

Lack of Antibacterial Activity of Commercial Supplies of Buchu Leaf and Pygeum Bark Against a Panel of Bacterial Pathogens

Original Research

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Chantelle Florenca, 4th year BPharm(Hons)¹

Ian E. Cock, BSc(Hons), PhD¹

Gary D. Grant, BPharm, PhD, GradCertHighEd¹

Matthew J. Cheesman, BSc(Hons), PhD, GradCertHighEd¹

¹ School of Pharmacy and Medical Sciences, Griffith University, Australia

ABSTRACT

Background: *Agathosma betulina* (Buchu) and *Pygeum africana* (Pygeum) are native Southern African plants with numerous traditional medicinal uses. However, studies on their antibacterial effects are limited. The discovery of new antibacterial treatment options has become important given the growing prevalence of bacterial resistance to common antibiotics.

Aims: To investigate the antibacterial effects of water, methanolic and ethyl acetate extracts prepared from different commercial plant materials against antibiotic-sensitive and antibiotic-resistant pathogens, and to determine their phytochemical and toxicity profiles.

Methods: Agar disc diffusion and liquid microdilution assays were used to determine the inhibitory effects of the Buchu and Pygeum extracts against common bacteria, including *Staphylococcus aureus*, MRSA, *Escherichia coli*, ESBL *E. coli*, *Klebsiella pneumoniae* and ESBL *K. pneumoniae*. Five different conventional antibiotics were also tested against the six bacterial species using these assays, serving as reference antibiotics. Qualitative phytochemical assays were used to identify phytochemical classes within each extract. *Artemia* nauplii assays were used to assess toxicities.

Results: All Buchu and Pygeum extracts prepared from the powdered commercial product, as well as the commercially supplied plant segments, were inactive in agar and liquid broth assays. All extracts were deemed to be non-toxic in *Artemia*.

Conclusion: Commercial supplies of Buchu and Pygeum were ineffective against all bacterial pathogens tested. While toxicity studies suggest they are safe to use in *Artemia*, the use of these products is brought into question due to their lack of antimicrobial activity. It is possible that extracts prepared from the fresh plant, compared to pre-processed plant material, may yield antimicrobial activities.

INTRODUCTION

The World Health Organisation (WHO) classifies multiple drug-resistant pathogens as one of the most serious threats to global health.¹ The prevalence of infections caused by

these microorganisms is rapidly increasing. The lack of antibiotic stewardship in clinical and community settings is a major cause of this threat.² An example of highly resistant bacteria includes the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*,

Klebsiella pneumoniae, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp.*), which are prevalent in hospitals.³ Perhaps the best-known antibiotic-resistant species is methicillin-resistant *Staphylococcus aureus* (MRSA), which possesses a mutated penicillin-binding protein that does not bind beta-lactam antibiotics.⁴ Additionally, extended spectrum beta-lactamase (ESBL) gram-negative bacteria including *E. coli* and *K. pneumoniae* harbour beta-lactamase enzymes that destroy most beta-lactam antibiotics, including penicillins, cephalosporins and aztreonam.⁵ There are several mechanisms by which bacteria develop resistance to antimicrobial drugs.⁶

Traditional plant-based medicines have recently been of great interest as an alternative to antibiotics due to the increasing threat of antimicrobial resistance. Many medicines with antimicrobial properties have been sourced from plants.⁷ However, relatively few of the estimated 500,000 plant species are yet to be investigated for their therapeutic properties. Many herbal preparations are sold and used without peer-reviewed evidence that substantiates claims pertaining to their efficacy. Two plant species that lack rigorous examination of their antibacterial efficacies are *Agathosma betulina* (Berg.) Pillans and *Pygeum africanum* Hook.f. Both species are native to Africa and have been used traditionally for a myriad of ailments. *Agathosma betulina*, commonly known as Buchu, is native to the Western Cape Province of South Africa in mountainous environments.⁸ Its use by the indigenous people of the western region of South Africa (Khoisan, Xhosa) dates back many centuries. Buchu has been found to play important roles in wound healing, analgesia, stomach ailments, flu-like symptoms and rheumatism, with studies reporting antimicrobial, anti-inflammatory and antioxidant medicinal properties.^{9,10} Whilst there are some reports of mild antibacterial activity of the raw plant material against *S. aureus*, *K. pneumoniae*, *E. coli* and *E. faecalis*, no studies have examined the effects of the commercially supplied Buchu leaf material or dried powders.¹⁰⁻¹²

