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Antibacterial Potentials of Methanol Extract of *Acalypha wilkesiana* Leaves on Selected Foodborne Pathogens

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ABSTRACT

Acalypha wilkesiana is one of the weed plants with many potential metabolites that are important medicinally. This study aimed to investigate the antibacterial activity of *A. wilkesiana* methanol leaves extract against some food-borne pathogens and identify its metabolites. The agar well diffusion method was used to study the growth inhibition of four (4) bacterial species; *Staphylococcus aureus*, *Bacillus cereus, Salmonella spp*, and *Escherichia coli* with an initial concentration of 250 µg/ml methanol leaves extract of *A. wilkesiana* and final concentration of 2000 µg/ml. The agar well diffusion revealed that concentrations of 2000 µg/ml were more effective than other concentrations of the extracts, (*S. aureus* 20+2.45), *Salmonella spp;* (20+0.82) *B. cereus* (19+3.27), and *E. coli* (18+4.16). The liquid chromatography mass spectrophotometer profile of the extract carried out showed the presence of important metabolites such as Scopoletin (7-hydroxycoumarins), (6beta,8betaOH)-6,8-Dihydroxy-7(11)-eremophilen-12,8-olide (Terpene lactones), Pyrethrin I (Pyrethrins), Quercetin (Flavonols), Quercitrin (Flavonoid-3-O-glycosides), Malonamic acid and succinamic acid among others. These identified metabolites were reported to have many medical importance. Therefore, the activities observed may be as a result of these metabolites. *A. wilkesiana* leaves could be explored further in the treatment of gastrointestinal disorders.

Keywords: *Acalypha wilkesiana,* Methanol, Antibacterial activity, Foodborne and LCMS,

INTRODUCTION

Acalypha wilkesiana, a small annual erect herb plant, can grow up to 0.6 m (Kirtikar, 2018). It is primarily found in the backyards of houses and waste places across the plains of India. *A. wilkesiana*, a traditional medicinal plant, is well-known by older generations in many countries, particularly in Africa and Asia. It grows as a weed in bushes, backyards, alongside roads, and other places such as home and crop premises (Seebaluck *et al*., 2015).

Despite thriving in wet, hot, and temperate tropical climates, there are no documented occurrences of this plant outside of these regions. While many local repositories do include this plant in their records, it is not widely utilized for medicinal purposes. The specific uses of this plant vary based on location and local traditional knowledge. In addition to its medicinal applications, this plant is also consumed as a leafy green vegetable, particularly in India and Oman, as a dietary supplement (Schmelzer, 2017).

A. wilkesiana has been found to have properties that can help alleviate insect bites, as stated by Kirtikar (2018). Insect bites can cause skin inflammation, blisters, and irritation due to the various types of poison they release upon stinging. *A. wilkesiana*, known for its skin-related healing properties, is also used to reduce soreness from insect bites. Its leaves are rich in antibacterial, antifungal, and antioxidant phytochemicals, which are beneficial for protecting the skin

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from external threats. Furthermore, the fluid released from crushed leaves serves as a lubricant for treating child constipation by inserting small ball-shaped pieces made from the leaves into the rectum, as noted by Sudhakar *et al*. (2020). The fluid contains an oily compound that helps to loosen the rectum for easier faeces release and provides protection to the injured rectum wall. Moreover, the fresh leaves contain a high concentration of volatile chemicals that can be used to alleviate headaches, epilepsy, earaches, and act as an expectorant. *A. wilkesiana* is used as natural remedy for health conditions due to its safety when consumed (Sudhakar *et al*., 2020)

The potential of *A. wilkesiana* plant extract to act as an anticancer agent was documented by Amarnath *et al*. (2014). *A. wilkesiana* shows promise in treating diabetes due to its anti-diabetic properties, as demonstrated by Nandhakumar *et al.* (2019). The hexane and methanolic extracts were found to reduce alpha amylase activity by 7.51% and 65.32% respectively. Alpha amylase is an enzyme responsible for breaking down starch into sugars. Subsequent in vivo tests on rats confirmed these findings. Diabetes was induced in the rats before administering the plant extract orally, resulting in a minimum 35% decrease in blood glucose levels. Furthermore, the levels of cholesterol, urea, and triglycerides also decreased after six hours.

Outbreaks of foodborne diseases pose a significant global food safety issue at present. The World Health Organization reports that around 600 million individuals fall ill annually due to consuming contaminated food, resulting in 420,000 deaths and the loss of 33 million healthy lives (WHO 2015). Conventional methods of managing microbial growth using synthetic medications generally have numerous side effects and are not universally affordable. Therefore, the use of herbal medicine cannot be overemphasized as an alternative to replace or supplement conventional synthetic drugs.

