Metabolite Profiling and Bioactivity Screening of Tylosema esculentum, Myrothamnus flabellifolius and Ozoroa paniculosa extracts using Ultra-Performance Liquid Chromatography–High-Resolution Mass Spectrometry (UPLC-HRMS)

Modiri D Setlhoka

201200117@buan.ac.bw

Botswana University of Agriculture and Natural Resources (BUAN)

Koketso Motlhanka

Botswana University of Agriculture and Natural Resources (BUAN)

Bakang K Kgasudi

Botswana University of Agriculture and Natural Resources (BUAN)

Eunicah Atamelang

Institute of Health Sciences (IHS)

Goodwell Modise

University of Botswana

Geremew Bultosa

Botswana University of Agriculture and Natural Resources (BUAN)

Molebeledi H D Mareko

Botswana University of Agriculture and Natural Resources (BUAN)

Mpho G Batlhophi

Botswana University of Agriculture and Natural Resources (BUAN)

Force T Thema

Botswana University of Agriculture and Natural Resources (BUAN)

Kereilemang K Nthoiwa

Fourth & Middle (PTY) Ltd

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Abstract

Natural resources provide vital support to communities, and indigenous plants remain particularly important, as they are widely used for food, medicine, and cultural practices. In the quest to integrate these resources into modern applications, exploring their phytochemical composition is essential for both conservation and utilization. This study compared the phytochemical profiles, antioxidant capacity, and antimicrobial activity of *Tylosema esculentum*, *Myrothamnus flabellifolius*, and *Ozoroa paniculosa* using water, 80% methanol, and 50% acetone extracts. LC-MS profiling revealed *M. flabellifolius* as the most chemically diverse, with major flavonoids including quercetin 3-O-glucuronide and quercitrin. Acetone extracts of *M. flabellifolius* exhibited the highest DPPH scavenging activity (57.13%) and strongest antibacterial effects against *E. coli*, *S. typhimurium*, and *S. aureus*. The latter extracts displayed particularly demonstrated dose-dependent antimicrobial activity, with inhibition zones reaching up to 20.1 mm for *E. coli* and 16.9 mm for *S. typhimurium*. Solvent type significantly influenced the phytochemical content and bioactivity of the extracts, with 50% acetone generally outperforming water and 80% methanol. Overall, the indigenous plants of Botswana demonstrated considerable potential as sources of bioactive compounds for use as natural antimicrobial agents in food packaging and preservation. In particular, *Myrothamnus flabellifolius* exhibited the most diverse phytochemical profile and the strongest antimicrobial properties. These findings highlight the plant's potential for development into natural antimicrobial products, warranting further investigation and application-focused research.

1. Introduction

Plants have gained significant attention in recent years due to their bioactive compounds, which are produced as secondary metabolites through various biosynthetic pathways, including the shikimic acid, malonic acid, mevalonic acid, and methylerythritol phosphate (MEP) pathways (Anmol et al. 2024; Cheikhyoussef et al. 2015). These metabolites are frequently associated with beneficial properties, such as antioxidants and antimicrobial activities (Takó et al. 2020; Al-Khayri et al. 2022). Consequently, numerous studies aimed to quantify phytochemical constituents and assess their biological properties across a variety of plant species (Al-Khayri et al. 2022; Cushnie and Lamb 2005; Zhang et al. 2025). In Botswana, indigenous plants have also been subjected to scientific scrutiny, with research focusing on species such as *Morama* (*Tylosema esculentum*) (Kobue-lekalake et al. 2022; Omotayo and Aremu 2021), *Myrothamnus flabellifolius*, and *Ozoroa paniculosa* (Kwape et al. 2016; Motlhanka and Mathapa 2012), which continue to be explored for their potential health benefits.

Morama (*Tylosema esculentum*), a leguminous plant predominantly found in Namibia, South Africa, and Botswana, has been traditionally consumed by indigenous communities, including the San and Bakgalagadi, who gather it from the wild (Gwamba et al. 2025). This plant is reported to contain a variety of phytochemicals, classified into phenolic acids, flavonoids, and phytoestrogens (Omotayo and Aremu 2021; Chingwaru et al. 2011). Recent studies have quantified the flavonoid content of Morama, revealing a concentration of 0.00023 mg catechin equivalents per gram (CE/g) (Kobue-lekalake et al. 2022). While Morama exhibits a lower flavonoid concentration compared to other local plants such as mogose

(*Bauhinia petersiana*) and kgengwe (*Citrulus lanatus* (*Thunb*.) *Mansf*.), its extracts have demonstrated antimicrobial activity against strains such as methicillin-resistant *Staphylococcus aureus, Corynebacterium diphtheriae*, and *Candida albicans* (Chingwaru et al. 2011).

Similarly, *Ozoroa paniculosa* has been recognized for its potential antibacterial and antioxidant properties. Traditional healers in the *Tswapong* region of Botswana utilize its roots to address various reproductive health issues, including swollen uterus conditions (Motlhanka et al. 2008). The therapeutic applications of this plant are often linked to its content anacardic acid, which is believed to exhibit anti-inflammatory effects. Motlhanka 2008 reported that water extracts of *O. paniculosa* displayed 90% free radical scavenging activity, suggesting its potential as an active ingredient in health products.

Myrothamnus flabellifolius has also been the subject of phytochemical studies. However, investigations into its antimicrobial and antioxidant activities are limited. Despite this, local communities have utilized extracts from this plant to treat respiratory ailments and skin infections (Chukwuma et al. 2019). The efficacy of these applications may be attributed to compounds such as galloylquinic acid, known for its wound-healing properties and inhibitory effects on HIV reverse transcriptase and DNA polymerase (Kamng'ona et al. 2011). Furthermore, other phytochemicals, including polyphenols such as gallic acid, alkaloids, tannins, and saponins, have been identified as contributors to its biological activities, with tannin content measured at 3.6%, predominantly as proanthocyanidins (Kwape et al. 2016). Cheikhyoussef et al. 2015 reported that ethanol extracts of *M. flabellifolius* yielded a total phenolic content of 372.42 ± 0.21 mg gallic acid equivalents per gram (GAE/g), comparable to methanol and water extracts.

This study is the first to use Ultra-Performance Liquid Chromatography–High Resolution Mass Spectrometry (UPLC-HRMS) to directly compare the phytochemical composition of *Tylosema esculentum*, *Myrothamnus flabellifolius*, and *Ozoroa paniculosa* from Botswana. Therefore, the present study aims to comprehensively compare the phytochemical profiles, antioxidants, and antimicrobial properties of *Tylosema esculentum, Myrothamnus flabellifolius*, and *Ozoroa paniculosa* extracts obtained with water, 80% methanol, and 50% acetone. The current paper reports findings on phytochemical profiles and the bioactivity of the three Botswana plants. This research seeks to enhance the understanding of the potential benefits these plants may offer and to promote their utilization in the food industry and modern medicine.

2. Materials and Methods

2.1 Experimental Design

The study employed a completely randomized block design, investigating three different plant species extracted for bioactive compounds using three solvents, being distilled water, 80% Methanol and a 50:50 acetone/water mixture.

2.2 Collection of Plant Materials

Fresh, whole *Myrothamnus flabellifolius* plants and *Ozoroa paniculosa* leaves were collected from Kanye village, Southern District, Botswana (24°59'17.02"S, 25°20'34.87"E) on 30 May 2024. *Tylosema esculentum* seeds were obtained from Ghanzi District (20°27'0"S, 24°52'60"E) at the end of the rainy season (March–May 2024). Plant materials were transported to the laboratory on the same day for processing. Botanical identification was performed by a botanist at the Botswana University of Agriculture and Natural Resources, and specimens were authenticated through comparison with reference collections at the National Herbarium and Gallery, Gaborone.

2.3 Preparation of Extracts

Plant extracts were prepared following method by Kopjar et al. 2009 with modifications. One gram of each dried plant sample was extracted using 10 mL of the respective solvent (distilled water, 80% methanol, and 50% acetone/water) at 60 °C for 30 min in a water bath, followed by static maceration at 4 °C for 12 h in the refrigerator. After extraction period, the mixture was centrifuged at 10000 rpm for 10 mins to obtain the aqueous extract, which was subsequently used for phytochemical evaluation. This method was employed to conserve heat liable metabolites in various plant parts (Okoduwa et al. 2016).