Pygeum africanum (commonly known as African cherry) is an evergreen tree found in regions of Africa. Its bark is used to treat benign prostatic hyperplasia, malaria, kidney disease, wound management, fever, lack of appetite and stomach pain.^{13,14} One study reported inhibitory effects of *P. africanum* extracts on the growth of *S. aureus* and MRSA on agar.¹⁵ However, no further work has been done with this species, and the antibacterial properties of commercially prepared *P. africanum* are yet to be verified. Consumers have

more access to pre-processed herbal formulations than in the past.¹⁶ However, information on how the herbal formulations were prepared are often unavailable. It should be noted that the Australian Therapeutic Goods Administration (TGA) lists *A. betulina* as a permissible ingredient in the Listed Medicines, where it states that pulegone is a mandatory component at a concentration no greater than 4%.¹⁷ The dry leaf, leaf oil (as a flavour or fragrance) and leaf powder are included in the Permissible Ingredients Determination. However, *P. africanum* is not listed as a permissible ingredient. Given the restrictions in physically transporting fresh plant materials from countries such as Africa due to stringent biosafety regulations imposed by Australian Customs, access to Buchu and Pygeum for the purpose of this experiment was restricted to online purchasing from suppliers of the processed material. This study aimed to test commercially available *A. betulina* and *P. africanum* material for antibacterial activity, using agar diffusion and microdilution broth assays. These plant materials were commercially sourced from two different suppliers. One provided the product in pre-extracted powdered forms, while the other products were provided as the leaves (Buchu) or bark (Pygeum) which more closely resembled the original source material but were also obtained from a commercial supplier.

METHOD

Plant Sources

Two different commercial samples of *A. betulina* (Buchu) leaf and *P. africanum* (Pygeum) bark were purchased online. Initially, pre-extracted powdered forms derived solely from the two plants were obtained. These powders had already been subjected to a solvent extraction of the plant material by the manufacturers who used undisclosed solvents. These solvents were subsequently removed by evaporation and the crude extracts shipped as the dried powders. The *A. betulina* leaf powder was purchased from Maple Life Sciences (India) and was named BU; while the *P. africanum* bark powder was obtained from Customised Health (Australia) and was named PY. To further explore the BU and PY plants, a supply of *A. betulina* leaves, supplied as Buchu tea leaves (and named BTL) was purchased from Tea Life (Australia). *Pygeum africanum* bark material (named PBK) was acquired from Ourland Herbs (Canada). Despite being unable to confirm how these plant materials had been processed prior to shipping, they were included in the study for comparison with the commercial pre-extracted powder preparations.

Antibiotics and Bacterial Species

Penicillin-G (potency of 1440–1680 µg/mg), erythromycin (potency ≥850 µg/mg), tetracycline (≥95% purity by HPLC), chloramphenicol (≥98% purity by HPLC) and ciprofloxacin (≥98% purity by HPLC) were purchased from Sigma-Aldrich (Australia) and were used as controls for the microdilution broth assays. Preloaded discs (Oxoid Ltd., Australia) containing penicillin (10 IU), erythromycin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg) and ciprofloxacin (1 µg) were used in disc diffusion studies as positive controls to further test susceptibilities on agar.

Escherichia coli (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), MRSA (ATCC 43300), *Klebsiella pneumoniae* (ATCC 13883) and ESBL *Klebsiella pneumoniae* (ATCC 700603) were purchased from the American Type Culture Collection (USA). The ESBL *Escherichia coli* strain was a wild-type clinical strain acquired from the Gold Coast University Hospital (Australia).

Plant Extract Preparation

Water, methanol and ethyl acetate were used in separate extractions for each of the plant samples.¹⁸ These solvents were of different polarities and therefore could extract different phytochemical subsets. In this way, a greater diversity of the compounds was extracted, and increased the likelihood that biologically active components would be isolated.¹⁹ The BTL leaves (approximately 15 mm in size) and the PBK bark fragments were processed with a laboratory grinder to formulate coarse powders. A mass of 1 gram of each plant was weighed into individual polypropylene tubes (Falcon Corning Life Sciences) and 50 mL of either deionised water, methanol (ChemSupply Australia) or ethyl acetate (Sigma-Aldrich) was added. The tubes were mixed continuously by rolling at 30 rpm for 24 hours at room temperature. To remove particulate plant matter, the samples were vacuum filtered using Whatman No. 1 filter paper and the filtrates were added to pre-weighed 50 mL polypropylene tubes. Organic solvents (methanol and ethyl acetate) were evaporated by air drying at 45°C in a chemical flow hood for 48–72 hours. Aqueous samples (–80°C) were dried via lyophilisation in an Alpha 1–4 LSC plus benchtop freeze dryer (Martin Christ) for 48–72 hours. The crude extracts were weighed to measure the extraction yield, then resuspended in 10 mL of deionised water containing 1% dimethyl sulfoxide (DMSO; Merck). Each extract was sonicated three times for 20 second pulses at 2 kHz, with 30 second rest intervals between pulses. The extracts were then sterilised via passage through 0.22 µm filters (Sarstedt) and aliquoted and stored at

–20°C until use.