This study aimed at investigating the antibacterial activity of *A. wilkesiana* methanol extract against foodborne bacteria.

MATERIALS AND METHODS

Plant Collection and Sources of Microorganisms

In December 2023, we acquired fresh red acalypha leaves from the Centre for Dry Land Agriculture (CDA) at Bayero University Kano. The plant was identified as *Acalypha wilkesiana* at Bayero University Kano and assigned Herbarium Number (BUKHAN) 0351. The leaves underwent thorough washing 3-4 times under running tap water and once with distilled water to eliminate all adhering substances such as dirt, soil, and contaminants. Following this, the leaves were dried in the shade for five days and then ground into powder using an electric blender, following the method described by Adesina *et al*., (2000). The foodborne microorganisms were sourced from the microbiology laboratory of Bayero University, Kano, and their presence was further confirmed using biochemical tests. These organisms were sub-cultured on agar slant and refrigerated for future use.

Preparation of Extract

The extraction of *A. wilkesiana* leaves was conducted following the method described by Usman *et al*. (2023) with some adjustments. Fifty grams (50 g) of the plant sample leaves were weighed and dissolved in 500 cm^3 of methanol solvent. The mixture was left to stand for 48 hours before being filtered using Whatman No.1 filter paper. The remaining residue was then evaporated using a water bath at 40 ºC to produce the methanol crude extract.

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Bioassay

Preparation of Extracts Concentrations

A stock solution with a concentration of 2000 µg/ml was created by mixing 2 g of plant extracts with 1ml of dimethyl sulfoxide (DMSO) in glass vial bottles, following the method described by Cheesbrough (2006). This solution was then diluted to obtain different concentrations (2000 µg/ml, 1000 µg/ml, 500 µg/ml, and 250 µg/ml).

Standardization of Inoculum

The bacterial isolates were adjusted to a turbidity of 0.5 McFarland standard (1.5 X 10^8 CFU/ml) by introducing sterile normal saline. The use of McFarland standards allowed for the standardization of microbial suspension turbidity, ensuring a consistent range of microorganism numbers. The McFarland standard was prepared by combining 0.05ml of barium chloride (1.17% w/v $BaCl_2.2H_2O$) and 9.95 ml of sulphuric acid [0.18M H_2SO_4 (1.0% w/v)], while maintaining constant stirring. To facilitate comparison, the standard was evaluated against a white background with a distinctive black line (Kalpana *et al.,* 2013).

Antimicrobial Assay

The method for the bioassay involved utilizing the agar well diffusion technique outlined by Cheesbrough (2006). A standardized 0.1ml of inoculum (1.5 x 10^8 CFU/ml) of the isolated organisms was applied on sterile Mueller Hinton Agar and spread using a sterile swab. Using a 6mm sterile cork borer, five wells were created on the agar plates containing the bacterial inoculum and 0.1 ml of the stock solution of the extracts at concentrations (2000, 1000, 500, and 250 µg/ml) were introduced into their respective wells. The fifth well received 0.1 ml of 250 µg/ml sodium benzoic acid to act as a positive control for the bacterial isolates. After 30 minutes of standing to allow extract diffusion, the inoculated plates were incubated at 37ºC for 24 hours. The resulting zones of clearance around the wells after incubation were observed, measured using a vernier caliper, and recorded in millimeters. Each experiment was performed in triplicate, and the mean result was determined for the test organisms (Cheesbrough, 2006).

Determination of Minimum Inhibitory Concentration and Minimum Bactericidal concentration

The lowest dose of the antimicrobial agent that prevented apparent microbial growth following an overnight incubation period is known as the minimum inhibitory concentration, or MIC (Andrews, 2002). The MIC was found using the doubling macro dilution broth method. To prepare the sterile Mueller Hinton broth for the bacterial isolates, two milliliters (ml) of the reconstituted crude extract at a concentration of 1000 µg/ml was added. Two milliliters of this extract concentration were then transferred to another test tube, and so on, until the seventh test tube was reached, resulting in extract concentrations ranging from 1000 to 65.2 µg/ml in various test tubes. Each test tube was inoculated with 0.1ml of an 18-hour culture of bacteria that had been previously adjusted to 0.5 McFarland standard. A positive control was a test tube with the broth and extract, while a negative control was a test tube with the broth and bacteria inoculum. After 24 hours of incubation at 37ºC, the inoculated culture tubes were checked for growth. The method showed that the minimum inhibitory concentration (MIC) was the lowest concentration of extract that did not exhibit any discernible growth when compared to the control (Andrews, 2002).

