

## Antibacterial Activity of Methanol Extracts of *Calypotes erosum* and *Racopilum africanum* and their Powder

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### ABSTRACT

Bryophytes have gained recognition as an exceptional source of novel natural products, including secondary metabolites with intriguing biological activities. This study explored the antibacterial potential of bryophytes (*Calypotes erosum* and *Racopilum africanum*) as sources of novel natural products with significant biological activities. The antibacterial properties of these moss plants' powder and methanol extracts were evaluated against six bacterial strains using the agar-well diffusion and broth-dilution methods. Quantitative phytochemical analysis revealed the presence of various compounds, including tannins, saponins, flavonoids, glycosides, alkaloids, and phenols, with *Calypotes erosum* exhibiting higher levels of these phytochemicals. Remarkably, the bryophyte powder exhibited more significant antibacterial efficacy compared with the methanol extracts. Particularly, *Calypotes erosum* powder at 1000 mg/ml demonstrated the highest zone of inhibition against *Escherichia coli* (80 mm) and *Salmonella typhi* (32 mm). However, no inhibition was observed when both moss plant powders were tested against *Plesiomonas shigelloides* at various concentrations. The results were compared with standard antibiotic discs, and all test organisms displayed sensitivity to *Racopilum africanum* (MIC < 0.1 mg/ml) and *Calypotes erosum* (MIC 1 mg/ml). This study suggests the potential of *Calypotes erosum* and *Racopilum africanum* powders as sources for future antibacterial drug development, highlighting their value in pharmaceutical research.

**Keywords:** Antibacterial, Bryophytes, Methanol, Phytochemicals, *Calypotes erosum*, *Racopilum africanum*

### INTRODUCTION

Bryophytes, often regarded as the most ancient land plants, constitute the second-largest group of terrestrial green plants following angiosperms. Taxonomically, they occupy a position between algae and pteridophytes (Asakawa, 2007). The global diversity of bryophytes is estimated to encompass approximately 15,000 to 25,000 species, categorically classified into three divisions: Marchantiophyta (liverworts), Anthocerotophyta (hornworts), and Bryophyta (mosses) (Goffinet and Shaw, 2009). These herbaceous plants primarily acquire water and essential mineral nutrients through their leaves, exhibiting a distinctive combination of characteristics that render them remarkably intriguing and unique. Despite their prevalence in humid environments, bryophytes are remarkably resistant to microbial infections, implying their ability to produce inducible antimicrobial compounds. Furthermore, research has unveiled their potential as sources of valuable antibiotics, prompting extensive exploration of these resources in search of potent, safe, and broad-spectrum antibiotics (Xie and Lou, 2009; Farina *et al.*, 2014). Bryophytes have gained recognition as an exceptional source of novel natural products, including secondary metabolites with intriguing biological activities, possibly applicable in pharmaceuticals (Asakawa, 1990; Singh *et al.*, 2007). The presence of antibiotic substances within bryophytes has been well-documented (Banerjee and Sen, 1979). These plants contain a wide range of compounds such as alkaloids (e.g., clavatoxine, clavatine, nicotine, lycopodine), polyphenolic acids (e.g., dihydrocaffeic), and

flavonoids (e.g., apigenin, triterpenes) (Almudena *et al.*, 2017). However, only a limited number of species have undergone thorough investigation.

The surging demand for plant-based medicines and the emergence of antibiotic-resistant bacteria have spurred scientific exploration into new plant-derived natural products. Bryophytes, owing to their medicinal attributes, represent a potential source of biologically active compounds. This, combined with their historical utilization as remedies for diverse ailments, underscores their significance in modern pharmacology (Sabovljević *et al.*, 2016). Bryophytes, armed with an array of potent chemical compounds, employ these substances as a defence mechanism. When fungal spores come into contact with a bryophyte thallus or leaves and the surface becomes moist, the plant releases phenolic compounds that inhibit spore germination. This mechanism may underlie the evolutionary success of bryophytes, enabling their survival for over 350 million years (Frahm, 2004).

*Calypotes erosum* Mull Hal and *Racopilum africanum* Mitt., are two bryophytes whose medicinal properties remain unexplored. This study investigated the phytochemical constituents and antibacterial activities of powder samples and methanol extracts derived from *Calypotes erosum* Mull Hal and *Racopilum africanum* Mitt.

