



Five *Ochna* species have high antibacterial activity and more than ten antibacterial compounds

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New measures to control infections in humans and other animals are continuously being sought because of the increasing resistance of bacteria to antibiotics. In a wide tree screening survey of the antimicrobial activity of extracts of tree leaves (www.up.ac.za/phyto), *Ochna pulchra*, a small tree found widely in southern Africa, had good antibacterial activity. We therefore investigated the antibacterial activity of acetone leaf extracts of some other available *Ochna* spp. Antibacterial activity and the number of antibacterial compounds in acetone leaf extracts of *Ochna natalitia*, *Ochna pretoriensis*, *O. pulchra*, *Ochna gamostigmata* and *Ochna serullata* were determined with a tetrazolium violet serial microplate dilution assay and bioautography against *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis* and *Pseudomonas aeruginosa*, bacteria commonly associated with nosocomial infections. The percentage yields of the extracts varied from 2.5% to 8%. The minimum inhibitory concentrations of the five species ranged from 40 µg/mL to 1250 µg/mL. *E. coli* was sensitive to all the extracts. The *O. pretoriensis* extract was the most active with minimum inhibitory concentrations of 0.065 mg/mL and 0.039 mg/mL against *E. coli* and *E. faecalis*, respectively. The *O. pretoriensis* extract also had the highest total activities of 923 mL/g and 1538 mL/g, indicating that the acetone extract from 1 g of dried plant material could be diluted to 923 mL or 1538 mL and would still kill these bacteria. Based on the bioautography results, the two most active species, *O. pretoriensis* and *O. pulchra*, contained at least 10 antibacterial compounds with similar R_i values. Some of these antibacterial compounds were polar and others were non-polar. Variation in the chemical composition of the species may have some taxonomic value. The order of activity of the species to the bacteria were *O. pretoriensis* > *O. pulchra* > *O. gamostigmata* > *O. serullata* > *O. natalitia*. Even before toxicity and bioavailability issues are considered, some *Ochna* spp. leaf extracts have the potential to be used in treating skin infections.

Introduction

Medicinal plants which form the backbone of traditional medicines have in the last few decades been subjected to many pharmacological studies. The search for novel antibacterial compounds in plants is relevant because of the increasing resistance of bacteria to synthetic antibiotics and the occurrence of fatal opportunistic infections.^{1,2}

The genus *Ochna* comprises about 86 species of evergreen shrubs belonging to the family Ochnaceae and occurs widely in Africa, Asia and Madagascar. This family of plants has been used as food and for medicinal purposes for many centuries. Members of this family are characterised by the presence of flavonoids, biflavonoids and terpenoids as main secondary metabolites. Several members of this genus are also used for the extraction of edible oils.^{3,4}

Previous phytochemical studies on some *Ochna* species found monomeric and dimeric flavonoids in the leaves of *Ochna integerrima*.^{5,6} Sibanda et al.⁷ isolated one anthrone and two vismiones from the root bark of *Ochna pulchra*. An isobiflavonoid, glycosides and saponins were also isolated from the stem bark of *Ochna afzeli*.^{8,9}

In a study by Reutrakul et al.⁹, *Ochna integerrima* extracts had good antiviral and antiparasitic activities. The study involved the isolation of biflavonoids with antimalarial activity; a flavonoid glycoside with anti HIV-1 activity was also isolated from the same species.⁹ An infusion of the roots and leaves of *O. integerrima* is used locally as an antidysenteric and antipyretic preparation.¹⁰ There appears to be no previous report on the antibacterial activity of any of the 86 species of *Ochna* to date.