2.4 Phytochemical Analysis

2.4.1 Determination of Total Phenolic Content (TPC)

The TPC was determined using the Folin-Ciocalteu method as described by Singleton and Rossi 1965. A 100 µL aliquot of Folin-Ciocalteu reagent was mixed with 500 µL of each plant extract and incubated in the dark for 15 mins. Following this, 2500 µL of saturated sodium carbonate (10.6 g/100 mL) was added, and the mixture was incubated for an additional 30 mins. The absorbance was then measured at 760 nm using a spectrophotometer. A calibration curve was established using gallic acid, with concentrations of 2, 4, 6, 8 and 10 mg GAE/g. The phenolic content was calculated based on the dry weight of the extracted plant material and expressed as mg of gallic acid equivalents per gram of dry weight (mg GAE/g). The regression equation for the calibration curve had an R^2 value of 0.9725 (Fig. 1)**.**

2.4.2 Determination of Total Flavonoid Content (TFC)

The total flavonoid content was assessed through a colorimetric assay involving aluminum chloride, as described by Ordonez et al. 2005. Each extract (1 mL) was combined with 1 mL of methanol, 0.5 mL of 1.2% aluminum chloride, 0.5 mL of 0.12 M potassium acetate, and 2.8 mL of distilled water. The mixture was incubated at room temperature for 30 minutes, after which the absorbance was measured at 415 nm. Results were expressed as mg of quercetin equivalents (QE) per gram of dry weight (mg QE /g). A calibration curve was established using quercetin standards with concentrations of 1, 2, 4, 6, and 8 mg QE /g. The regression equation for the calibration curve had an R^2 value of 0.9856 (Fig. 2).

2.4.4 Ultra-Performance Liquid Chromatography (UPLC) (UPLC-HRMS) Analysis

2.4.4.1 Sample preparation for Metabolite Profiling

Approximately 0.25 g of sample was weighed into a 15 ml falcon tube and extracted with 10 mL 50% methanol/1% formic acid in water with vortex mixing and sonication. After centrifugation, the supernatants were diluted 5x into 50% methanol/0.1% formic acid and transferred to glass vials for analysis.

2.4.4.2 UPLC-HRMS Instrumentation and Chromatographic Conditions

Metabolite profiling was conducted using a Waters Acquity Ultra-performance Performance Liquid chromatography Chromatography (UPLC) system coupled to a Cyclic Quadrupole time-of-flight (qTOF) mass spectrometer (Waters, Milford, MA, USA). The column eluate was first passed through a Photodiode Array (PDA) detector before introduction into the mass spectrometer, allowing for the simultaneous collection of UV and MS data.

Chromatographic separation was achieved on a Waters HSS T3 column (2.1 × 150 mm, 1.7 µm particle size, a reversed phase C-18) maintained at 60 °C. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The following linear gradient was applied at a flow rate of 0.3 mL/min: 0-1 min, 0% B; 1-12 min, 0-28% B; 12-12.83 min, 28-40% B; 12.83-14.33 min, 40-100% B; followed by a 2-minute re-equilibration at 0% B. The injection volume was 0.5 µL.

The mass spectrometer was operated in electrospray ionization (ESI) negative mode. The source conditions were as follows: desolvation temperature at 275 °C, desolvation gas flow at 650 L/h, and a cone voltage of 15 V. Data were acquired in high-resolution mode from *m/z* 100 to 1500. For metabolite identification, data-independent acquisition (MSE mode) was employed, collecting alternating low-collision-energy (4 V) and high-collision-energy (ramped from 40–100 V) scans to obtain both precursor and fragmentation data. Leucine enkephalin was used as the lock mass for real-time mass correction, and the instrument was calibrated with sodium formate prior to analysis.

2.4.4.3 Data Processing and Metabolite Identification

After data compression, centroiding, and lock mass correction, the data were processed using MS-DIAL and MS-FINDER software (RIKEN Center for Sustainable Resource Science) for peak deconvolution, alignment and metabolite identification (Tsugawa et al. 2015; Lai et al. 2018). Compounds were quantified in a relative manner against a calibration curve established using a range of ellagic acid standards from 0.2 to 5 mg/L.

2.5 Antioxidant Activity

2.5.1 Determination of Radical Scavenging Activity (RSA)

The RSA was evaluated using the DPPH method according to Brand-Williams et al. 1995. A 100 µL solution of DPPH was combined with 2.9 mL of ethanol. Subsequently, 500 µL of each plant extract was added, and the mixture was incubated in the dark at room temperature for 20 mins. Absorbance was measured at 517 nm, and DPPH scavenging activity was calculated using the formula:

$$\textbf{DPPH Scavenging Activity (\%)} = (A0 - A1) \div A0\ (100)$$

Where:

- A0 = Absorbance of the control solution (without the sample)
- A1 = Absorbance of the sample solution (with the sample)

2.5.2 Ferric Reducing/Antioxidant Power (FRAP)

The FRAP assay was performed based on the method outlined by Bakour et al. 2019 . Freshly prepared FRAP reagent (3 mL) was warmed to 37 °C for 4 min., mixed with 40 µL of the plant extract and the absorbance was measured at 734 nm. The mixture was incubated at 37 °C for 20 minutes. A calibration curve was created using known concentrations of Fe^{2+}, with concentrations of 0, 200, 400, 600, and 800 µM. Results were expressed as µM Fe^{2+}. The regression equation for the calibration curve had an R^2 value of 0.9982 (Fig. 3).

2.6 Antimicrobial Activity

Antibacterial activity was assessed using the disc diffusion method by Black & Black 2018 with slight modifications. After autoclaving Mueller Hinton agar at 121 °C for 15 mins, the agar was cooled and poured into petri dishes. Selected isolates of *S. typhimurium*, *E. coli*, and *S. aureus* were swabbed over the entire surface to inoculate the agar. Sterile Whatman No. 3 filter paper discs (4 mm diameter) were dipped in 20 µL of each plant extract and dried at room temperature for 1 hr. The discs were then placed on the surface of the inoculated agar. Control discs were prepared by saturating them in 20 µL of each solvent used for extraction, that is acetone, methanol and water for comparison. Gentamicin (CN10) was used as a positive control. The plates were refrigerated at 8 °C for 1 hr to allow diffusion of the extracts before incubation at 37 °C for 24 hrs. After incubation, the inhibition zones were measured in millimeters (mm)

2.7 Determination of Minimum Inhibitory Concentration (MIC)

The MIC of the selected plant extracts was assessed using the broth dilution method Abdulhamid et al. (2018). Various concentrations (10-50 mg/mL) of the extracts, known to have antimicrobial properties against the test bacteria, were prepared in test tubes containing Mueller Hinton Broth (MHB). Each tube was inoculated with the bacteria, and the samples were incubated at 37°C for 24 h. The MIC was determined by identifying the lowest concentration of the extract that exhibited no turbidity, indicating bacterial growth inhibition

2.8 Data Analysis

Data obtained from this study were analyzed using ANOVA with statistical software SPSS. Results were expressed as mean ± standard deviation. Mean separation was performed using the least significant

difference test (LSD), with significance determined at $p < 0.05$.

3. Results

The results presented in this study include the phytochemical profiles and antioxidant activity, are detailed in Tables 1, 2 as well as antimicrobial activity, outlined in Table 3. Additionally, Figures 1, 2 and 3, illustrate the relevant findings. The results are summarized as follows:

3.1 Phytochemical Content and Antioxidant Activity

Table 1 Total flavonoids, phenols, FRAP, and DPPH percentages of *T. esculentum, M. flabellifolius,* and *O. paniculosa* extracts.

Extract	Flavonoids (mg QE/g)	Phenols (mg GAE/g)	FRAP (µM)	DPPH (%)
MOR_{ac}	19.91 ± 0.12^{a}	109.85 ± 0.30^{c}	2949.33 ± 12.45^{c}	39.79 ± 0.13^{b}
MOR_{meth}	24.18 ± 0.09^{b}	212.92 ± 0.20^{a}	3112.67 ± 10.77^{b}	21.34 ± 0.02^{c}
MOR_{H2O}	16.78 ± 0.15^{d}	150.92 ± 0.25^{b}	2026.00 ± 11.10^{e}	N/A
MRY_{ac}	27.74 ± 0.20^{f}	137.05 ± 0.30^{d}	3296.00 ± 15.00^{a}	57.13 ± 0.14^{a}
MRY_{meth}	28.08 ± 0.10^{f}	207.99 ± 0.25^{a}	3259.33 ± 12.95^{a}	41.30 ± 0.05^{b}
MRY_{H2O}	27.49 ± 0.15^{e}	106.92 ± 0.20^{c}	2337.67 ± 10.30^{d}	N/A
ORZ_{ac}	25.28 ± 0.12^{e}	194.12 ± 0.25^{b}	2766.00 ± 11.50^{c}	39.67 ± 0.11^{b}
ORZ_{meth}	25.80 ± 0.10^{e}	188.32 ± 0.30^{b}	1802.67 ± 10.00^{f}	39.40 ± 0.10^{b}
ORZ_{H2O}	17.09 ± 0.15^{d}	175.99 ± 0.20^{b}	1639.33 ± 11.50^{g}	N/A

Values with different superscripts within a column indicate significant differences ($p < 0.05$).