Bacterial Subcultures

Frozen bacterial glycerol stocks were initially streaked on agar plates to ensure culture purity. The *E. coli* and *K. pneumoniae* strains were initially streaked on Mueller-Hinton (MH) agar, whilst the ESBL strains were streaked on Brilliance ESBL agar plates (Thermo Fisher Scientific). *Staphylococcus aureus* was streaked on MH agar supplemented with 2% NaCl, while MRSA was streaked on Brilliance MRSA 2 agar (Thermo Fisher Scientific). All bacteria were cultured at 37°C except for MRSA, which was cultured at 35°C to prevent heteroresistance (growth of both susceptible and resistant staphylococci) from arising in MRSA cultured at temperatures above 35°C.²⁰ Individual bacterial colonies of the *E. coli* and *K. pneumoniae* strains were inoculated into 40 mL of fresh MH broth media, while *S. aureus* and MRSA were inoculated into the same media supplemented with 2% NaCl. The inoculated broth was incubated for 18 hours until the cell count reached approximately 10⁸ cells/mL. All antibacterial test conditions conformed to the Clinical and Laboratory Standards Institute (CLSI) standardised methods.²⁰

Antibacterial Assays

A modified Kirby-Bauer disc diffusion method was used to evaluate antibacterial activity on agar.¹⁸ Growth inhibition was measured to the nearest whole millimetre as the zone of inhibition (ZOI) for each disc. ZOI values for samples that did not inhibit growth were reported as 6 mm (the diameter of the filter discs applied to the agar surface). Liquid dilution broth assays were used to determine MIC values for all extracts and antibiotics as previously described.²¹ Extract MIC values >5000 µg/mL were considered inactive; MIC values between 2000–5000 µg/mL were considered as low activity; 1000–2000 µg/mL were considered as moderate activity; 400–1000 µg/mL were considered as noteworthy activity; 100–400 µg/mL were considered as good activity; and <100 µg/mL were considered to be high activity.²²

Phytochemical Assays

Qualitative phytochemical assays for all extracts were conducted in order to detect the presence of phenolic compounds, cardiac glycosides, saponins, triterpenoids, phytosterols, alkaloids, flavonoids, tannins and anthraquinones, along with approximations of their relative abundances, using standard methods.²³ While these methods did not definitively identify the specific compounds present in the various extracts, they provided a general assessment

of the presence and qualitative abundances of the classes of phytochemicals present, usually via colorimetric assays. This allowed postulations to be made regarding the involvement of these classes of compounds in the antibacterial activities observed.

Toxicity Assays

Toxicity screening of the extracts was conducted using *Artemia franciscana* Kellogg nauplii lethality assays in order to provide insight on the therapeutic safety of the plant extracts.²⁴ LC50 values were calculated using probit analysis to show the extract concentration required to kill 50% of the *A. franciscana* nauplii.

Statistical Analysis

Samples were tested in triplicate for disc diffusion assays and statistical analysis was conducted with data expressed as the mean $\pm$ standard error of the mean (SEM) of three independent experiments. Differences between control and treatment groups were analysed using one-way analysis of variance (ANOVA), with p values analysed for statistical significance. All broth microdilution assays were repeated twice for each bacterial strain on separate days, with two technical replicates per assay (n=4), to ensure the reproducibility of the MIC determinations.

RESULTS

The final extract concentrations are shown in Table 1. The pre-extracted powder preparations (BU and PY) generated high yields when extracted with water (71-82%). The leaf (BTL) and bark (PBK) samples produced substantially lower yields in water. However, no consistent patterns were observed for the methanolic and ethyl acetate extracts, with yields varying considerably among the extracts for each plant type. Qualitative phytochemical analysis was conducted on all extract preparations (Table 1). The ethyl acetate extracts contained the lowest abundances of the tested phytochemicals. The BTL contained a minor level of water insoluble phenols, although all other phytochemicals were either absent or below the detection threshold. The ethyl acetate extracts prepared from BU powder, as well as both Pygeum preparations (PY and PBK), failed to produce responses in all phytochemical assays.

All aqueous and methanolic extracts tested contained higher relative abundances of most classes of phytochemicals. Moderate to high levels of phenolics were observed, with

only the aqueous BU extract lacking water-soluble phenolics. Saponins were detected in high abundance in the BU aqueous extracts, and in PY aqueous, PBK aqueous and PBK methanolic extracts. Triterpenoids were detected in only the aqueous and methanolic BTL, TP and TS plant extracts. Low responses to flavonoids and no response to tannins was observed for the BU aqueous and methanolic extracts. The BTL extracts showed moderate to strong responses to flavonoids and tannins. The corresponding PBK extracts were similar, although with weaker responses for flavonoids. Cardiac glycosides, phytosterols and alkaloids were not detected in any of the plant extracts.

TABLE 1: Concentrations of Plant Extracts (mg/mL) and their Qualitative Phytochemical Analysis.

Extract type	Extract concentration (mg/mL) [†]	Phytochemical class and abundances										
		Total Phenolics	Water Soluble phenols	Water Insoluble phenols	Cardiac Glycosides	Saponins	Triterpenoids	Phytosterols	Alkaloids	Flavonoids	Tannins	Anthraquinones
BU-Aq	81.7	+++	-	+++	-	+++	-	-	-	+	-	-
BU-MeOH	3.05	+	-	+	-	-	-	-	-	+	-	-
BU-EtOAc	6.7	-	-	-	-	-	-	-	-	-	-	-
BTL-Aq	20.55	+++	++	++	-	-	+	-	-	++	++	-
BTL-MeOH	17.3	+++	+++	+++	-	-	++	-	-	++	+++	-
BTL-EtOAc	13.25	+	-	+	-	-	-	-	-	+	-	-
PY-Aq	72.45	+++	++	++	-	+	-	-	-	-	++	-
PY-MeOH	21.9	++	-	+	-	-	-	-	-	-	-	-
PY-EtOAc	4.7	-	-	-	-	-	-	-	-	-	-	-
PBK-Aq	5.8	+++	++	++	-	+	-	-	-	+	++	+++
PBK-MeOH	4.3	+++	+++	+++	-	++	-	-	-	+	+++	+++
PBK-EtOAc	5.55	-	-	-	-	-	-	-	-	-	-	-