## MATERIALS AND METHODS

### Plant Sample/Test Microbes Collection and Preparation

Moss Plants (*Calypotes erosum* and *Racopilum africanum*) materials were collected in May 2019 from their natural habitat within Olabisi Onabanjo University, Ago-Iwoye, Ogun State (6° 92' 33" N and 3° 87' 25" E) and identified at the Department of Plant Science, Olabisi Onabanjo University, Ago-Iwoye. Each plant was collected and carefully sorted out from other extraneous materials, washed and air dried and afterward finely ground into powder. 10 g of the samples were measured and macerated in absolute methanol for 72 h after which it was filtered using Whatman filter paper 1. The filtrate was kept in a steam bath and allowed to dry. The dry mass (concentrate) was kept until use for antibacterial assay.

The six bacterial species used were *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Bacillus cereus* ATCC 10702, *Plesiomonas shigelloides* ATCC 51903, *Proteus vulgaris* and *Salmonella typhi*. The last two species (*Proteus vulgaris* and *Salmonella typhi*) were sub-cultured to get pure culture. The test organisms were procured from the Department of Microbiology Laboratory, Babcock University, Ilishan, Ogun State.

### Quantitative Determination of Phytochemicals

The phenolic content of both samples was assessed using a spectroscopic method outlined by Singh *et al.* in (2007), following the extraction of phenolic components using ether. The determination of alkaloids and saponins content was conducted via gravimetric analysis after extraction using specific solvents, following established protocols (Singh *et al.*, 2007). To evaluate flavonoid content, 80 % methanol was repeatedly used for extraction, and gravimetric assessment was based on the methodology detailed by Boham and Kocopai (1974). Tannins content was determined through a spectroscopic method previously utilized by Ajuru *et al.* (2017), while glycosides were quantified employing the spectrometric technique as outlined by El-Olemyl *et al.* (1994).

### Antibacterial Assay and Determination of Zone of Inhibition

In the antibacterial assay, an overnight bacterial broth culture was utilized. Both the agar-well diffusion and broth-dilution methods were employed. Firstly, nutrient agar plates were prepared, sterilized at 121 °C for 15 minutes, and allowed to cool to approximately 40 °C. Once cooled, the agar was poured into sterile Petri dishes, where it solidified, following the procedures outlined by Perez *et al.* (1990) and King and Brown, (2001). On the solidified agar plates, the bacterial broth was evenly swabbed using a swab stick. Three holes were then created in the agar using a cork-borer with a 10 mm diameter. Subsequently, various concentrations of the sample (1000, 100, and 10 mg/ml) were introduced into the holes in triplicate. The plates were then incubated for 24 hours. In a similar fashion, different

amounts of the sample powder (1000, 100, and 10 mg) were weighed and dissolved in 10 ml of sterile water to obtain concentrations of 1000, 100, and 10 mg/ml respectively. These solutions were then mixed with sterile nutrient agar (250 ml), poured into Petri dishes, and allowed to solidify. Once solidified, a single hole was created in the agar using a cork-borer with a 10 mm diameter. The various bacterial broths were introduced into the hole in triplicate (Ulka and Karadge, 2010). Finally, the zones of inhibition were measured with a transparent ruler (in millimetres). Sensitivity discs of standard antibiotic drugs were used as positive controls.

### Determination of Minimum Inhibitory Concentration (MIC) Of Methanol Extract

The minimum inhibitory concentration (MIC) was determined through a 10-fold dilution method, resulting in concentrations of 0.1, 1, 10, 100, and 1000 mg/ml respectively. These concentrations were introduced into a 96-well plate and incubated for 24 h (NCCLS, 2008). Subsequently, the MIC of the test (sample + organism + broth) was determined by comparing its turbidity with that of the control (organism + broth). The MIC was defined as the lowest concentration of the extract that inhibited any visible bacterial growth.

### Data Analysis

Analysis was done using MS Excel and SPSS version 20. Student's t-test was done to evaluate statistical differences in the phytochemical yield of the two bryophytes; a two-way Analysis of Variance (ANOVA) was used to compare concentration factors and extract types. LSD and Duncan tests were used to determine specific mean differences at  $P < 0.05$  level of significance.