Where: MOR_{ac} - *Tylosema esculentum* extracted with 50% acetone; **MOR_{meth} -** *Tylosema esculentum* extracted with 80% methanol; **MOR_{H2O} -** *Tylosema esculentum* extracted with water; **MRY_{ac} –** *Myrothamnus flabellifolius* 50 % acetone; **MRY_{meth} –** *Myrothamnus flabellifolius* extracted with 80 % methanol; **MRY_{H2O} –** *Myrothamnus flabellifolius* extracted with water; **ORZ_{ac} -** *Ozoroa. paniculosa* extracted with 50% acetone; **ORZ_{meth} -** *Ozoroa. paniculosa* extracted with 80% methanol; **ORZ_{H2O} -** *Ozoroa. paniculosa* extracted with water. **N/A** means not analysed

Table 2 Major phytochemical compounds detected using LC-MS from Botswana indigenous plants of *T. esculentum, M. flabellifolius,* and *O. paniculosa*.

Feature ID	Tentative Identification	Avg Mz	Avg Rt (min)	Molecular Formula	Plant Source (Relative Distribution %)
616	3-(3,4-dimethoxyphenyl)-5-(1-hydroxyethyl)-1,2-oxazole-4-carboxylic acid	311.123	4.867	$C_{11}H_{11}NO_4$	MOR (99.7%)
822	Theogallin	463.087	8.927	$C_{14}H_{16}O_{10}$	MRY (99.1%)
743	Lithospermoside	330.117	2.838	$C_{14}H_{19}NO_8$	MOR (99.9%)
743	Lithospermoside	330.117	4.535	$C_{14}H_{19}NO_8$	MOR (100%)
553	Dehydroabietic acid	301.216	13.96	$C_{20}H2_8O_2$	MRY (100%)
1453	6-{[5,7-dihydroxy-4-oxo-2-(3,4,5-trihydroxyphenyl)-4H-chromen-3-yl] oxy}-3,4,5-trihydroxyoxane-2-carboxylic acid	495.077	7.657	$C_{21}H_{18}O_{14}$	ORZ (95.2%)
1384	Quercetin 3-O-glucuronide	479.081	8.333	$C_{21}H_{18}O_{13}$	MRY (71.4%)
1223	Quercitrin	449.108	6.476	$C_{21}H_{20}O_{11}$	MRY (100%)
1943	Balanophotannin A;(+) Balanophotannin A	769.089	7.597	$C_{34}H_{24}O_{21}$	MRY (99.9%)
1740	Quercetin 3-(2-galloylglucoside)	617.113	8.061	C28H24O16	MRY (100%)
500	CNP0442054 (Aralkylamines)	289.140	11.98	$C_{13}H_{16}N_6O_2$	MRY (100%)
499	CNP0442054 (Aralkylamines)	289.140	12.09	$C_{13}H_{16}N_6O_2$	MRY (100%)
103	3,4-Methylenedioxybenzaldehyde	168.064	2.838	$C_8H_6O_3$	MOR (100%)
26	4-ethenylbenzene-1,2-diol	137.059	9.665	$C_8H_8O_2$	ORZ (99.9%)
665	2-(3,4-dihydroxyphenyl)-3,5,6,7-tetrahydroxy-4H-chromen-4-one	319.045	7.740	$C_{15}H_{10}O_8$	ORZ (96.5%)
1826	Fucoxanthin	659.429	13.96	$C_{42}H_{58}O_6$	MRY (100%)
1371	Colensane and clerodane diterpenoids	475.266	12.49	$C_{29}H_{42}O_3$	MRY (100%)
1106	Colupox b	417.264	12.49	$C_{25}H_{36}O_5$	MRY (99.9%)
908	8-Hydroxy-3,4,9,10-tetramethoxypterocarpan	361.127	9.665	$C_{19}H_{20}O_7$	ORZ (100%)
876	2-methyl-1-(4-methylphenyl)-5-oxo-4-(thiophen-2-ylmethylidene)-3-	354.116	6.739	$C_{20}H_{19}NO_3S$	MOR (100%)

	pyrrolecarboxylic acid ethyl ester				
657	Phytuberin	317.171	13.19	$C_{17}H_{26}O_4$	MRY (100%)
699	Tazarotenic acid	324.105	6.569	$C_{19}H_{17}NO_2S$	MOR (100%)
375	Nopalinic acid	263.125	11.18	$C_{10}H_{18}N_2O_6$	MRY (100%)
104	3-Methoxyanthranilate	168.065	4.535	$C_8H_8NO_3$-	MOR (100%)
1633	Kaempferitrin	579.169	9.016	$C_{27}H_{30}O_{14}$	ORZ (99.9%)
154	5-Hydroxyindole-3-acetic acid	192.064	6.739	$C_{10}H_9NO_3$	MOR (100%)
822	Theogallin	345.082	4.501	$C_{14}H_{16}O_{10}$	MOR (97.4%)
1824	Sorocein B	659.226	2.838	$C_{40}H_{34}O_9$	MOR (100%)

Results in Table 2 highlight major compounds tentatively identified from three indigenous plants of Botswana (*M. flabellifolius, O. paniculosa* and *T. esculentum*) through LC-MS technique. The identified compounds are presented with their mass-to-charge ratio (m/z), retention time (RT), molecular formula and their plant source (relative distribution %). Notably data confirms *M. flabellifolius* (MRY) as the most diverse plant, with flavonoids identified as one of major classes detected, for instance quercetin **3-O-glucuronide** (ID 1384) **a**nd **quercitrin** (ID 1223), were predominantly dectected from *M. flabellifolius*. Likewise, in *O. paniculosa* (ORZ), **kaempferitrin** (ID 1633) was identified as major flavonoid. Other flavonoid compounds identified in this species include tetrahydroxy-chromen-one derivative (m/z 319.045) and an 8-Hydroxypterocarpan (m/z 361.127). In contrast, the phytochemical profile of *T. esculentum* (MOR) was less diverse and dominated by lithospermoside (m/z 330.117, IDs 743 & 744). Hydrolyzable tannins like theogallin were detected in both *M. flabellifolius* and *T. esculentum*.

The UPLC-MS chromatographic profiles in Figure 4 demonstrate that *Myrothamnus flabellifolius* is the most phytochemically diverse plant, with multiple distinct peaks that correspond to a broad variety of secondary metabolites. Additionally, of all the plant material analyzed, the seed coat of the morama bean (*Tylosema esculentum*) bears the least diverse phytochemicals.

3.2 Antimicrobial Activity

Fig 5 highlights *M. flabellifolius* extracts as the most effective against three bacterial species tested. For instance, The disc diffusion assay demonstrates that *M. flabellifolius* 50 % acetone extracts resulted in mean inhibition zone of 18.8 mm against *S. aureus* whereas the same solvent for *T. esculentum* extracts yielded inhibition zone of 10.8 mm against *S. aureus*.

The effect of concentration was dose-dependent and clearly observable across all active extracts (Figure 6). For the most potent concentration (200 mg/mL) of each extract against *S. typhimurium*, *S. aureus* and *E. coli*, mean inhibition zones (mm) resulted in higher inbition zones. Compared to all other plant extract

groups, gentamicin (0.01 mg/mL), which was used as a positive control, was significantly different ($p<0.05$).

Table 3 Inhibition levels of *Escherichia coli, Salmonella typhimurium and Staphylococcus aureus* on *Myrothamnus flabellifolius*, *Tylosema esculentum* and *Ozoroa paniculosa* plants extracts.