We have found that plants used traditionally to treat infections have very low antibacterial activity in *in vitro* assays. The low activity exhibited may be related to the extractant available to traditional healers because aqueous extracts of plants have very low activity.^{11,12} Consequently, we started investigating the antimicrobial activity of acetone leaf extracts of more than 600 South



African trees. We used only acetone for extraction because it is the best extractant for antimicrobial compounds from plants, based on parameters such as efficacy of extraction, diversity of compounds extracted, ease of removal of solvent after extraction, human safety and safety to microorganism in bioassays.¹¹ In these studies, *Ochna pretoriensis* had good antibacterial activity (Pauw and Eloff, unpublished results). The good activity of *O. pretoriensis* motivated the present investigation of the antibacterial activity and the number of antibacterial compounds present in other, easily available *Ochna* species: *Ochna natalitia*, *O. pretoriensis*, *O. pulchra*, *Ochna gamostigmata* and *Ochna serrulata*.

Materials and methods

Plant collection, drying and storage

Leaves of the five plant species investigated were collected in summer at the National Botanical Gardens in Pretoria, the Lowveld National Botanical Gardens in Nelspruit and the University of Pretoria Botanical Gardens. The origin of each tree is documented in the database of the botanical garden and voucher specimens were deposited in different herbaria (Table 1). Only clean dry leaves without any lesions or dirt were selected. Leaves were not washed with water to limit the possibility of extracting some water-soluble compounds and especially to limit the possibility of fungal growth on moist leaves. The leaves were dried in the dark at room temperature and pulverised into a fine powder (with particles less than 1 mm in diameter) and stored in closed glass bottles in the dark until use.

Extraction

The dry leaf powder (2.0 g) was extracted with 20 mL acetone (technical quality; Merck Pharmaceuticals, Pretoria, South Africa), which is very effective in extracting antimicrobial compounds from plants.¹¹ The resulting suspension was shaken vigorously in 50-mL polyester centrifuge tubes and centrifuged for 15 min at 4000 rpm (Hettich Centrifuge, Rotofix 32A, Labotec, Johannesburg, South Africa). The extracts were decanted into preweighed glass vials through Whatman No. 1 filter papers and concentrated to dryness under a stream of cold air. The dried extracts were made up to a concentration of 10 mg/mL (stock solution) in acetone to be used in subsequent assays and stored at 5 °C in tightly stoppered glass tubes.

Phytochemical analysis

Samples of 10 µL from the different stock solutions (10 mg/mL) were loaded onto thin layer chromatography (TLC) plates (Merck, Kieselgel 60 F254) and developed in three mobile systems used in the phytomedicine laboratory of the University of Pretoria. The mobile systems used to develop the plates were EMW (ethyl acetate:methanol:water) 40:5.4:4 (v/v/v), CEF (chloroform:ethyl acetate:formic acid) 5:4:1 (v/v/v) and BEA (benzene:ethanol:ammonia hydroxide) 90:10:1 (v/v/v).¹²

After development, the plates were visualised under UV light and sprayed with vanillin-sulphuric acid reagent spray (0.1 g vanillin in 28 mL methanol and 1 mL sulphuric acid) and heated at 110 °C until optimum colour development was achieved.

Test organisms

Microbial cultures used in the determination of minimum inhibitory concentrations (MIC) and bioautography were obtained from the Department of Medical Microbiology at the University of Pretoria. The bacterial cultures were maintained on Müller-Hinton agar at 4 °C and in Müller-Hinton broth at 37 °C prior to use for the determination of MICs and bioautography. The bacterial strains used in this study were: *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 27853) and *Pseudomonas aeruginosa* (ATCC 25922). These are the most important nosocomial pathogens in South Africa¹³ and the specific strains used are recommended for comparing antibiotics.¹⁴

Bioautographic assay

Chromatograms were prepared as described above. The plates were then sprayed with actively growing bacterial cultures until wet and incubated at 37 °C under 100% relative humidity.¹⁵ Following overnight incubation, the plates were sprayed with 2 mg/mL tetrazolium violet solution (Sigma, Pretoria, South Africa) and incubated for between 30 min and 60 min; the colour change was monitored to detect growth and inhibition of the test organisms after scanning in the bioautograms. Clear zones against a purple background on the plates indicate inhibition of bacterial growth by antibacterial compounds present in the extracts.¹⁶

TABLE 1: Common names (English and Afrikaans), collection locations and voucher specimen numbers of the five species of *Ochna* investigated.