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The Minimum Bactericidal Concentration

This is the antimicrobial agent's lowest concentration at which an organism cannot grow. A sterile Mueller Hinton Agar plate was inoculated with a 0.1 ml aliquot from tubes that did not exhibit obvious bacterial growth after the minimum inhibitory concentration was determined. The bacterial isolate was then allowed to develop for 24 hours at 37◦C. The minimum bactericidal concentration (MBC) was determined by taking the lowest concentration at which no growth was seen, as shown by Andrews (2002).

LCMS Profile Analysis

The method reported by Bashir *et al*., (2022) was adopted. The extract was dissolved in Hplc grade methanol and filtered through polytetrafluoroethylene (PTFE) membrane filter with 0.45 µm size. Ten microliter (10.0 µl) of the filtrate was loaded into C18 Sunfire column (5.0 µm 4.6 mm x 150 mm) of the LC system. The separation was achieved at a flow rate of 1.0 ml/min, both the sample and column temperature were kept at 25°C. A gradient of mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in Acetonitrile) was used. (Table 1).

Table 1: Solvent Gradient.

Time	0.1% formic acid in water (%A)	0.1% formic acid in ACN (%B)
0	95	5
1	95	5
13	5	95
15	5	95
17	95	5
19	95	5
20	95	5

From ratio of A/B 95:5 this ratio was maintained for further 1 min, then A/B 5:95 for 13min, to 15 min. then A/B 95:5 to 17 min, 19 min and finally 20 min. the Photodiode Array (PDA) detector was set at 210-400nm with resolution of 1.2nm and sampling rate at 10 points/sec. The mass spectra were acquired with a scan range from m/z 100 – 1250 after ensuring the following settings: ESI source in positive and negative ion modes; capillary voltage 0.8kv (positive) and 0.8kv (negative); probe temperature 600°C; flow rate 10 mL/min; nebulizer gas, 45 psi. MS set in automatic mode applying fragmentation voltage of 125 V. The data was processed with Empower 3. The compounds were identified on the basis of the fragmentation pattern by comparing with data base for organic compounds SDBM Data base (Bashir *et al*., 2022).

RESULTS AND DISCUSSION

The antibacterial activity of methanol extract of *Acalypha wilkesiana* leaves on food borne bacteria is presented in Table 2. The results show that the extract inhibited all the test organisms. A. *wilkesiana* extract had higher inhibitory effect on *Salmonella spp* (20± 0.82) and *S. aureus* (20± 2.45) followed by *B cereus* (19± 3.27) and then *E. coli* (18± 4.16) which had the least activity. The antibacterial activity of methanol extract of *A. wilkesiana* against foodborne bacteria have been evaluated in the study. The antibacterial activity of methanol extract of *A. wilkesiana* against foodborne bacteria is reported here for the first time.

Previous studies conducted by Mansur *et al.* (2024), Iyekowa *et al.* (2016), Erebor *et al.* (2015), Anokwuru *et al.* (2014), Onocha and Dusanya (2010), Marwah *et al.* (2007) and Shurwalker *et al.* (2004), established that *Acalypha* species have anti-microbial, anti-trypanosomal, wound healing and antioxidant potential. The results of this study support the fact that *A. wilkesiana* has a broad-spectrum activity since it inhibited both gram positive *S. aureaus, Bacillus cereus* and gram-negative *Salmonella spp.* and *E. coli*. Our results corroborate the work of Iyekowa *et al.* 2016) which reported antibacterial activity of methanol extract of *A. wilkesiana* against all

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the clinical isolates tested with inhibition zones of 10.50 – 23.00mm. Omotayo *et al.* (2017) also observed that *S. aureus* was more susceptible than *E. coli* a gram-negative bacterium. This also correspond with the works of Mansur *et al.* (2024), Onocha and Olusanya (2010) and Oladunmoye (2006) where they demonstrated that *S. aureus* was suppressed by the methanolic extracts of *A. wilkesiana*. Results of this study was also supported by the work of Alade and Irobi (1993), who stressed that extracts of high polarity of *A. wikesaena* showed antibacterial activity than others.