Treatment	Concentration (mg/mL)	Microorganism	Inhibition zone	Statistical grouping
CN10 Gentamicin	0.01	*Escherichia coli*	22.23 ± 0.40	A
CN10 Gentamicin	0.01	*Staphylococcus aureus*	22.87 ± 0.53	A
CN10 Gentamicin	0.01	*Salmonella typhimurium*	22.83 ± 0.43	A
Myrothamnus (50% Acetone)	200	*Escherichia coli*	19.97 ± 0.09	B
Myrothamnus (50% Acetone)	200	*Staphylococcus aureus*	17.55 ± 0.12	C
Myrothamnus (50% Acetone)	200	*Salmonella typhimurium*	17.17 ± 0.20	C
Myrothamnus (50% Acetone)	100	*Staphylococcus aureus*	14.10 ± 0.10	D
Myrothamnus (50% Acetone)	100	*Salmonella typhimurium*	14.48 ± 0.30	D
Myrothamnus (50% Acetone)	100	*Escherichia coli*	12.30 ± 0.23	E
Myrothamnus (80% Methanol)	200	*Escherichia coli*	12.60 ± 0.20	E
Myrothamnus (80% Methanol)	200	*Salmonella typhimurium*	11.00 ± 0.35	F
Myrothamnus (80% Methanol)	200	*Staphylococcus aureus*	10.57 ± 0.25	F
Myrothamnus (50% Acetone)	50	*Staphylococcus aureus*	11.00 ± 1.16	F
Myrothamnus (80% Methanol)	100	*Salmonella typhimurium*	9.82 ± 0.10	G
Myrothamnus (80% Methanol)	100	*Staphylococcus aureus*	9.47 ± 0.09	G
Myrothamnus (50% Acetone)	50	*Salmonella typhimurium*	10.57 ± 0.13	FG
Myrothamnus (50% Acetone)	50	*Escherichia coli*	10.23 ± 0.21	G
Ozoroa paniculosa (50% Acetone)	200	*Staphylococcus aureus*	12.67 ± 0.18	D/E

Tylosema esculentum (80% Methanol)	200	*Staphylococcus aureus*	10.80 ± 0.12	F
Myrothamnus (80% Methanol)	100	*Escherichia coli*	9.33 ± 0.33	G
Myrothamnus (50% Acetone)	25	*Staphylococcus aureus*	9.53 ± 0.76	G
Ozoroa paniculosa (50% Acetone)	200	*Salmonella typhimurium*	10.37 ± 0.29	FG
Ozoroa paniculosa (50% Acetone)	200	*Escherichia coli*	10.37 ± 0.17	G
Tylosema esculentum (80% Methanol)	200	*Escherichia coli*	11.70 ± 0.06	F
Tylosema esculentum (80% Methanol)	200	*Salmonella typhimurium*	9.87 ± 0.07	G
Myrothamnus (80% Methanol)	50	*Staphylococcus aureus*	7.60 ± 0.27	H
Myrothamnus (80% Methanol)	50	*Escherichia coli*	8.32 ± 0.15	H
Myrothamnus (80% Methanol)	50	*Salmonella typhimurium*	7.57 ± 0.29	H
Tylosema esculentum (50% Acetone)	200	*Staphylococcus aureus*	11.23 ± 0.12	F
Tylosema esculentum (50% Acetone)	200	*Escherichia coli*	10.20 ± 0.23	G
Tylosema esculentum (50% Acetone)	200	*Salmonella typhimurium*	10.07 ± 0.17	G
Myrothamnus (50% Acetone)	25	*Salmonella typhimurium*	8.90 ± 0.82	G/H
Myrothamnus (50% Acetone)	25	*Escherichia coli*	9.67 ± 0.67	G
Ozoroa paniculosa (50% Acetone)	100	*Staphylococcus aureus*	12.22 ± 0.39	D/E
Ozoroa paniculosa (50% Acetone)	100	*Salmonella typhimurium*	8.67 ± 0.17	G/H
Ozoroa paniculosa (50% Acetone)	100	*Escherichia coli*	8.10 ± 0.27	H
Tylosema esculentum (50% Acetone)	100	*Staphylococcus aureus*	10.77 ± 0.15	F

Tylosema esculentum (50% Acetone)	100	*Salmonella typhimurium*	7.60 ± 0.06	H
Tylosema esculentum (50% Acetone)	100	*Escherichia coli*	7.47 ± 0.25	H
Tylosema esculentum (80% Methanol)	100	*Escherichia coli*	10.30 ± 0.24	FG
Tylosema esculentum (80% Methanol)	100	*Staphylococcus aureus*	8.89 ± 0.06	GH
Tylosema esculentum (80% Methanol)	100	*Salmonella typhimurium*	7.90 ± 0.06	H
Ozoroa paniculosa (80% Methanol)	200	*Escherichia coli*	9.00 ± 0.58	GH
Ozoroa paniculosa (80% Methanol)	200	*Salmonella typhimurium*	7.93 ± 0.67	H
Ozoroa paniculosa (80% Methanol)	200	*Staphylococcus aureus*	8.00 ± 0.00	H
Tylosema esculentum (80% Methanol)	50	*Staphylococcus aureus*	9.47 ± 0.09	G
Tylosema esculentum (80% Methanol)	50	*Escherichia coli*	9.57 ± 0.26	G
Tylosema esculentum (80% Methanol)	50	*Salmonella typhimurium*	6.27 ± 0.27	I
Ozoroa paniculosa (50% Acetone)	50	*Staphylococcus aureus*	8.30 ± 0.18	H
Ozoroa paniculosa (50% Acetone)	50	*Escherichia coli*	7.27 ± 0.27	HI
Tylosema esculentum (50% Acetone)	50	*Staphylococcus aureus*	8.82 ± 0.10	GH
Tylosema esculentum (50% Acetone)	50	*Salmonella typhimurium*	6.40 ± 0.06	I
Tylosema esculentum (50% Acetone)	50	*Escherichia coli*	6.53 ± 0.22	I
Myrothamnus (80% Methanol)	25	*Staphylococcus aureus*	6.70 ± 0.15	I
Ozoroa paniculosa (80% Methanol)	100	*Staphylococcus aureus*	6.93 ± 0.54	I
Ozoroa paniculosa (80% Methanol)	100	*Salmonella typhimurium*	6.33 ± 0.33	I

Ozoroa paniculosa (80% Methanol)	100	Escherichia coli	6.80 ± 0.49	I
Tylosema esculentum (50% Acetone)	25	Staphylococcus aureus	7.73 ± 0.15	HI
Tylosema esculentum (50% Acetone)	25	Escherichia coli	6.17 ± 0.17	I
Tylosema esculentum (80% Methanol)	25	Staphylococcus aureus	7.17 ± 0.17	HI

Different subscript letter means significantly different at p<0.05

4. Discussion

The UPLC-MS metabolite profiling, while from a separate extraction, provides fundamental chemical rationale for the observed bioactivities. In this study, superior antimicrobial and antioxidant activity of *M. flabellifolius* extracts could be attributed to their rich and diverse phytochemical profile. The UPLC-MS analysis (Table 2 and Fig. 4) revealed that *M. flabellifolius* contained the most diverse group of bioactive compounds, including known antimicrobial flavonoids like quercetin 3-O-glucuronide and quercitrin, as well as terpenoids such as dehydroabietic acid (Kumar et al. 2023; Cushnie and Lamb 2005; Sinha et al. 2022). In a study conducted by Wang et al. 2018 quercitin at MICs 50X and 10X resulted in structural abnormalities such as cell lysis and uneven endochylema density leading to cell death in *E. coli* and *S. aureus*, respectively. Similarly, in this study other identified metabolites include colensane/clerodane diterpenoids (m/z 475.266), and phytuberin (m/z 317.171). The presence of these compounds is known for their antimicrobial and antifungal activities (Nguyen et al. 2021; Murthy et al. 2008), further supporting MRY extracts as an ideal antimicrobial agent. Evidently, this phytochemical profile corresponded to higher total flavonoids and phenolics from the extracts (Table 1). For instance, the highest flavonoid content was observed in *M. flabellifolius* extracted with 80% methanol (28.08 ± 0.10 mg QE/g), closely followed by *M. flabellifolius* 50% acetone extracts (27.74 ± 0.20 mg QE/g). (Table 1). Moreover, the same extracts showed potent antioxidant capacity (FRAP: 3296.00 µM; DPPH: 57.13%). The coexistence of substances with strong antioxidant activity and direct antimicrobial qualities points to the possibility of multi-target bioactivity which possibly cause disruption of microbial membranes and provide oxidative stress mitigation (Cushnie and Lamb 2005lçin 2012; Motlhanka 2008; Sinha et al. 2022; Wu et al. 2024), leading to significant inhibition zones up to 19.97 mm for *E. coli*, 17.7 ± 0.4 mm for *S. typhimurium*, while 17.55 ± 0.3 mm was observed for *S. aureus* (Table 2, Fig. 5, Fig. 6). In addition, MIC of 25 mg/mL for *M. flabellifolius* (Acetone/Water 50%) extract against *S. typhimurium*, *E. coli* and *S. aureus* was observed (Fig. 7). Meanwhile, 80% methanol/water extracts of *Myrothamnus* also displayed relatively good antimicrobial activity although less than those of 50% acetone for the same plant (Fig. 7). On the other hand, the antimicrobial activity of *O. paniculosa* and Morama extracts was moderate, with the acetone/water extracts outperforming the methanol/water extracts. Moreover, in all the organisms tested, *E. coli* consistently showed the highest MIC values across all extracts, indicating greater

