ruminants.

Minimum Bactericidal Concentration (MBC) Assay

To determine the minimum concentration of an antibacterial agent necessary to eradicate a bacterium, the MBC is a subculture of undergrowth microbes treated with samples of leaves and water extracts from the MIC. After incubation, each bacteria in Table 1 observed either was grown, in a thin or thick growth layer. *A. baumannii* and *E. faecalis* exhibit thin growth at 150 mg/mL and 300 mg/mL of pumpkin leaf water extract, with no further colony development.

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Table 1. MIC (IC_{50}) value and MBC of *C. moschata* leaves water extracts

Selected microbes	Gram	IC_{50} (mg/mL)	MBC (mg/mL)
B. subtilis	+	No activity	ND
E. faecalis	+	20	300
S. aureus	+	180	*
S. enterica	-	82.38	*
A. baumannii	-	8	150
K. pneumoniae	-	240	*

Abbreviations: * indicates growth (visible colony); ND indicates not determined

These may result from the antibiotic activity mechanism that prevents bacteria from synthesizing proteins, affecting their cells' survival (colony-forming units) (Reygaert, 2018). An agent that inhibits protein synthesis is a chemical that stops or slows down the development or proliferation of cells by interfering with the processes that directly produce new proteins. Certain antibiotic classes function by obstructing the bacterial protein synthesis's 30s or 50s subunits.

In general, there are various stages at which protein synthesis inhibitors function, such as the beginning, the development of the 70s, and the elongation process of polypeptide synthesis. Reeju Sharma (2020) lists the antibiotics aminoglycosides (kanamycin, amikacin), macrolides (erythromycin, azithromycin), tetracycline (doxycycline), oxazolidinone (linezolid), and chloramphenicol (chloromycetin) among those that hinder or obstruct bacterial protein synthesis

Thin Layer Chromatography (TLC)

Table 2 displays the results of aluminium TLC silica gel separations of aqueous extracts from *C. moschata* leaves. Cucurbitacins, tannins, and polyphenols can be found in cucurbits (Rolnik & Olas, 2020). The results showed that vanillin identified terpenoid and tannin in visible light with a yellow-red colour spot. When a purple stain developed in the aqueous extract of pumpkin leaves, anisaldehyde was discovered as a terpenoid (Ibiba et al., 2020).

Moreover, while reacting with DPPH and Dragendoff's, respectively, the pumpkin leaf water extracts showed the potential availability of alkaloids (orange-brown colour spot) and antioxidants (yellow colour spot). Raw pumpkin leaves were found to have more robust DPPH radical scavenging capacities than the fruit and seeds by Mashitoa et al. (2021). In addition, screening for phytochemistry showed that the fraction and extract of pumpkin leaves contain alkaloids and tannins, which are secondary metabolites.

Table 2. TLC observation of *C. moschata* leaves water extracts

Spray reagent	Color of spot		Leaves Sample R_f value	Bioactive compound
	Visible	Heated		
Vanillin	Dark red	Yellow red	0.74	Tannin
	Dark red	Yellow red	0.70	Tannin
Anisaldehyde	Light blue	Purple	0.78	Triterpenes
DPPH	Light pink	Yellow	0.60	Antioxidant
	Light blue	Yellow	0.27	Antioxidant
Dragendorff's	Light blue	Orange-brown	0.15	Alkaloid

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The phytochemicals in the sample leaves water extracts are responsible for their antibacterial properties. The Cucurbita genus of plants contains a high concentration of antioxidants and phytochemicals. The phytochemicals cucurbitacins, saponins, carotenoids, phytosterols, and polyphenols are the most important ones found in cucurbits. The pharmacological properties, such as antioxidant, anticancer, antidiabetic, hepatoprotective, antibacterial, anti-obesity, diuretic, anti-ulcer activity, and antigenotoxic, are attributed to these bioactive phytoconstituents. Terpenoids, abundant in cucurbits, damage bacterial and fungal membranes and have inhibitory effects. (Rolnik & Olas, 2020). Alkaloid-containing pumpkins can help regulate microbial infections, raise or lower blood pressure, and stimulate the neurological system (Lusiana et al., 2018). Additionally, polyphenol-tannin showed synergistic antibacterial activity against both bacteria and fungi. Consequently, the water extracted from pumpkin leaves, which contains bioactive substances such as triterpenes, tannin, terpenoids, antioxidants, and alkaloids, may be an antibacterial against different microorganisms.