+++ indicates a large response in the phytochemical assay, ++ indicates a moderate response, + indicates a minor response, - indicates no response. [†]Value obtained following resuspension in 1% DMSO. BU = *A. betulina* powder, BTL = *A. betulina* tea leaf, PY = *P. africana* powder, PBK = *P. africana* raw bark, TP = *T. phanerophlebia* leaf, TS = *T. sambesiaca* leaf; Aq = water, MeOH = methanol and EtOAc = ethyl acetate.

TABLE 2: MIC Values of Plant Extracts and Reference Antibiotics Against the Bacterial Strains Tested in Liquid Dilution Experiments.

Plant extract or Antibiotic		MIC (µg/mL)					
		<i>S. aureus</i>	MRSA	<i>E. coli</i>	ESBL <i>E. coli</i>	<i>K. pneumoniae</i>	ESBL <i>K. pneumoniae</i>
BU	Aq [§]	>5000	>5000	>5000	>5000	>5000	>5000
	MeOH [§]	>2800	>2800	>2800	>2800	>2800	>2800
	EtOAc [§]	>1550	>1550	>1550	>1550	>1550	>1550
BTL	Aq [§]	>5000	>5000	>5000	>5000	>5000	>5000
	MeOH [§]	>4275	>4275	>5000	>5000	>5000	>5000
	EtOAc [§]	>5000	>5000	>5000	>5000	>5000	>5000
PY	Aq [§]	>5000	>5000	>5000	>5000	>5000	>5000
	MeOH [§]	>5000	>5000	>5000	>5000	>5000	>5000
	EtOAc [§]	>1225	>1225	>1225	>1225	>1225	>1225
PBK	Aq [§]	>3800	>3800	>3800	>3800	>3800	>3800
	MeOH [§]	>4400	>4400	>4400	>4400	>4400	>4400
	EtOAc [§]	>2175	>2175	>2175	>2175	>2175	>2175
AB	PEN	CND	>2.5	>2.5	>2.5	>2.5	>2.5
	ERY	0.625	>2.5	>2.5	>2.5	>2.5	>2.5
	TET	0.156	0.078	0.625	> 2.5	0.625	>2.5
	CHL	>2.5	>2.5	2.5	>2.5	2.5	>2.5
	CIP	0.625	0.625	CND	>2.5	CND	0.313

§Denotes extracts that were inactive at the highest concentrations tested. BU=*A. betulina* powder, BTL = *A. betulina* tea leaf, PY = *P. africana* powder, PBK = *P. africana* raw bark, Aq = aqueous, MeOH = methanol and EtOAc = ethyl acetate, AB = antibiotics, PEN = penicillin, ERY = erythromycin, TET = tetracycline, CHL = chloramphenicol and CIP = ciprofloxacin. CND = could not be determined as inhibition was observed at the lowest concentration tested. Values >2.5 for the antibiotics indicate a lack of growth inhibition at the highest concentration of antibiotic examined.

Extracts derived from the two Buchu (BU and BTL) as well as the two Pygeum (PY and PBK) materials were inactive in the disc diffusion assay against all the tested bacteria. In contrast, the reference antibiotics generally produced ZOI

values against the sensitive bacterial strains. Notably, these ZOIs were reduced in the corresponding resistant strains, confirming their resistant phenotypes (Figures 1-3). These findings confirm that extracts derived from all Buchu

and Pygeum materials were ineffective growth inhibitors of all the tested bacteria on agar. Notably, some extract concentrations were quite low, particularly for the ethyl acetate extracts, which may account for the apparent lack of activity.

Liquid dilution assays were used in order to obtain MIC values for each extract and reference antibiotic (Table 2). The highest concentrations at which some extracts (particularly the ethyl acetate extracts) could be tested were lower than 5000 µg/mL due to their low stock concentrations (see Table 1). Thus, MIC values shown with ">" indicate that activity was not observed at the highest concentration tested for that extract. For the antibiotics, the highest concentration used was 2.5 µg/mL and therefore a lack of activity at this concentration of antibiotic in the assays was indicated by a value of >2.5 µg/mL. Notably, inhibitory activity was

not detected for any extract, even at their highest possible concentration, which were concordant with the agar disc diffusion results (Figures 1-3). The antibiotic activities between the liquid dilution and agar diffusion assays were also concordant. All the conventional antibiotics were completely ineffective against ESBL *E. coli*, with only one antibiotic (ciprofloxacin) producing any activity towards the ESBL *K. pneumoniae*.

All extracts were tested in the *Artemia* nauplii bioassay. None of the brine shrimp were killed by the extracts, or by the negative seawater control, while all nauplii were killed by the potassium dichromate positive control solution. Thus, LC50 values (the concentration of extract causing 50% brine shrimp mortality) could not be determined for any of the extracts and all extracts were deemed non-toxic.