resistance. This phenomenon is well documented, as outer membranes in gram negative bacteria like *E. coli limits* the penetration of many antimicrobial compounds, making them less susceptible to plant extracts (Dzotam and Kuete 2017; Fankam et al. 2011). Notably, the antimicrobial activity (Table 3) seems to improve with an increase in concentration from 25mg/mL to 200mg/mL across the plant extracts and bacteria. This potency, while less than the positive control gentamicin (MIC 0.01 mg/mL), which served as a benchmark for strong activity, suggests a robust, dose-dependent response antimicrobial effect. The effect of concentration is consistent with findings of previous studies, that demonstrated that many plant extracts exhibit enhanced antimicrobial activity with increasing concentration (Murthy et al. 2005; Nazzaro et al. 2013; Omotayo and Aremu, 2021). The positive control, CN10 Gentamicin, demonstrated the highest activity, with inhibition zones ranging from 22.0 mm to 22.8 mm for all tested pathogens, confirming its effectiveness as an antibiotic agent. On the other hand, *Myrothamnus* (80% methanol/water) and Morama (50% acetone/water) have higher MIC values, which suggests that their chemical components are less effective or that the extraction process influences the availability of the compound (Plaskova and Mlcek, 2023). Furthermore, these findings correlate with results by Matotoka and Masoko 2024 who reported that *M. flabellifolius Welw*. leaves possessed antibacterial properties with minimum inhibitory concentration greater 630 µg/mL for different bacterial strains tested.

In contrast, the limited efficacy of *T. esculentum* (Morama) seed coat and *O. paniculosa* leaf extracts may be due to combination of less diverse phytochemical profile and extraction effiency. This is evidenced by their low antioxidant activity, for instance, *O. paniculosa* water extract had mean FRAP of 1639.33 µM (Table 1), and consistently weak antimicrobial performance, even at the highest concentration of 200 mg/mL (Fig. 6). However, while less diverse to *M. flabellifolius* extracts, *O. paniculosa* UPLC-HRMS results showed better phytochemical profile than *T. esculentum* extracts. For example, in Table 2 powerful bioactives such as 4-ethenylbenzene-1,2-diol, myricetin (2-(3,4-dihydroxyphenyl)-3,5,6,7-tetrahydroxy-4H-chromen-4-one) and kaempferitrin was tentatively identified in *O. paniculosa*. Myricetin has been shown to have strong antiviral activity against SARS-CoV-2 by blocking its primary protease (M^{pro} inhibitors) and lowering pulmonary inflammation (Xiao et al. 2021), suggesting that its presence in *O. paniculosa* may have pharmacological significance. Notably, results in Table 1 demonstrates potency of *Ozoroa paniculosa*: For instance, *T. esculentum* extracted using 50% acetone recorded lower total phenolic content (109.85 ± 0.30 mg GAE/g) as compared to *O paniculosa* which recorded 194.12 ± 0.25 mg GAE/g. This support results in Table 3 reporting higher inhibitions zones as compared to *T. esculentum* seed coat extracts in which less metabolites were identified. *T. esculentum* extracts generally displayed limited microbial activity, particularly at lower concentrations, where no inhibition was observed in both 50% acetone and 80% methanol extracts at 25 mg/mL (Fig. 6). The best performance at 200 mg/ml yielded inhibition zones of 11.7 ± 0.06mm and 11.23 ± 0.12 mm against *E. coli* and *S. aureus*, respectively (Table 3, Fig. 6). In contrast, *O. paniculosa* recorded mean inhibition zone of 10.37 ± 0.17 mm at 200 mg/mL for the same organisms using 50% acetone (Table 3), indicating only modest antimicrobial potential, possibly attributed to its lower concentrations of phytochemicals extracted through lower temperature extraction technique in this study. Critically, the lower temperature and cold extraction

technique employed in this study, while gentle, has inherently lower extraction efficiency. This method likely failed to liberate a sufficient concentration of the antimicrobial constituents, particularly the less soluble compounds, from these already phytochemically sparse materials (Okoduwa et al. 2016). The limited activity of these extracts reinforces the idea that phytochemical richness and diversity are key drivers of potent antimicrobial action. The UPLC-HRMS profile of *T. esculentum* was dominated almost exclusively by lithospermoside (Table 2, Fig. 4), indicating a lack of the synergistic compound diversity necessary for potent, broad-spectrum activity.

The significant variation in bioactivity between extracts underscores the critical importance of solvent selection. Across all plants, the 50% acetone/water solvent consistently yielded more active extracts than 80% methanol/water. For example, the DPPH results indicated that *M. flabellifolius* extracted with 50% acetone extracts provided more effective radical scavenging capabilities, exemplified by a DPPH percentage of 57.13 compared to 41.30 for *M. flabellifolius* extracted with 80% methanol and 39.40% for *O. paniculosa* extracted with 80% methanol, respectively (Table 1). This antioxidant capacity could synergistically enhance the antimicrobial activity of the extracts, as oxidative stress is a critical factor influencing microbial survival and resistance (Motlhanka, 2008). Acetone/water is an effectively balanced solvent, capable of extracting a wider spectrum of medium polarity to polar compounds, including many antimicrobial flavonoids and phenolics (Khan et al. 2022; Bakour et al. 2019). The notably reduced efficacy of the 80% methanol extracts, particularly for *O. paniculosa* where it resulted in no observable activity, suggests it was less effective at solubilizing the specific active compounds, potentially missing key hydrophilic metabolites (Kumar et al. 2023; Nguyen et al. 2021). This confirms that solvent choice is not merely about yield but fundamentally shapes the bioactive profile of the final extract. Thus, the current study demonstrated that phytochemical richness, which is influenced by the extraction parameters as well as the intrinsic plant source, determines the strong antimicrobial activity of plant extracts. One promising option for the development of a natural antimicrobial agent is *M. flabellifolius*. To fully utilise its bioactive potential for use in natural medicine and food preservation, future research should concentrate on refining the extraction procedure, possibly using a technique like microwave-assisted extraction with 50% acetone.

5. Conclusion

This work evaluated the phytochemical profiles, antioxidant capacity, and antimicrobial activity of *Tylosema esculentum*, *Myrothamnus flabellifolius*, and *Ozoroa paniculosa* extracts. The findings demonstrate substantial bioactivity, with *Myrothamnus flabellifolius* exhibiting the highest phytochemical diversity and strongest antimicrobial and antioxidant effects. The significant variations in phytochemical content based on extraction methods highlight the importance of optimizing these protocols to maximize the yield of bioactive compounds. Further research is essential to elucidate the specific mechanisms of action, bioavailability, and potential applications of these extracts in food preservation and therapeutic formulations. Notably, *Myrothamnus flabellifolius* was identified as the most effective antimicrobial agent due to its rich phytochemicals content, indicating its potential as an active ingredient in food packaging and other food preservation strategies, as well as in the use of natural medicine.

Overall, the findings of this study provide a compelling basis for the continued exploration of these extracts, emphasizing their valuable contributions to food safety and health applications. By optimizing extraction methods and investigating further applications, it will be possible unlock the full potential of these bioactive compounds.

Declarations

CONFLICTS OF INTEREST

The authors declare no conflicts of interest in this manuscript.

ETHICAL APPROVAL

Not applicable

Author Contribution

M.D.S : Conceptualization, methodology, investigation, formal analysis, writing – original draft.K.M : Investigation, data curation, writing – review & editing.B.K.K : Investigation, Validation, writing – review & editing.E.A : Microbiological analysis, validation, visualization.G.M. : Formal analysis support, writing – review & editing.G.B : Critical revision of the manuscript, formal analysis support.M.H.D.M : Methodology guidance, writing – review & editing.M.G.B : Microbiolgical analysis, Visualization, writing – review & editing.F.T.T : Writing – review & editing.K.K.N : Validation, writing – review & editing.