## RESULTS

### The Quantitative Phytochemical Analysis of the Plant Samples

In Table 1, the composition of phytochemicals in the two tested bryophytes is presented as percentages. Alkaloids were found to be the predominant constituent in both bryophytes, followed by saponins, with flavonoids being the least abundant. Notably, *Calypotes erosum* exhibited a significantly higher ( $p < 0.05$ ) percentage of all the tested phytochemicals.

### Antibacterial Analysis of *Calypotes erosum*

Table 2 shows the results of the zone of inhibition caused by both the dry powdered sample and the methanol extract of *Calypotes erosum* across three concentration ranges. The dry powdered sample inhibited the growth of all the test organisms, with the most substantial inhibition observed against *E. coli* at 80 mm. In contrast, the methanol extract exhibited inhibitory effects on the test organisms, except for *Staphylococcus aureus*. The dry powdered sample, on the whole, yielded larger zones of inhibition compared to the

methanol extract. The result also shows that there was no interaction effect in the zone of inhibition produced by the plants against *B. cereus* and *S. aureus* suggesting that the differences in the mean zones of inhibition produced by the dry powdered sample and the methanolic extract of the plant against the two bacterial strains is consistent across the concentration range and vice versa. The dry powder was found to significantly elicit a higher zone of inhibition (values) in both strains while also exhibiting a dose-dependent response across the concentration range with the highest concentration (1000 mg/ml) producing the highest zone of inhibition ( $p < 0.05$ ) than the other two levels with comparable values in the *Bacillus* strain while the dose-dependent response observed in the *Staphylococcus* strain was not significantly different from each other.

However, there was interaction effect observed in *P. vulgaris*, *S. typhi*, *E. coli* and *P. shigelloides*, the interaction was such that in *Proteus*, comparable dose-dependent increase was observed in the zone of inhibition produced by the methanolic extract which are significantly different from the constant values produced across the concentration range in the dry powdered samples; in contrast, the trend observed in the *S. typhi*, *E. coli* and *P. shigelloides* strains shows a dose-dependent increase where the values produced in the dry powdered sample are significantly different from those produced in the methanol extracts. Thus, in summary, the dry powdered sample appeared to produce the highest significant zone of inhibition against all the tested bacterial strains except *P. vulgaris*.

**Table 1:** The Quantitative Phytochemical Analysis of the Plant Samples.

Phytochemicals (%)	<i>Calymperes erosum</i>	<i>Racopilum africanum</i>
Tannins	0.0086	0.0073
Saponins	0.383	0.372
Flavonoids	0.0057	0.0038
Glycosides	0.198	0.188
Alkaloids	0.446	0.418
Phenols	0.273	0.249

Table 3 presents the result of the zone of inhibition produced by the dry powdered sample and the methanol extract of *R. africanum* at the three concentration range, while Table 4 shows the minimum inhibitory concentration of the Bryophytes against the test organisms. The result shows that the dry powder sample inhibited growth in all the test organisms except *P. vulgaris* and also there was an interaction effect in the zone of inhibition produced by the plant against only *P. vulgaris* across the concentrations suggesting that the differences in the mean zones of inhibition produced by the dry powdered sample and the methanolic extract of the plant against the bacterial strain

was inconsistent across the concentration range and vice versa.

Further examination of the cell means shows a dose-dependent increase in the zone of inhibition produced by the methanolic extract against *P. vulgaris*, with the highest mean zone of inhibition produced at a concentration of 1000 µg/ml. This value is significantly different from the other concentrations within the extract and also across the dry powdered samples. The activity of the two forms of the plant samples against the other bacterial strains showed no significant interaction effect; however, the main effects suggest that higher zones of inhibition were produced by the powdered samples against *B. cereus*, *S. aureus* and *S. typhi*, though this effect was not significant ( $p < 0.05$ ) in *B. cereus* while the methanolic extract presented higher zones of inhibition against *E. coli* and *P. shigelloides*. In most cases however, a significant dose-dependent response was observed in *B. cereus*, *S. aureus*, and *P. shigelloides*, while same similar trend was observed in *S. typhi* and *E. coli* albeit not significant at  $p < 0.05$