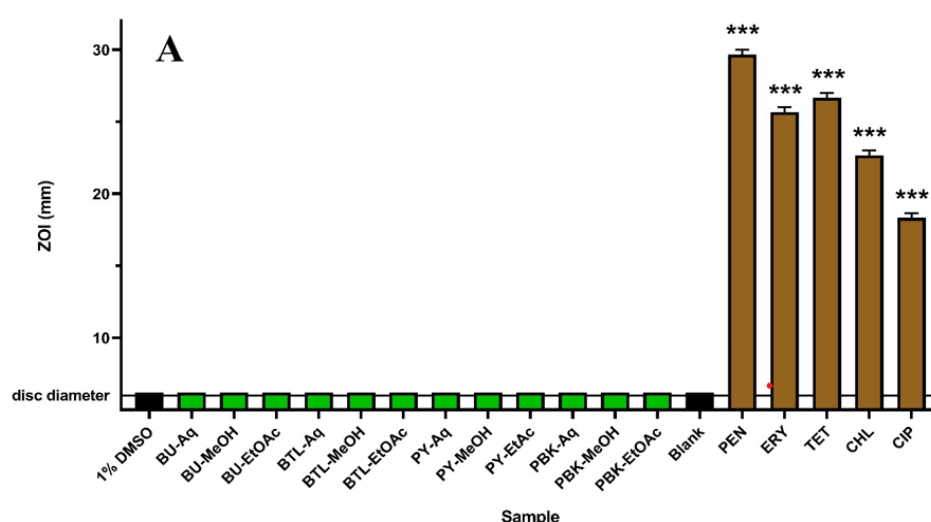


FIGURE 1. Agar Disc Diffusion Assays for Plant Extracts and Reference Antibiotics Against *S. aureus* (A).

The ZOI values were measured in millimetres (mm) and the disc sizes were 6mm, as indicated on the y-axis. Negative control discs contained 1% DMSO (for crude extracts) or blank discs (for antibiotics) and are shown by the black bars. Inactive extracts are in green, while antibiotics are shown as brown bars. Each value is expressed as the mean $\pm$ SEM of triplicate assays of each extract and antibiotic. Results are significantly different to the negative control (1% DMSO for extracts; blank for antibiotics) if $p < 0.05$ (*), < 0.01 (**) or < 0.001 (***). BU = *A. betulina* powder, BTL = *A. betulina* tea leaves, PY = *P. africana* powder, PBK = *P. africana* raw bark, Aq = aqueous, MeOH = methanol, EtOAc = ethyl acetate, PEN = penicillin, ERY = erythromycin, TET = tetracycline, CHL = chloramphenicol and CIP = ciprofloxacin.

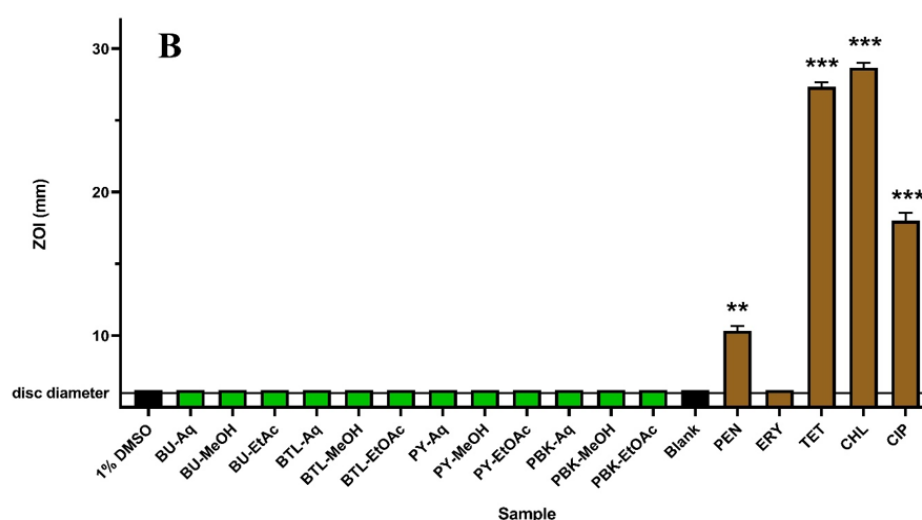


FIGURE 2. Agar Disc Diffusion Assays for Plant Extracts and Reference Antibiotics Against MRSA (B).

The ZOI values were measured in millimetres (mm) and the disc sizes were 6mm, as indicated on the y-axis. Negative control discs contained 1% DMSO (for crude extracts) or blank discs (for antibiotics) and are shown by the black bars. Inactive extracts are in green, while antibiotics are shown as brown bars. Each value is expressed as the mean $\pm$ SEM of triplicate assays of each extract and antibiotic. Results are significantly different to the negative control (1% DMSO for extracts; blank for antibiotics) if $p < 0.05$ (*), < 0.01 (**) or < 0.001 (***). BU = *A. betulina* powder, BTL = *A. betulina* tea leaves, PY = *P. africana* powder, PBK = *P. africana* raw bark, Aq = aqueous, MeOH = methanol, EtOAc = ethyl acetate, PEN = penicillin, ERY = erythromycin, TET = tetracycline, CHL = chloramphenicol and CIP = ciprofloxacin.