Plant species	Common names	Location where collected	Herbarium and voucher number
<i>Ochna natalitia</i> (Meisn.) Walp	Showy Ochna Pronkrooihout	Lowveld National Botanical Gardens	Lowveld NBG Herbarium 30/1969
<i>Ochna pretoriensis</i> E.Phillips	Magalies Ochna Magalies rooihout	Pretoria National Botanical Gardens	HGWJ Schweickerdt Herbarium 114801
<i>Ochna pulchra</i> Hook.f	Peeling-bark Ochna Lekkerbreek	Pretoria National Botanical Gardens	HGWJ Schweickerdt Herbarium 148021
<i>Ochna serrulata</i> (Hochst) Walp.	Carnival Ochna Fynblaarrooihout	Pretoria National Botanical Gardens	HGWJ Schweickerdt Herbarium 114820
<i>Ochna gamostigmata</i> Du Toit	Small Forest Ochna Kleinbosrooihout	Kirstenbosch National Botanical Gardens	HGWJ Schweickerdt Herbarium 114796



Minimum inhibitory concentrations

To determine the MIC values of the different plant extracts on the test organisms, the microplate serial dilution assay developed by Eloff¹⁷ was used. This experiment was performed in triplicate and repeated twice for verification. The MIC was recorded as the lowest concentration of the plant extract at which the bacterial growth was inhibited. The MIC values were read after both a 12-h incubation and a 36-h incubation with the plant extracts to determine if the activity was bacteriostatic only or also bactericidal. Gentamycin was used as a positive control to confirm the sensitivity of the system. To compare the activity of different plants the total activity in mL/g was calculated by dividing the total mass in mg extracted from 1 g of plant material by the MIC value in mg/mL.¹⁸

Results and discussion

Quantity extracted

The percentage yields in acetone from the five species were: *O. gamostigmata* (8%) followed by *O. pulchra* (7.5%), *O. serullata* (7%), *O. pretoriensis* (6%) and lastly *O. natalitia* (2.5%). The yields were in the same order as acetone extracts from leaves of different members of the Combretaceae.¹

Phytochemical analysis

The intermediate polarity solvent system CEF separated the compounds in all these plant extracts more efficiently than did EMW and BEA (Figure 1). The efficient extraction and separation of phytochemicals is largely dependent on the type of solvent(s) used during extraction, particularly its polarity.¹⁵

There were similarities between the TLC chromatograms of *O. pretoriensis* and *O. pulchra* extracts; each had 10 similar bands, which is indicative of similar chemical compositions. The chromatograms may be useful in the taxonomy of the plants examined.¹⁹ Some of the spots were yellow when viewed in daylight and light blue when viewed under UV light (365 nm). This difference may be as a result of the presence of a strong chromophoric group which is often observed with plant phenolics (especially flavonoids and their glycosides).

Whilst there were some major differences in the TLC fingerprints of the five species, there were also identical bands in similar mobile phases with the same R_f values across a number of species, because of similar biogenetic pathways and metabolite pool sizes in the genus.

Bioautography

TLC-based bioautography is a useful technique that allows the localisation of antimicrobial compounds present in the crude plant extracts.¹⁵ The clear spots on the bioautograms indicate the location of the active compounds.¹⁶ Bioautography showed that *O. pretoriensis* and *O. pulchra* had four major active bands (Figure 1). Because EMW separated compounds

that could not be separated by the other solvent systems, there were at least 10 different antibacterial compounds present in the extracts of *O. pretoriensis* and *O. pulchra*. CEF was the best overall solvent for the separation of bioactive compounds in this study. There were five prominent active bands present in the *O. gamostigmata* extract. Three of the active bands had the same R_f value as that of bands characteristic of flavonoids – yellow under daylight and light blue under 365-nm wavelength UV light – which may mean that the same compounds occur in different species. There were also major differences between the chromatograms from different plant species sprayed with vanillin–sulphuric acid. These differences may have some taxonomic significance.¹⁹