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Data Availability

All data supporting the findings of this study are available within the article

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Figures

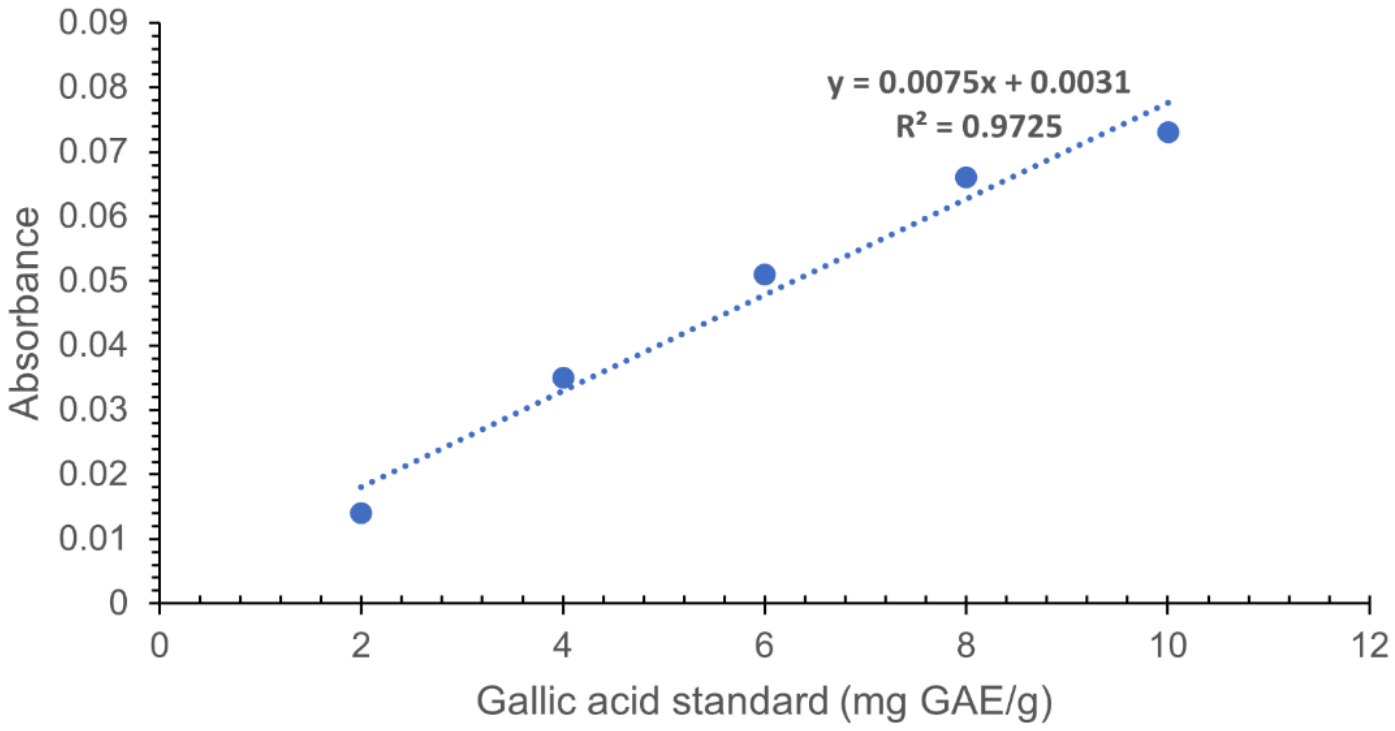

Figure 1

Gallic acid standard curve for the quantification of TPC

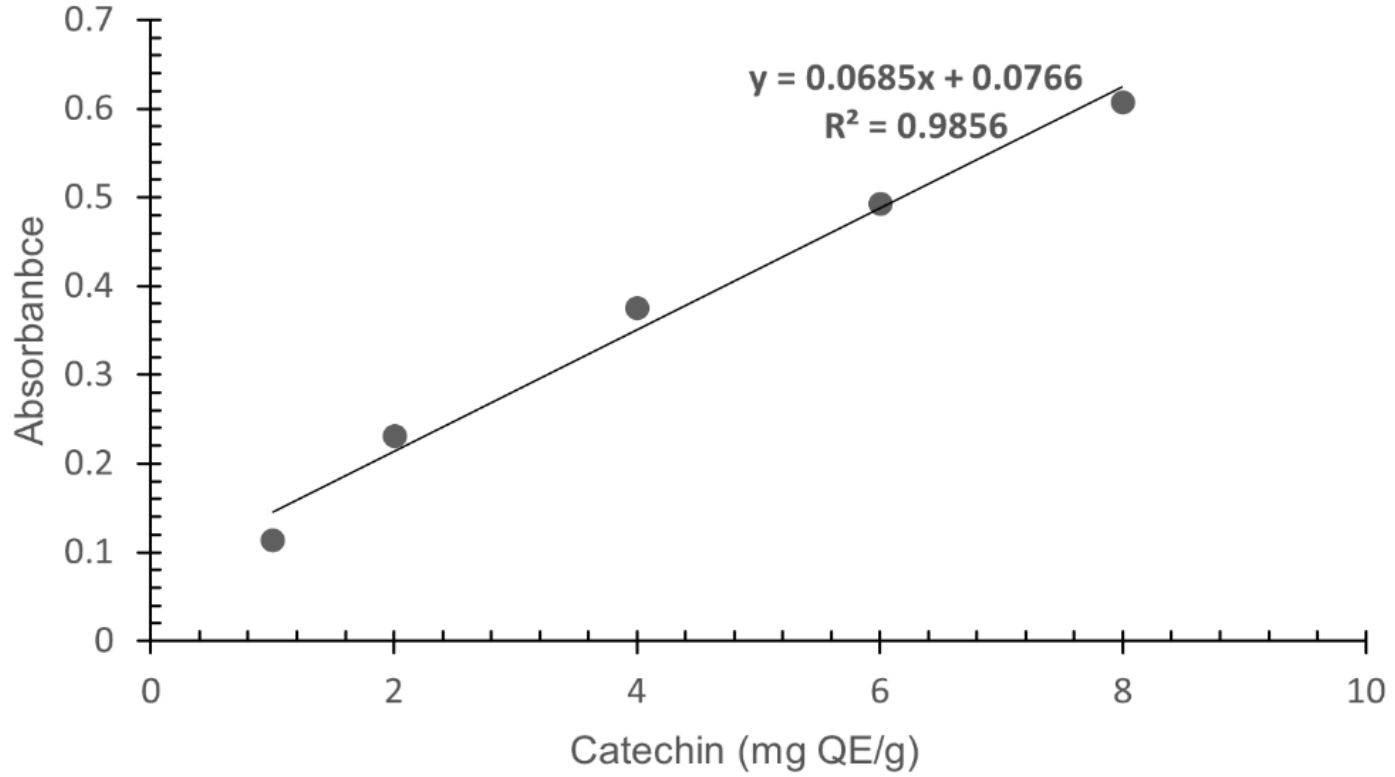

Figure 2

Catechin standard curve for the quantification of TFC.