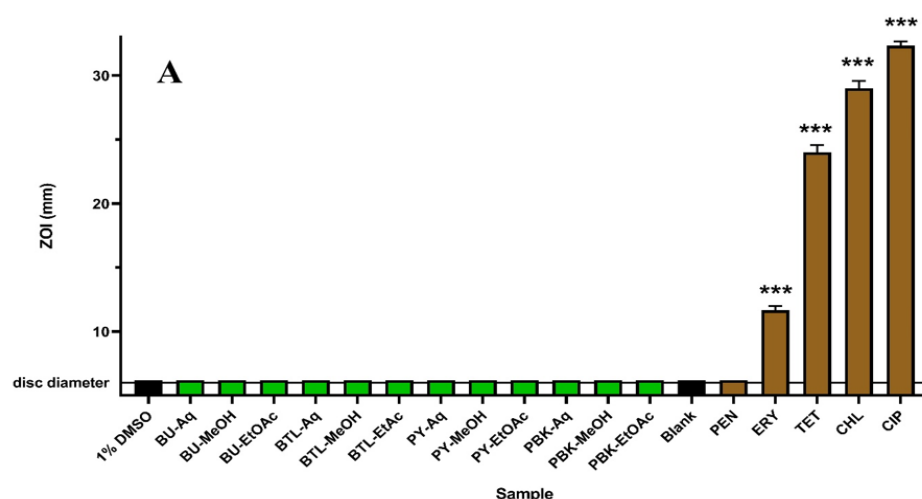


FIGURE 3. Agar Disc Diffusion Assays for Plant Extracts and Reference Antibiotics Against *E. coli* (A).

The ZOI values were measured in mm and the disc sizes were 6 mm, as indicated on the y-axis. Negative control discs contained 1% DMSO (for crude extracts) or blank discs (for antibiotics) shown by the black bars. Inactive extracts are in green, while antibiotics are shown as brown bars. Each value is expressed as the mean $\pm$ SEM of triplicate assays of each extract and antibiotic. Results are significantly different to the negative control (1% DMSO for extracts; blank for antibiotics) if $p < 0.05$ (*), < 0.01 (**) or < 0.001 (***). BU = *A. betulina* powder, BTL = *A. betulina* tea leaves, PY = *P. africana* powder, PBK = *P. africana* raw bark, Aq = aqueous, MeOH = methanol, EtOAc = ethyl acetate, PEN = penicillin, ERY = erythromycin, TET = tetracycline, CHL = chloramphenicol and CIP = ciprofloxacin.

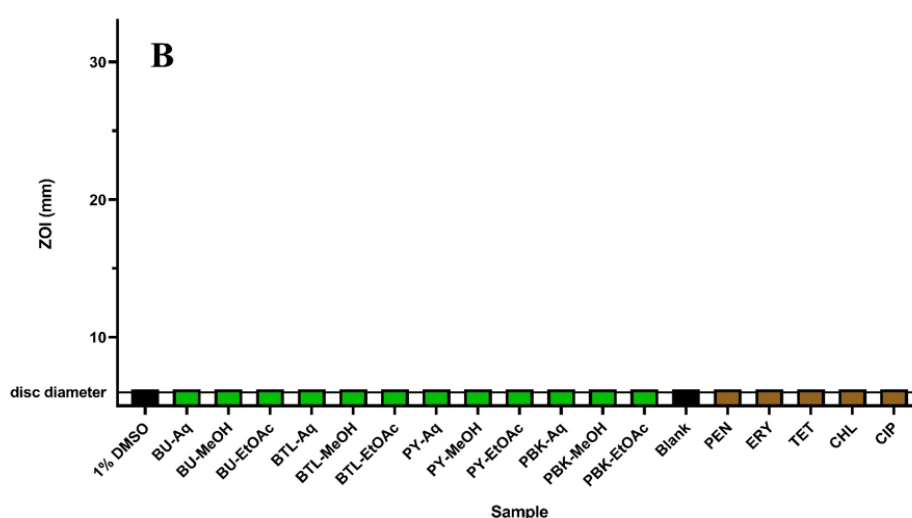


FIGURE 4. Agar Disc Diffusion Assays for Plant Extracts and Reference Antibiotics Against ESBL *E. coli* (B).

The ZOI values were measured in mm and the disc sizes were 6 mm, as indicated on the y-axis. Negative control discs contained 1% DMSO (for crude extracts) or blank discs (for antibiotics) shown by the black bars. Inactive extracts are in green, while antibiotics are shown as brown bars. Each value is expressed as the mean $\pm$ SEM of triplicate assays of each extract and antibiotic. Results are significantly different to the negative control (1% DMSO for extracts; blank for antibiotics) if $p < 0.05$ (*), <0.01 (**) or <0.001 (***). BU = *A. betulina* powder, BTL = *A. betulina* tea leaves, PY = *P. africana* powder, PBK = *P. africana* raw bark, Aq = aqueous, MeOH = methanol, EtOAc = ethyl acetate, PEN = penicillin, ERY = erythromycin, TET = tetracycline, CHL = chloramphenicol and CIP = ciprofloxacin.