The source of the bacterial strains used in this study, and the concentration of plant extract are factors which can influence the biological activity of the plant extract. It was also observed that the extract was dose dependent as concentration increased the sensitivity also increased and vice versa (Ify *et al.,* 2021). Our results show that *Acalypha wilkesiana* extract possess measurable antibacterial activities against some microorganisms incriminated in foodborne illnesses.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of methanol extract of *A. wilkesiana* on food borne bacteria is shown in Table 3. The result shows that minimum inhibitory concentration and minimum bactericidal concentration values of the extract against *Salmonella spp* and *Staphylococcus aureus* were 250 µg/ml and 500 µg/ml respectively while the MIC and MBC values for *E. coli* and *B. cereus* were 500 µg/ml and 1000µg/ml respectively. This implies that this methanol extract had a greater effect on destroying these organisms.

Since some of these organisms have been implicated in gastrointestinal diseases, these results provide an insight into the acclaimed therapeutic effect of this plant in gastrointestinal related diseases. It was also reported by Ify *et al.* (2021) that the leaf and young shoot are used as vegetable, eaten with rice dishes popularly used for the treatment of gastrointestinal disorders.

Table 2: Antibacterial activity of methanol extract of *Acalypha wilkesiana* against food borne Bacteria

Bacterial isolates	**Concentration (µg/ml) and zone of inhibition in mm (Mean**+ SD)				
	2000	**1000**	**500**	**250**	**Control SBA**
S. aureus	20+2.45	17+4.19	14+2.67	12+0.82	20+4.16
Bacillus cereus	19+3.27	16+0.82	14+1.63	11+0.81	23+1.63
Escherichia coli	18+4.16	16+4.89	12+2.45	11+2.67	20+1.62
Salmonella spp	20+0.82	18+0.82	13+1.63	10+2.45	24+0.81

Key: SBA: Sodium Benzoic Acid, SD = Standard Deviation

Table 3: Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of methanolic extract of *Acalypha wilkesiana* on some bacterial isolates (µg/ml)

Bacterial isolates	**MIC**	**MBC**
Salmonella spp	250	500
Bacillus cereus	500	1000
Escherichia coli	500	1000
Staphylococcus aureus	250	500

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Table 4: Tentative Compounds Identified from *Acalypha wilkesiana* methanol Extract using LCMS

S/No	Analyser/Ionization mode	Compound Mass (Da)	M+H/M-H(m/z)	Tentative Identification
1	QqQ/ESI (-)	192	191.147	Scopoletin (7-hydroxycoumarins)
2	QqQ/ESI (-)	266	265.150	(6beta,8betaOH)-6,8-Dihydroxy-7(11)-eremophilen-12,8-olide (Terpene lactones)
3	QqQ/ESI (-)	328	327.490	Pyrethrin I (Pyrethrins)
4	QqQ/ESI (-)	302	301.138	Quercetin (Flavonols)
	QqQ/ESI (-)	448	447.313	Quercitrin (Flavonoid-3-O-glycosides)
5				
6	QqQ/ESI (+)	103	104.272	Malonamic acid
7	QqQ/ESI (+)	117	118.116	succinamic acid

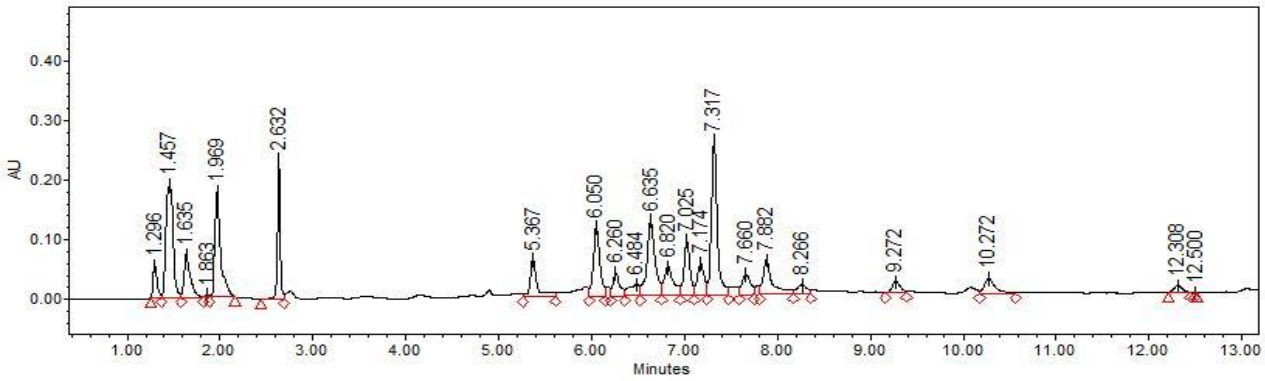

Figure 1: Total ion chromatogram of *Acalypha wilkesiana* Methanol Extract.