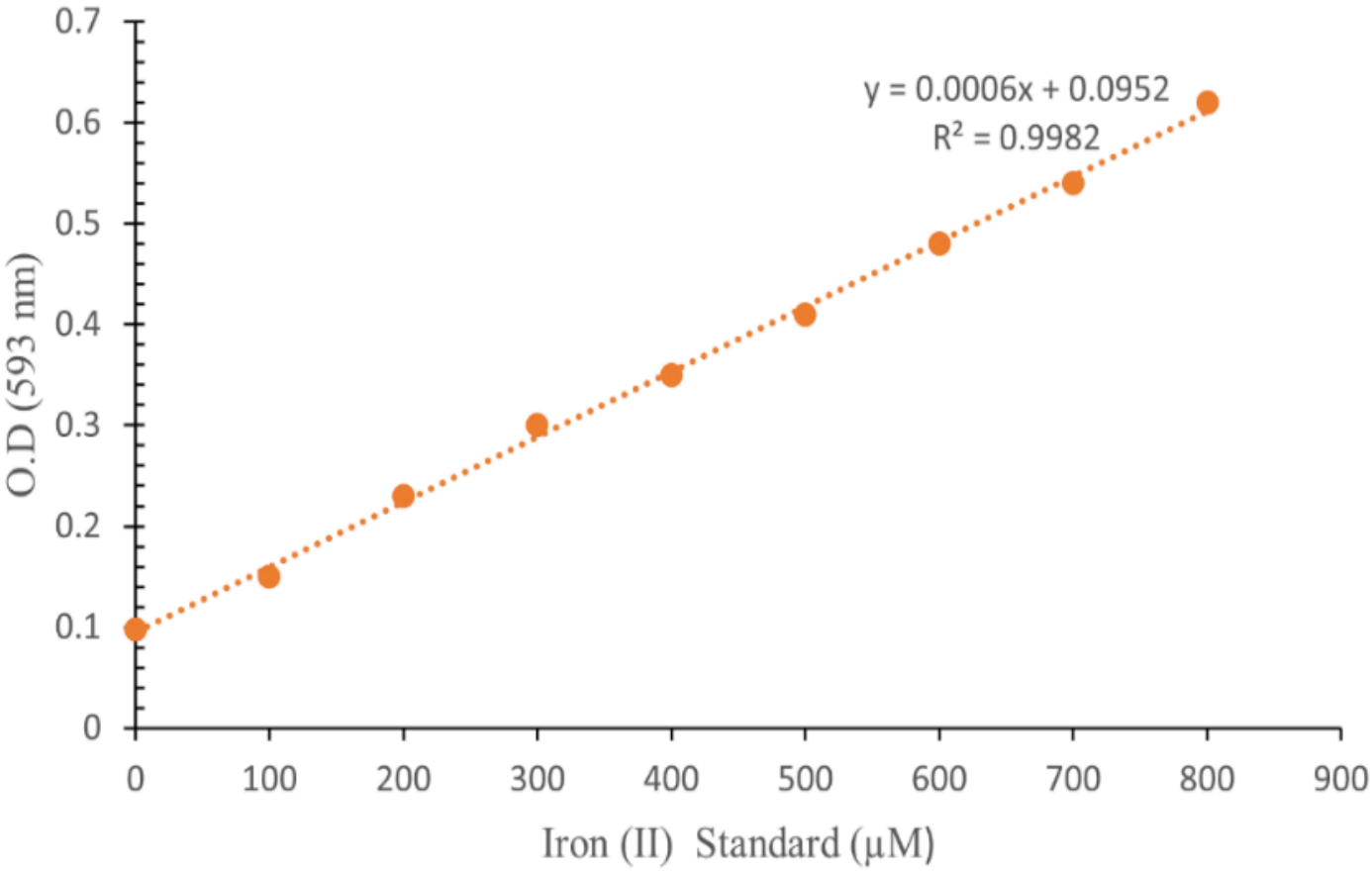

Figure 3

Iron (II) standard curve for FRAP.

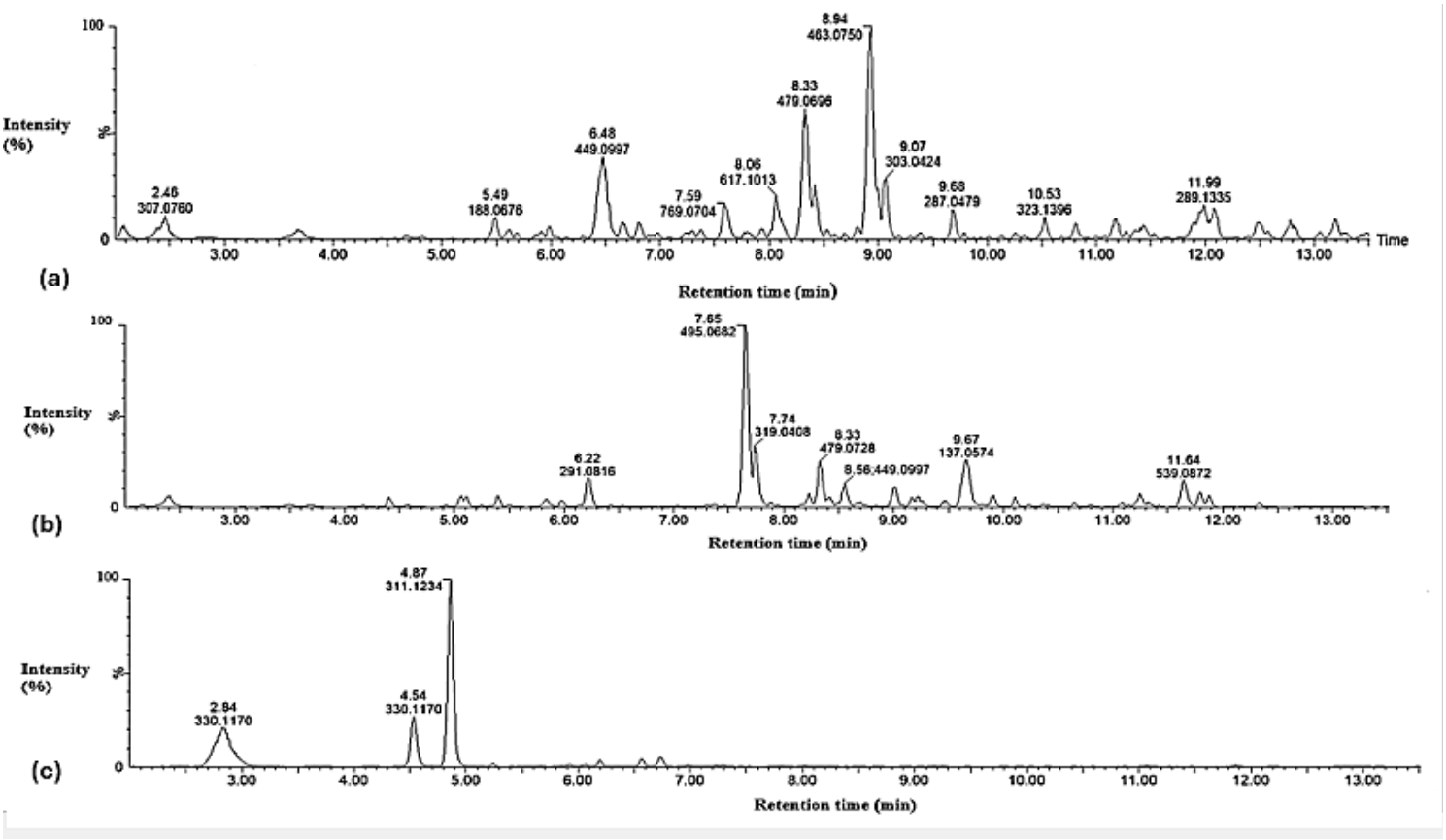

Figure 4

The Ultra-Performance Liquid Chromatography (UPLC-MS)-chromatographic profiles of methanolic leaf extracts from *Myrothamnus flabellifolius*(a), *Ozoroa paniculosa*(b), and *Tylosema esculentum* seed coat (c).

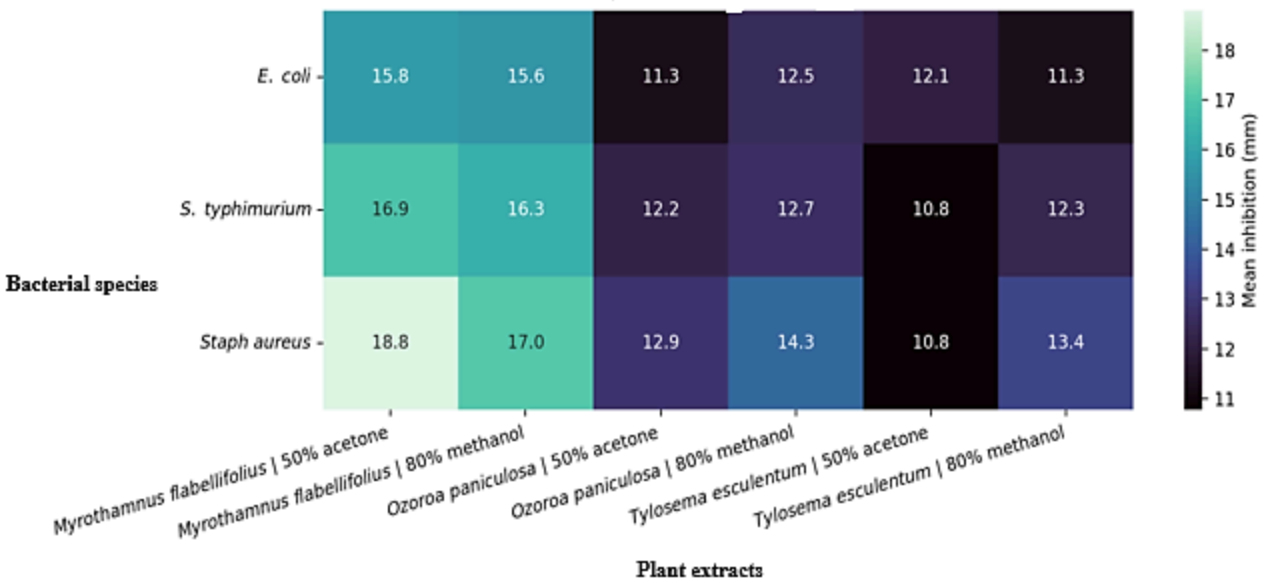

Figure 5

Comparison of mean inhibition zones (mm) produced by acetone/water and methanol/water extracts of *Myrothamnus flabellifolius*, *Tylosema esculentum*, and *Ozoroa paniculosa* against different bacterial species.