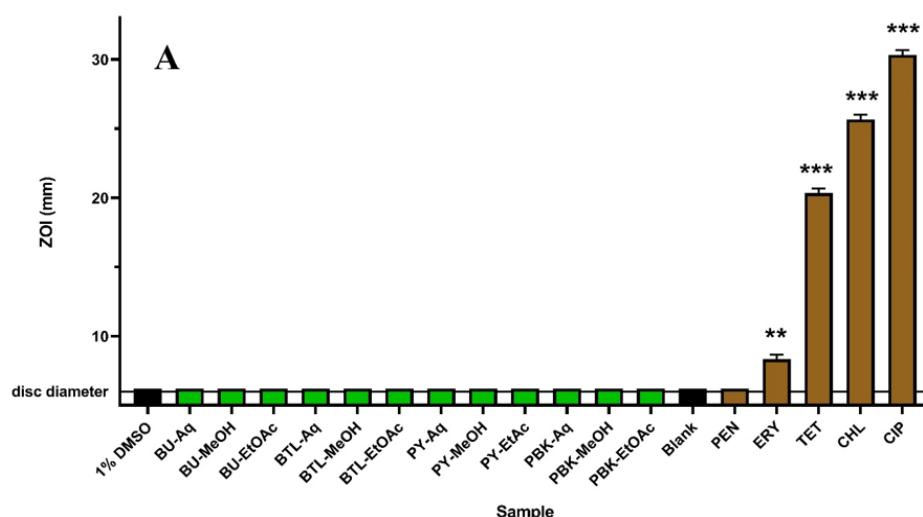


FIGURE 5. Agar Disc Diffusion Assays for Plant Extracts and Reference Antibiotics Against *K. pneumoniae* (A).

The ZOI values were measured in mm and the disc sizes were 6 mm, as indicated on the y-axis. Negative control discs contained 1% DMSO (for crude extracts) or blank discs (for antibiotics) and are shown by the black bars. Inactive extracts are in green, while antibiotics are shown as brown bars. Each value is expressed as the mean $\pm$ SEM of triplicate assays of each extract and antibiotic. Results are significantly different to the negative control (1% DMSO for extracts; blank for antibiotics) if $p < 0.05$ (*), <0.01 (**) or <0.001 (***). BU = *A. betulina* powder, BTL = *A. betulina* tea leaves, PY = *P. africana* powder, PBK = *P. africana* raw bark, Aq = aqueous, MeOH = methanol, EtOAc = ethyl acetate, PEN = penicillin, ERY = erythromycin, TET = tetracycline, CHL = chloramphenicol and CIP = ciprofloxacin.

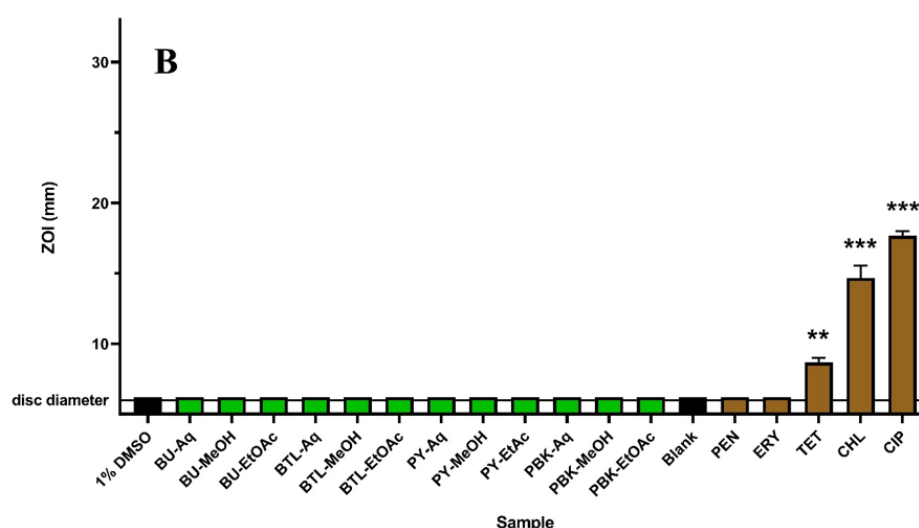


Figure 6. Agar Disc Diffusion Assays for Plant Extracts and Reference Antibiotics Against ESBL *K. pneumoniae* (B).

The ZOI values were measured in mm and the disc sizes were 6 mm, as indicated on the y-axis. Negative control discs contained 1% DMSO (for crude extracts) or blank discs (for antibiotics) and are shown by the black bars. Inactive extracts are in green, while antibiotics are shown as brown bars. Each value is expressed as the mean $\pm$ SEM of triplicate assays of each extract and antibiotic. Results are significantly different to the negative control (1% DMSO for extracts; blank for antibiotics) if $p < 0.05$ (*), <0.01 (**) or <0.001 (***). BU = *A. betulina* powder, BTL = *A. betulina* tea leaves, PY = *P. africana* powder, PBK = *P. africana* raw bark, Aq = aqueous, MeOH = methanol, EtOAc = ethyl acetate, PEN = penicillin, ERY = erythromycin, TET = tetracycline, CHL = chloramphenicol and CIP = ciprofloxacin.