## DISCUSSION

Generally, bryophytes have been reported to serve as a source for a wide variety of chemical compounds that are known to have numerous potentials (Pants and Tewari, 1990). Oligosaccharides, polysaccharides, sugar alcohols, amino acids, fatty acids, aliphatic compounds, phenylquinones, aromatic and phenolic substances are reportedly present in bryophytes (Pants and Tewari, 1990). The presence of alkaloids, glycosides, flavonoids and saponin as part of the (bryophyte species) chemical constituent is an indication that the plants have some medicinal potential. According to previous reports (Dan *et al.*, 2008; Nantachit *et al.*, 2010), the alkaloids and flavonoids found in bryophyte extracts exhibit potent antibacterial and antifungal properties against various pathogenic microorganisms. Glycosides have shown potential in treating heart ailments (Pawar and Arumugam, 2011). Furthermore, saponins exhibit expectorant properties that can help manage inflammation in the upper respiratory tract and also possess anti-diabetic properties (Neto *et al.*, 2011.).

Although there is limited literature available on the use of bryophytes as antibacterial agents despite their global distribution, it is widely acknowledged that almost all bryophytes can resist attacks from pathogenic bacteria and fungi (Basile *et al.*, 1998; Asakawa, 2001). The test organisms were more sensitive to *Racopilum africanum* than they were to *Calymperes erosum* (MIC=1) (Table 4). The test plants from this work showed promising degrees of inhibitory effects on the test organisms because mosses are a rich source of secondary metabolites with antimicrobial activity (Asakawa, 1981, Sabovljevic *et al.*, 2006; Asakawa, 2007; Mellegard *et al.*, 2009).

Table 2: Antibacterial activity of *Calymperes erosum*

Factors	<i>Bacillus cereus</i>			<i>Staphylococcus aureus</i>			<i>Proteus vulgaris</i>		
	DP	ME	Mean	DP	ME	Mean	DP	ME	Mean
1000	22.33±2.52	12.33±0.58	<b>17.33±5.72<sup>a</sup></b>	23.67±5.51	10.00±0.00	<b>16.83±8.26<sup>a</sup></b>	10.00±0.00 <sup>b</sup>	15.33±1.53 <sup>a</sup>	<b>12.67±3.08</b>
100	16.33±3.79	11.00±1.00	<b>13.67±3.83<sup>b</sup></b>	17.67±2.08	10.00±0.00	<b>13.83±4.40<sup>a</sup></b>	10.00±0.00 <sup>b</sup>	13.33±0.58 <sup>a</sup>	<b>11.67±1.86</b>
10	14.33±1.53	10.00±0.00	<b>12.17±2.56<sup>b</sup></b>	17.00±2.65	10.00±0.00	<b>13.50±4.18<sup>a</sup></b>	10.00±0.00 <sup>b</sup>	11.00±1.00 <sup>a</sup>	<b>10.50±0.84</b>
Mean	<b>17.67±4.33<sup>a</sup></b>	<b>11.11±1.17<sup>b</sup></b>		<b>19.44±4.53<sup>a</sup></b>	<b>10.00±0.00<sup>b</sup></b>		<b>10.00±0.00</b>	13.22±2.11	
Streptomycin 30 ug	15			12			15		
Ciprofloxacin 10 ug	19			30			25		
Erythromycin 10 ug	25			31			20		
Gentamycin 10 ug	15			12			20		
Factors	<i>Salmonella typhi</i>			<i>Escherichia coli</i>			<i>Plesiomonas shigelloides</i>		
	DP	ME	Mean	DP	ME	Mean	DP	ME	Mean
1000	32.33±2.52 <sup>a</sup>	11.67±1.15 <sup>d</sup>	<b>22.00±11.45</b>	80.00±10.00 <sup>a</sup>	11.00±1.00 <sup>c</sup>	<b>45.50±38.32</b>	27.67±2.52 <sup>a</sup>	11.67±0.58 <sup>d</sup>	<b>19.67±8.91</b>
100	24.00±1.73 <sup>b</sup>	11.33±0.58 <sup>d</sup>	<b>17.67±7.03</b>	20.67±1.15 <sup>b</sup>	10.33±0.57 <sup>c</sup>	<b>15.50±5.72</b>	21.67±1.53 <sup>b</sup>	10.67±0.58 <sup>d</sup>	<b>16.17±6.11</b>
10	18.00±2.65 <sup>c</sup>	10.33±0.58 <sup>d</sup>	<b>14.17±4.54</b>	13.67±4.04 <sup>bc</sup>	10.00±0.00 <sup>c</sup>	<b>11.83±3.25</b>	18.67±1.15 <sup>c</sup>	10.00±0.00 <sup>d</sup>	<b>14.33±4.80</b>
Mean	<b>24.78±6.55</b>	<b>11.11±0.93</b>		<b>38.11±32.03</b>	<b>10.44±0.73</b>		<b>22.67±4.27</b>	10.78±0.83	
Sparfloxacin 10 ug	14			14			12		
Pefloxacin 10 ug	32			12			15		
Amoxacillin 30 ug	20			12			13		
Ciprofloxacin 10 ug	30			30			23		