Minimum inhibitory concentrations

The MIC values of the plant extracts ranged from 0.039 mg/mL to 1.25 mg/mL after a 12-h incubation and from 0.078 mg/mL to 1.25 mg/mL after the 36-h incubation (Table 2A). *O. pretoriensis* was the most active against *E. coli* and *E. faecalis* with MIC values of 0.065 mg/mL and 0.039 mg/mL, respectively. *O. pulchra* was the most active against *P. aeruginosa* and *S. aureus* with MIC values of 0.065 mg/mL and 0.078 mg/mL, respectively. *E. coli* was sensitive to all the extracts. The order of activity of the species to the tested organisms was *O. pretoriensis* > *O. pulchra* > *O. gamostigmata* > *O. serullata* > *O. natalitia*.

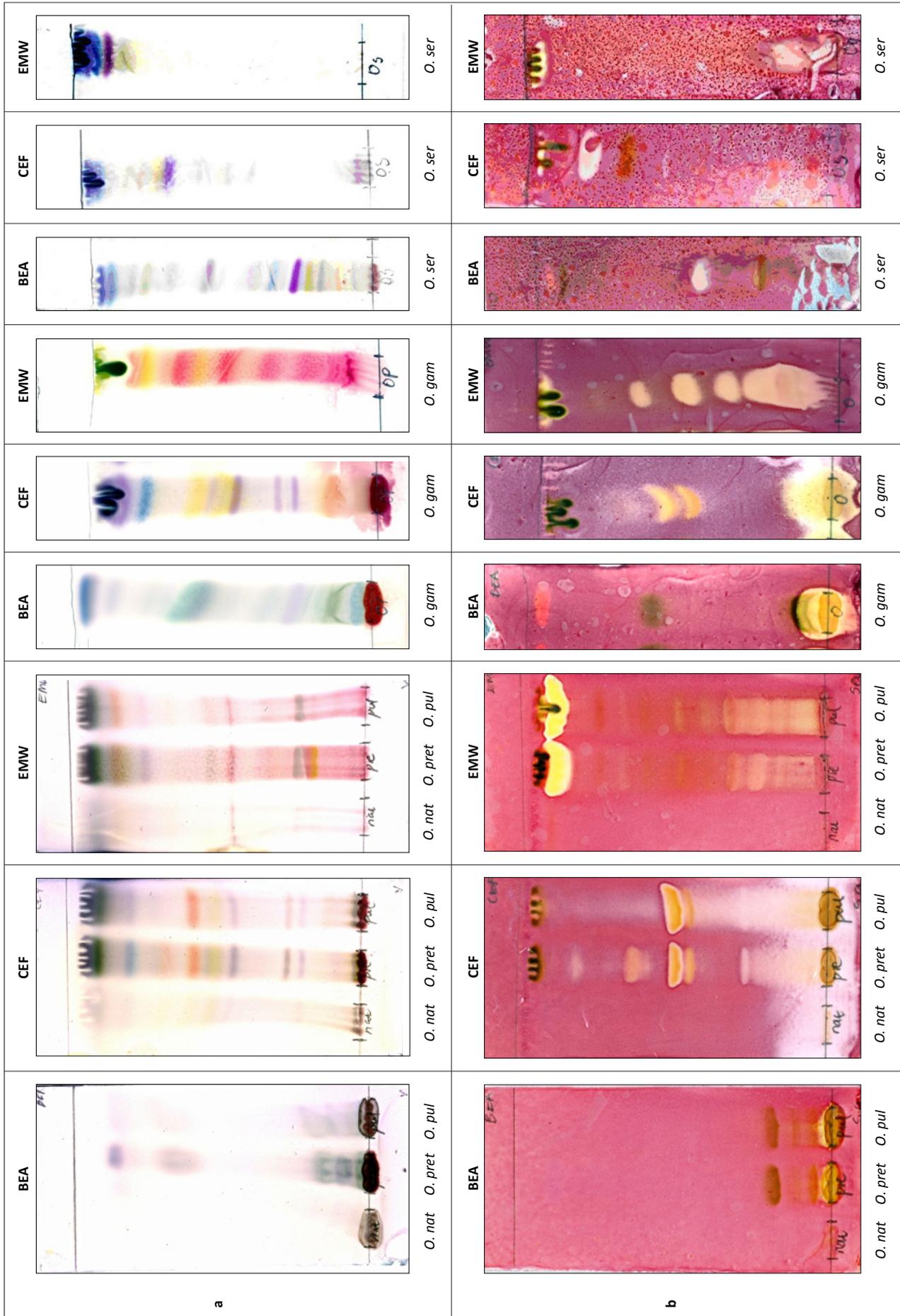
Different patterns of sensitivity of the test organisms to the plant extracts were noted after 12 h and 36 h (Table 2), which indicates that some of the bioactive compounds in the extracts are bacteriostatic and some are bactericidal. The MIC of *O. gamostigmata* was 0.31 mg/mL at 12 h against both *E. coli* and *E. faecalis*; the MIC remained the same after 36 h, indicating that the bioactive compounds had bactericidal activity. In contrast, the MIC of *O. pulchra* against *P. aeruginosa* was 0.063 mg/mL after 12 h, but rose to 0.1 mg/mL at 36 h, which indicates that the activity may be partly bacteriostatic.