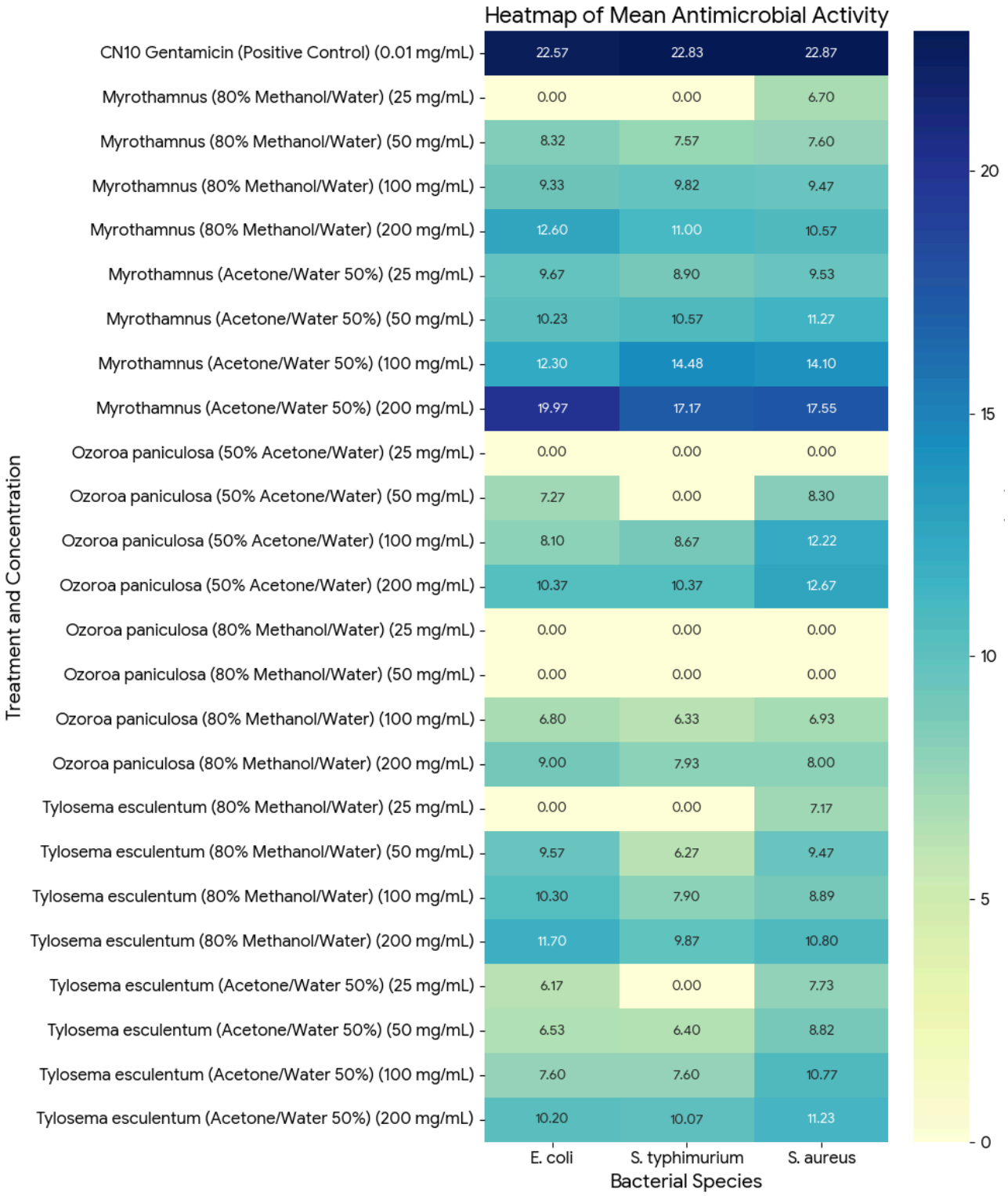

Figure 6

Antimicrobial activity of *Myrothamnus flabellifolius*, *Tylosema esculentum* and *Ozoroa paniculosa* extracts against *Escherichia coli, Salmonella typhimurium and Staphylococcus aureus* bacterial species.

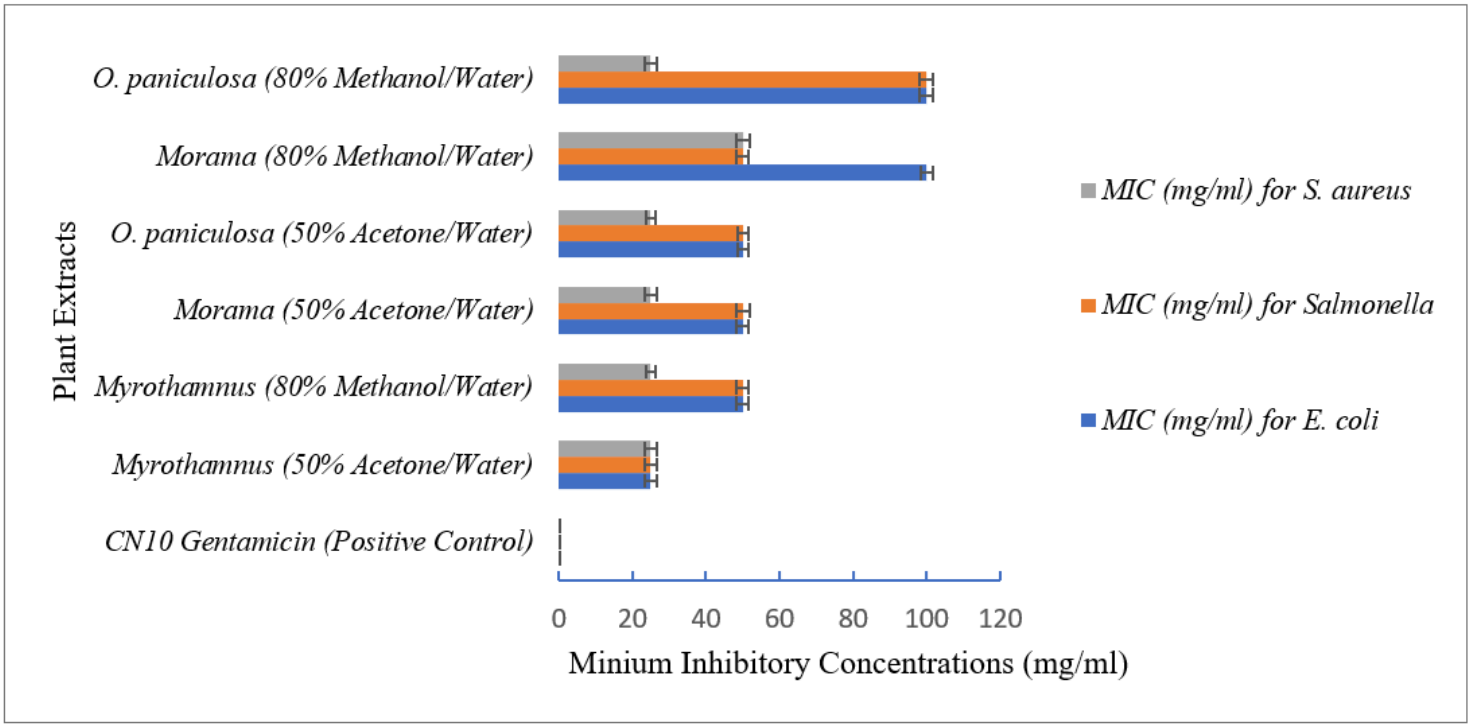

Figure 7

Minimum Inhibitory Concentrations (MIC) levels of *Myrothamnus flabellifolius*, *Tylosema esculentum* and *Ozoroa paniculosa*extracts on *Escherichia coli, Salmonella typhimurium and Staphylococcus aureus*.