Note: column and row means (bolded) shows the mean differences in the main factors separately when there is no interaction effect where means with same letters across either the rows (plant treatment type) or column (concentration) are not significantly different from each other ( $p > 0.05$ ); while the cell means (not bold across both column and row) shows the mean differences in the interaction effect where means with same alphabet across all cell means in each microorganisms are not significantly different ( $p > 0.05$ ). DP- Dry Powder, ME-Methanol extract

Table 3: Antibacterial activity of *Racopilum africanum*

Factors	<i>Bacillus cereus</i>			<i>Staphylococcus aureus</i>			<i>Proteus vulgaris</i>		
	DP	ME	Mean	DP	ME	Mean	DP	ME	Mean
1000	15.00±3.00	11.67±2.08	<b>13.33±2.94<sup>a</sup></b>	18.33±2.89	13.67±2.08	<b>16.00±3.41<sup>a</sup></b>	10.00±0.00 <sup>b</sup>	13.67±2.08 <sup>a</sup>	<b>11.83±2.40</b>
100	10.33±0.58	11.00±1.00	<b>10.67±0.82<sup>b</sup></b>	16.67±1.15	11.33±0.58	<b>14.00±3.03<sup>b</sup></b>	10.00±0.00 <sup>b</sup>	11.00±0.00 <sup>b</sup>	<b>10.50±0.55</b>
10	10.00±0.00	10.00±0.00	<b>10.00±0.00<sup>b</sup></b>	14.33±2.08	10.00±0.00	<b>12.17±2.71<sup>b</sup></b>	10.00±0.00 <sup>b</sup>	10.33±0.58 <sup>b</sup>	<b>10.17±0.41</b>
Mean	<b>11.78±2.86<sup>a</sup></b>	<b>10.89±1.36<sup>a</sup></b>		<b>16.44±2.56<sup>a</sup></b>	<b>11.67±1.94<sup>b</sup></b>		<b>10.00±0.00</b>	<b>11.67±1.87</b>	
Streptomycin 30 ug	15			12			15		
Ciprofloxacin 10 ug	19			30			25		
Erythromycin 10 ug	25			31			20		
Gentamycin 10 ug	15			12			20		
Factors	<i>Salmonella typhi</i>			<i>Escherichia coli</i>			<i>Plesiomonas shigelloides</i>		
	DP	ME	Mean	DP	ME	Mean	DP	ME	Mean
1000	17.67±2.52	12.33±1.53	<b>15.00±3.46<sup>a</sup></b>	13.67±1.53	16.67±4.73	<b>15.17±3.54<sup>a</sup></b>	13.33±1.53	14.33±0.58	<b>13.83±1.17<sup>a</sup></b>
100	16.67±2.08	10.67±1.15	<b>13.67±3.61<sup>a</sup></b>	11.67±2.89	14.67±3.06	<b>13.17±3.13<sup>a</sup></b>	10.00±0.00	12.67±0.58	<b>11.33±1.51<sup>b</sup></b>
10	15.33±3.21	10.33±0.58	<b>12.83±3.43<sup>a</sup></b>	10.00±0.00	13.00±2.65	<b>11.50±2.35<sup>a</sup></b>	10.00±0.00	11.33±0.58	<b>10.67±0.82<sup>b</sup></b>
Mean	<b>16.56±2.51<sup>a</sup></b>	<b>11.11±1.36<sup>b</sup></b>		<b>11.78±2.28<sup>b</sup></b>	<b>14.78±3.49<sup>a</sup></b>		<b>11.11±1.83<sup>b</sup></b>	<b>12.78±1.39<sup>a</sup></b>	
Sparfloxacin 10 ug	14			14			12		
Pefloxacin 10 ug	32			12			15		
Amoxacillin 30 ug	20			12			13		
Ciprofloxacin 10 ug	30			30			23		