Numerous bacteria, including Gram-positive and Gram-negative bacteria and fungi, are susceptible to the antimicrobial effects of polyphenols (Manso et al., 2021). Tannis were detected from tlc via dark red spot when sprayed with vanillin reagent (Ismaeel et a.,l 2020). Tannins exhibit antibacterial activity by forming complexes with proteins through covalent and non-covalent interactions. It has been shown that condensed tannins can adhere to the cell walls of ruminal bacteria, stopping their proliferation and inhibiting their ability to produce proteases (Othman et al., 2019). Tannins can inhibit both Gram-positive and Gram-negative bacteria, and most tannins have a bacteriostatic effect instead of a bactericidal one. Certain tannins primarily attack the outer membrane of Gram-negative bacteria, disrupting the lipopolysaccharide that gives these bacteria their resistance to antibiotics (Farha et al., 2020). Tannins are macromolecular polyphenols that have a robust antibacterial impact on Gram-negative bacteria because they contain many phenolic hydroxyls. Tannis that was detected in TLC can be one of the compounds that had antibacterial properties.

Furthermore, due to the alkaloids in the leaf extracts have functional groups in addition to a proton-donating amine hydrogen atom and a proton-receiving nitrogen atom, they can form hydrogen bonds with enzymes, receptors, and proteins. Alkaloids inhibit bacterial growth via multiple mechanisms, such as blocking bacterial synthesis of proteins and nucleic acids, altering the permeability of the bacterial cell membrane, causing damage to the cell wall and membrane, suppressing bacterial metabolism, and blocking efflux pumps (Yan et al., 2021).

Triterpenoids are also found in C. moschata leaves. Previous research has shown that oleanolic acid, a pentacyclic triterpenoid molecule derived from plants, can kill *E. faecalis* by

rupturing the bacteria's cell membranes and causing a decrease in the viability of the bacteria's cells when exposed to 32–64 µg/mL of the chemical (Yang et al., 2020). Terpenes are known to have antibacterial qualities against bacteria that are resistant to antibiotics as well as those that are susceptible to them. These effects are primarily caused by terpenes promoting cell rupture and inhibiting DNA and protein synthesis (Álvarez-Martínezet al., 2021).

The bioactive compound in *C. moschata* leaves water extract

A balance between the three polarities of the plate (stationary phase), the mobile phase, and the bioactive chemical affected every successful TLC separation result. Because of the hydroxyl groups on the surface of the TLC silica gel plates, which are utilised as polar adsorbents, the polar chemicals found in leaf water extracts will stick close to the source because they have a significant affinity for the TLC silica plate. In proximity to the solvent front, the less polar molecules tend to advance (Lisa Nichols, 2016). The solvent tries to move the spot along with the alkaloid and antioxidant components along the plate, while the polar silica gel tries to retain them in place. As a result, less polar chemicals such as tannin (0.70, 0.74), triterpenes (0.78), and antioxidants (0.60) from pumpkin leaves have high Retention Factor (Rf) values (Table 2). Although the pumpkin leaf water extract's modest Rf values of 0.15 indicate the presence of alkaloids, the extract's antioxidant Rf values of 0.27 indicate the presence of polar molecules that firmly bind to the adsorbent.

An effective solvent typically yields Rf between 0.3 and 0.7. Many variables, including layer thickness, moisture on the TLC plate, temperature, mobile phase depth, TLC plate type, and solvent parameters, can influence each compound's Rf value (Michael Judge, 2018). Consequently, various Rf values may arise from identifying a chemical using a different mobile phase. The solvent utilized (ethyl acetate: methanol: water with a ratio of 6:3:1 (v/v/v). can separate the compounds rather well, even if it may not be the most effective one due to the Rf values of several compounds in leaves water extract being over the standard range and tailing.

Mixtures of solvents are employed to achieve optimum separation of TLC. Ethyl acetate solvent is a low polar compound, methanol is a medium polar compound, and water is a polar compound. A solvent's mobile phase (elute) strength is primarily related to how strongly it adsorbs onto the adsorbent, and because silica adsorbents are polar; thus, eluting strength increases with solvent polarity (Hage, 2018). It is important to remember that even a small addition of a polar solvent can greatly boost the mixture's eluting capacity when employing solvent combinations. To aid in the movement of the compounds, a little amount of a polar solvent, such as water, can be added.

Conclusion

This study demonstrated the availability of phytochemicals like tannin, triterpenes, antioxidants, and alkaloids in the aqueous extract of *C. moschata* leaves. The phytochemicals found in a sample of C. moschata leaves can act as an antibacterial to prevent the growth of *A. baumannii* (150 mg/mL) and *E. faecalis* (300 mg/mL) at respective concentrations.

These findings imply that *C. moschata* leaf water extracts could be a valuable source of natural antimicrobials for feed additives. In conclusion, pumpkin leaf water extract may be used as an alternate feed or feed additive for small ruminants with their antibacterial properties that can help in ruminant health. To increase our understanding, a higher concentration of pumpkin leaf water extract should be employed in the future to investigate the precise mechanism by which the bioactive chemical inhibits the growth of bacteria

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