Total activity and reciprocals of the MICs were calculated for all extracts (Table 2B and Figure 2). The total activity is important because it shows the quantity and quality of the active compounds present in the plant extracts.²⁰ Total activity is also dependent on the quantity of material extracted from the dried plant leaves; the higher the total activity, the greater is the potential for application of the specific plant extract.¹⁸

O. pretoriensis, *O. pulchra* and *O. gamostigmata* had high activity with the total activity of *O. pretoriensis* being 1538 mL/g (against *E. faecalis*), *O. pulchra* 1153 mL/g (against *P. aeruginosa*) and *O. gamostigmata* 769 mL/g (against *P. aeruginosa*).

Conclusions

Three of the five *Ochna* species investigated – *O. pretoriensis*, *O. gamostigmata* and *O. pulchra* – had significant antibacterial activity based on their MIC values and bioautography. The bioactive compounds in two of the most active species,



O. nat, *Ochna natalitia*; *O. pret*, *Ochna pretoriensis*; *O. pul*, *Ochna pulchra*; *O. gam*, *Ochna gamostigmata*; *O. ser*, *Ochna serullata*.

FIGURE 1: Chromatograms and bioautograms of the five *Ochna* species developed in BEA (benzene:ethanol:ammonia hydroxide), CEF (chloroform:ethyl acetate:formic acid) and EMW (ethyl acetate:methanol:water) solvent systems and (a) sprayed with vanillin–sulphuric acid reagent spray to show the separation of phytochemicals present in the extracts and (b) sprayed with actively growing *Staphylococcus aureus* to detect compounds with antibacterial activity (i.e. where the active bacteria did not change colour).

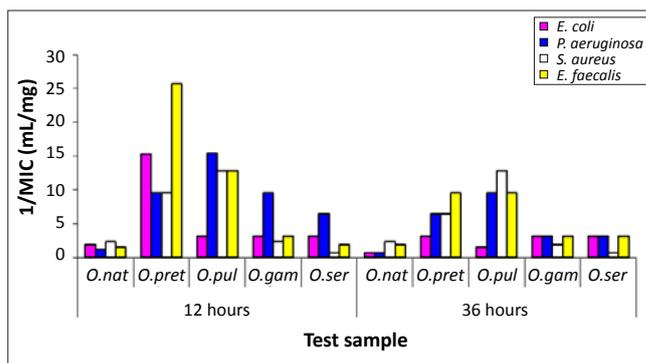
TABLE 2a: Minimum inhibitory concentrations (mg/mL) of the acetone extracts of five *Ochna* species and the positive control, gentamycin, against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus faecalis* after 12-h and 36-h incubations.