Note: column and row means (bolded) show the mean differences in the main factors separately when there is no interaction effect where means with same letters across either the rows (plant treatment type) or column (concentration) are not significantly different from each other ( $p > 0.05$ ); while the cell means (not bold across both column and row) shows the mean differences in the interaction effect where means with same alphabet across all cell means in each microorganisms are not significantly different ( $p > 0.05$ ).

RacPow-Powder Sample, RacMet-Methanolic Extract

**Table 4:** Minimum inhibitory concentration (MIC) of the Bryophytes on the test organisms (mg/ml)

	<i>Calymperes erosum</i>	<i>Racopilum africanum</i>
<i>Bacillus cereus</i>	1	<0.1
<i>Staphylococcus aureus</i>	1	<0.1
<i>Proteus vulgaris</i>	1	0.1
<i>Salmonella typhi</i>	1	<0.1
<i>Escherichia coli</i>	1	<0.1
<i>Plesiomonas shigelloides</i>	1	<0.1

From this study, specific antibacterial compounds present in the methanol and powder samples of the test bryophytes are more effective against specific bacteria, however, the powder was found to be more effective on the test bacteria than the methanol extract. This observation is consistent with the report of Oyesiku and Caleb (2015), that specific antibacterial compounds isolated are more effective against specific bacteria species. It is commonly reported that concerning the antimicrobial activity of methanol extract, it possesses greater antifungal than antibacterial activity (Milan *et al.*, 2008), however, methanol extract exhibits better antibacterial activity against Gram (+) bacteria than Gram (-). Methanol extracts of *C. erosum* and *R. africanum* proved more effective against *P. vulgaris* than their powders. In accordance with Parihar *et al.* (2010) on the antibacterial efficacy of methanolic extracts of lower plants, the powder of *C. erosum* stimulated a higher zone of inhibition in both *B. cereus*, *S. aureus* and *S. typhi* with a dose-dependent trend across the tested concentrations range. Furthermore, the methanol extract produced higher zones of inhibition against *E. coli* and *P. shigelloides* with a considerably high value against *S. aureus*. Most of the active substances in medicinal plants are in aromatic or saturated organic compounds and some of the substances are easily extracted by polar and non-polar solvents (Cowan, 1999; Altuner *et al.*, 2011). However, the performance of the powder in this study suggests that some bryophytes are capable of releasing these potent antimicrobial properties without necessarily using a solvent. In the past, researchers have examined different organic solvent extractions of bryophytes, but in this study, the powder showed a very promising antibacterial potential. Powder (at 1000 mg/ml) of *C. erosum* elicited the overall highest zone of inhibition (80 mm) on *E. coli* followed by *C. erosum* powder (1000 mg/ml) on *Salmonella typhi* with 32 mm zone of inhibition. No inhibition was recorded when *C. erosum* and *R. africanum* (powder) was screened against *P. vulgaris* across the three concentrations (10, 100, and 1000 mg/ml). The trend in the MIC also suggests that all the test organisms were highly sensitive to *C. erosum* and *R. africanum*.

## CONCLUSION

In summary, previous research has explored organic solvent extracts of bryophytes, but our study unveiled the remarkable antibacterial potential of bryophyte powders. At a dose of 1000 mg the bryophyte powders displayed substantial inhibition against all tested bacterial strains, except for *P. vulgaris*, where methanolic extracts appeared more effective at the same concentration. This highlights the need for additional screening of bryophyte powders for antibacterial properties. Furthermore, there is great potential in harnessing the phytochemical components of these powders for both pharmaceutical and agricultural applications. Additionally, the identification and characterization of their bioactive compounds hold promise for future research.

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