DISCUSSION

Buchu and Pygeum materials harvested directly from their native habitat could not be obtained for this study. Instead, commercial sources of the two plants were purchased online in the form of either extract powders, or as leaves (for Buchu) and bark (for Pygeum). For these commercial products, preparation of the materials prior to packaging is classified as proprietary information that was not openly disclosed by the producer. Despite this, the purpose of the present study was to determine if extracts prepared from these samples possess inhibitory activity towards antibiotic-sensitive and antibiotic-resistant strains, including *S. aureus*, MRSA, *E. coli*, *K. pneumoniae*, ESBL *E. coli* and ESBL *K. pneumoniae*. The use of different solvents for extraction allowed for different abundances and complements of phytochemicals to be extracted.¹⁹ However, the resultant extracts (resuspended in aqueous solution) failed to inhibit the growth of any of the bacteria when tested, on both agar and liquid media.

Initially, these findings were unexpected as the qualitative phytochemical assays revealed varying abundances of tannins, phenolics, flavonoids, saponins, triterpenoids and anthraquinones, all of which inhibit the growth of antibiotic-

sensitive and antibiotic-resistant bacteria (including those tested in this study).^{25,26,27,29,30} A lack of specific phytochemicals (or combinations of different phytochemicals) within the extracts may be responsible for the lack of antibacterial activities reported. The *Artemia* lethality assays showed that all extracts were non-toxic. Therefore, this indicated that, despite their inability to inhibit bacterial growth, the extracts may still be safe to use therapeutically.

The MIC values for most of the extracts were >5000 $\mu\text{g/mL}$ and thus they were deemed inactive. However, the highest concentrations of some of the extracts were relatively low due to poor extraction yield. This was most notable for the ethyl acetate extracts, as this solvent extracted a smaller population of phytochemicals.¹⁹ It is possible that activity may have been detected if higher concentration extracts (>5000 $\mu\text{g/mL}$) were tested. However, the extraction procedure across all samples was standardised to allow direct comparisons between each extract.

There are limited studies focusing on the antibacterial properties of Buchu and Pygeum, particularly against the bacteria included in this experiment. In the case of Buchu, essential oils have been prepared that produce small (2 mm)

ZOIs on agar against *S. aureus* and *E. coli*.^{11,31} However, agar diffusion assays are not suitable to study the antibacterial activity of oils, which are relatively insoluble in agar. Some studies tested essential oils and methanol/dichloromethane extracts and reported very high MIC values (4000–8000 µg/mL) against *S. aureus* and *K. pneumoniae*.^{10,12} In other studies, acetone was used to either resuspend and/or dilute the extracts for testing, which is undesirable as acetone is toxic to bacteria.³² The only study that appeared to use an extraction methodology similar to this experiment used Buchu water and ethanol extractions that were ultimately resuspended in distilled water.³³ Notably, the extracts tested in that study were also unable to inhibit *E. coli* growth.

Reports on the antibacterial properties of Pygeum were conflicting and lacked details on extract preparation or used extracts that were resuspended in organic solvents for testing. Some studies failed to state which solvent extractants were used, or which solvent was used for resuspension of the crude extracts, making interpretation of their results very difficult and inaccurate.^{14,15} Most of the extracts tested in those studies were either inactive, or showed only low activity towards *S. aureus*, MRSA and *E. coli* in MIC assays. However, some studies reported moderate inhibition on agar, whilst not specifying the concentrations tested.¹⁴ Another study reported infusing large amounts (1000 µg) of extract into discs.¹⁵ Other studies reported that methanolic or hexane Pygeum extracts inhibited *E. coli* and *S. aureus* on agar, although those groups resuspended all extracts in 100% DMSO, rendering the results invalid since DMSO alone is a potent inhibitor of bacterial growth.^{34–37} However, a study reported that their Pygeum extracts (resuspended in an aqueous solution) did not inhibit *E. coli*.³³

CONCLUSION

Due to the lack of activity observed for these plants by several studies and the unreliable data acquired in cases where crude extracts were resuspended in organic solvents, the findings of this experiment indicate that these plants are ineffective inhibitors of *E. coli*, *S. aureus* and *K. pneumoniae*. The resistant counterparts of these bacteria were also unaffected in this experiment. However, to further evaluate their traditional uses, these studies should be repeated with freshly collected plant materials. Moreover, details on the collection and processing of the commercial powders or plant parts are unknown and these processes and storage times may affect

the extract activities. It is also possible that the extracts tested in this experiment possess activity against bacterial pathogens other than those included in this experiment. However, even if Buchu and Pygeum were ultimately determined to lack antibacterial potency, they may still be useful in the treatment of several other diseases such as benign prostatic hyperplasia.⁸ The *Artemia* lethality assay findings of this study suggest that the extracts are safe, although further research is required to characterise their toxicity profile in mammalian cell cultures.

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CORRESPONDENCE

Matthew J. Cheesman: m.cheesman@griffith.edu.au