Bacteria	Minimum inhibitory concentration (mg/mL)											
	12 hours						36 hours					
	<i>O. natalitia</i>	<i>O. pretoriensis</i>	<i>O. pulchra</i>	<i>O. gamostigmata</i>	<i>O. serullata</i>	<i>O. natalitia</i>	<i>O. pretoriensis</i>	<i>O. pulchra</i>	<i>O. gamostigmata</i>	<i>O. serullata</i>	Gentamycin	
Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	
<i>E. coli</i>	0.520	0.18	0.065	0.02	0.312	0.00	0.312	0.00	0.312	0.00	0.312	0.00
<i>P. aeruginosa</i>	0.833	0.36	0.104	0.04	0.065	0.02	0.104	0.04	0.156	0.00	0.104	0.04
<i>S. aureus</i>	0.416	0.18	0.104	0.04	0.078	0.00	0.416	0.18	1.250	0.00	0.078	0.00
<i>E. faecalis</i>	0.625	0.00	0.039	0.00	0.078	0.02	0.312	0.00	0.520	0.18	0.312	0.00

s.d., standard deviation.

TABLE 2b: Total activity (mL/g) of the acetone extracts of five *Ochna* species against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus faecalis* after 12-h and 36-h incubations.

Bacteria	Total activity (mL/g)									
	12 hours					36 hours				
	<i>O. natalitia</i>	<i>O. pretoriensis</i>	<i>O. pulchra</i>	<i>O. gamostigmata</i>	<i>O. serullata</i>	<i>O. natalitia</i>	<i>O. pretoriensis</i>	<i>O. pulchra</i>	<i>O. gamostigmata</i>	<i>O. serullata</i>
<i>E. coli</i>	48	923	288	256	224	30	192	120	256	224
<i>P. aeruginosa</i>	30	576	1153	769	448	20	384	958	256	224
<i>S. aureus</i>	60	461	958	192	56	60	384	958	153	56
<i>E. faecalis</i>	213	1538	958	256	134	106	512	641	256	224



O. nat., *Ochna natalitia*; *O. pret.*, *Ochna pretoriensis*; *O. pul.*, *Ochna pulchra*; *O. gam.*, *Ochna gamostigmata*; *O. ser.*, *Ochna serullata*.

FIGURE 2: The activity volume or inverse minimum inhibitory concentration (MIC) of different extracts of *Ochna* species after 12-h and 36-h incubations, indicating the volume to which one milligram of the extract can be diluted and still inhibit growth of the different pathogens. Results for 36 h indicate bactericidal activity. Bars from left to right indicate activity of different plant extracts against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus faecalis*.

O. pretoriensis and *O. pulchra*, were similar. *O. pretoriensis* had the highest total activity of 1538 mL/g against *E. faecalis* and 923 mL/g against *E. coli*. Publications on other species in the *Ochna* genus have reported the isolation of flavonoids and biflavonoids with antimalarial and anti-HIV activities. We report for the first time, the antibacterial activities, the number of antibacterial compounds present and the R_f values of antibacterial compounds of some *Ochna* species. Because of their high total activity it appears that acetone leaf extracts of several *Ochna* species could be valuable in treating infections caused by these nosocomial pathogens. *O. pulchra* is notoriously difficult to cultivate and would not be a good candidate for the development of a herbal medicine. *O. pretoriensis* is easier to cultivate and therefore is a better candidate for an in-depth investigation. We also have isolated and characterised several of the compounds responsible for the antibacterial activity of *O. pretoriensis*; these results will be published elsewhere.

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Competing interests

We declare that we have no financial or personal relationships or competing interests which may have inappropriately influenced us in writing this article.

Authors' contributions

T.J.M. performed the research and wrote the first draft of the manuscript. J.N.E. identified the project, guided the research, and revised and submitted the manuscript.



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