The LCMS profile was conducted using the waters LCMS equipment and the total ion chromatogram was presented in figure 1 and the summary table of the tentative compounds was also presented in figure 1, where analytes were determined at both positive and negative mode. Many analytes were detected and some tentative compound identified were previously reported to be active against clinical conditions ranging from antioxidant, anti-inflammation, anti-cancer, anti-bacteria, anti-diabetes among others. They are grouped into four classes; Terpenes, Flavanols, Flavonoids and organic acids.

The tentative analytes are: Scopoletin (7-hydroxycoumarins), (6beta,8betaOH)-6,8-Dihydroxy-7(11)-eremophilen-12,8-olide (Terpene lactones), Pyrethrin I (Pyrethrins), Quercetin (Flavonols), Quercitrin (Flavonoid-3-O-glycosides), Malonamic acid and succinamic acid. The antibacterial activity observed from this study could be as a result of the presence of terpene derivatives as a reviewed by Wiart *et al*. (2023), where their study identifies terpenes isolated from the medicinal Angiosperms of Asia and the Pacific with antibacterial and/or antifungal activities. About 300 antibacterial and/or antifungal terpenes were identified during the study period. Terpenes with a MIC $\leq$ 2 µg/mL are mostly amphiphilic and active against Gram-positive bacteria, which is similar to our findings (Wiart *et al.,* 2023). Gram-negative bacteria, compared to Gram-positive bacteria, are more resistant to plant natural products and antibiotics because they are packed in a

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hydrophilic and negatively charged shield of lipopolysaccharides (Denyer *et al.,* 2002). However, water, nutrients as well as hydrophilic, and, to a lesser extent, amphiphilic xenobiotics with a molecular mass below 600 g/mol, cross this outer layer through large transmembrane protein channels known as porins or aquaporins (Berg *et al*., 2010). The terpene derivatives identified in our study was 266 g/mol which is much lower than 600 g/mol, this could be the mechanism of the activity observed. Other mechanism proposed by Wiart *et al.* (2023) are specific and/or nonspecific, nonspecific involves the destabilization of cytoplasmic membranes in Gram-positive bacteria by lipophilic and amphiphilic terpenes as seen with linear terpenes (Moura *et al*., 2004)) while specific mechanism inhibit bacterial and fungal efflux pumps or inhibit glycoprotein in HepG2/ADR cells, and as such, might be able to inhibit bacterial and/or fungal efflux pumps as in *S. aureus* (Wiart *et al*., 2023).

Furthermore, flavonol have a long history as antibacterial and other ailment, quercetin which is a flavonol was identified from *A. wilkesiana* methanol extract and the activity recorded in this study could also be linked to the presence of this very important metabolite. Among an array of pharmacological activities that quercetin exhibits, its antimicrobial property is the most important one. This is because of its safety status as designated by the United States Food and Drug Organization (FDA) (Majumdar and Mandal 2024) and its multi-target-based mode of action against bacterial systems (Siriwong *et al*., 2016; Kannan *et al*., 2022).

Other metabolites present in this extract are organic acids, which are natural compounds found in various foods, vegetables and plants. A wider spectrum of its application includes antibacterial activity and high food safety (GRAS) and are extensively used in the food industry as antimicrobial agents (Ji *et al*., 2023). In addition, organic acids have little impact on the sensory properties of products and are a low-cost, easy-to-apply option in the food industry (Mani-López *et al*., 2012) where they can be used in animal production, as growth promoters, and in the food industry for the hygiene of equipment directly related to food (Castro *et al*., 2020)]. The molecular mechanisms of organic acid inhibition include energy competition, bacterial outer membrane permeabilization, increased intracellular osmotic pressure, and the inhibition of biomolecule synthesis. Undissociated organic acid molecules are lipid-soluble and can enter the cell by free diffusion and dissociate to produce acid ions (ROO^-) and protons (H^+). (Ji *et al*., 2023).

CONCLUSION

The findings of this study have indicated that the methanol extract of *A. wilkesiana* has antibacterial activity, particularly on *S. aureus* and *Salmonella* spp which have long been known as common causes of food poisoning. *A. wilkesiana* also contains bioactive metabolites like Scopoletin (7-hydroxycoumarins), (6beta,8betaOH)-6,8-Dihydroxy-7(11)-eremophilen-12,8-olide (Terpene lactones), Pyrethrin I (Pyrethrins), Quercetin (Flavonols), Quercitrin (Flavonoid-3-O-glycosides), Malonamic acid and succinamic acid which have physiological effects in humans from the LCMS analysis. The potential activity of the extract observed could be as a result of different important metabolites. Therefore, it may be useful in drug development for general antimicrobial management. The metabolites should be isolated and purified for further studies.

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