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IN VITRO ANTIBACTERIAL SCREENING OF *PAPPEA CAPENSIS* EXTRACTS USING the *p*-IODONITROTETRAZOLIUM CHLORIDE (INT) ASSAY

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Abstract

The medicinal plants used by herbalists have received a large amount of attention recently from the scientific world due to dilemmas such as re-emergence of resistant organisms and devastating pandemics. In this study *Pappea capensis* used by traditional healers for the treatment of many diseases including cancer in Limpopo was screening for its antimicrobial activity using *p*-iodonitrotetrazolium chloride (int) assay. Three solvents (acetone, methanol and water) were used to elute the components possessed by the plant.

The antibacterial screening was performed on *Staphylococcus aureus* and *Klebsiella pneumoniae*. The acetonic and methanoic extracts were shown to have antibacterial effect against both Gram positive and Gram negative organisms used. Both extracts (acetonic and methanoic) indicated that potential antimicrobial compounds were in the high polarity fraction. Water was found to be the only solvent mostly used by traditional healers to make medicinal mixtures for their patients. Water was found to possess more antibacterial effect against gram negative organism than the gram positive organism. Even though the traditional healers use water only and claim that their patients are cured, however this study showed that acetonic and methanoic extracts have better antimicrobial activity than the water extract.

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Introduction

Plants have played a vital role in providing necessary compounds for the treatment of many diseases for many years. It is therefore important to assess if *Pappea capensis* possesses any of antimicrobial agents. The discovery of the antimicrobial agents (antibiotics) reduced the volume of widespread diseases globally[1,2]. However, due to non-adherence and failure to control the use of these antibiotics this has led to the development of drug resistant microorganisms [3]. The efficacy of many antimicrobial agents is being threatened by the emergence of the microbial resistant organisms to existing chemotherapeutic treatment [4]. Bacterial strains resistant to a lot of antimicrobial agents have made the search for the new and novel drugs to be the highest priority in the sciences fraternity [5, 6]. The emergence of methicillin-resistance *Staphylococcus aureus,* drug resistance tuberculosis, multi-drug resistance and extensively resistance tuberculosis to name a few [7] have necessitated the discovery of novel compounds to be developed as drugs Furthermore, it's been reported that other antimicrobial agents are associated with allergy, hypersensitivity, immune suppression, and side effects [8]. Most known novel drugs (Amphotericin B and azole) for the chronic treatment of chronic fungal infection also were clouded by the development of resistant Candida species [7]. Previous studies done on drug resistance proved the need for the development of new and novel antimicrobial agents that would alleviate the emergence and re-emergence of drug resistance organisms' globally[3, 5, 9]. Studies have found that plant-derived products represent an attractive source of antimicrobial agents due to their accessibility and affordability, especially for the Sub-Saharan countries[10]. The use of plants by traditional healers and most people globally necessitated the scientific screening for their toxicity and safety for human consumption[10, 11]. In this study *p*-iodonitrotetrazolium chloride (INT) assay was used to test and screen the inhibitory effects on the different microorganisms mostly isolated from cancer patients.

The following organisms *Staphylococcus aureus* (ATCC 25923) and *Klebsiella pneumoniae* (ATCC700603) representing Gram-positive and Gram-negative bacteria, respectively, were used for antimicrobial screening against three extracts namely: ethanoic, water and methanolic of *Pappea capensis*. It was also important to include a known antibacterial agent as a positive control for these assays in order to provide direct comparison for the different extracts and to ensure that the assay was performed successfully. Both the gentamycin and vancomycin at 2ug/ml where used for the Gram-negative and Gram-positive organisms.

Methods

Plant material

The plant material (*Pappea capensis* on figure.1) was authenticated by Dr Zietzmann Bloemfontein Museum and scientists at the National Botanical Gardens in Pretoria, South Africa (PC Zietsman & A Makhoahle 5448 the specimen is housed at the herbarium of the National Museum, Bloemfontein (NMB)). The collected bark and wood materials were dried at room temperature and pulverised by a mechanical mill and weighed. It was then stored at room temperature until analysis.

Three 360g wood samples were weighed out for extraction with 1080 ml acetone, water or methanol, respectively. Volumes were adapted according to the consistency of the sample. Then the rest of the solvent was added, and solutions allowed to seep out for 24 hours.

Filtering was performed after 24 hours, then the solids were removed from each solution by filtration with a Millipore funnel with medium filter paper (Bright sign nr102) connected to a Millipore vacuum pump. Where needed, samples were centrifuged in 50ml conical tubes.

Removing Solvents: Most extracts contained both aqueous and organic solvents and we employed both Freeze-drying steps with a Virtis Freeze drier to remove aqueous solvent as well as a Rotary evaporate (55°C) to remove organic solvents. After repeated steps of both freeze-drying and vacuum evaporation the samples were moved to pre-weighed containers and the yield determined.

Determination of antibacterial activity of extracts using the p-Iodonitrotetrazolium chloride (INT) assay:

The antibacterial activity of plant extracts was tested on *Staphylococcus aureus* and *Klebsiella pneumoniae*, representatives of Gram-positive and Gram-negative bacteria, respectively. Bacterial cultures were grown on Mueller-Hinton (MH) agar plates at 37˚C. An overnight streak plate was used to inoculate MH broth (Merck, USA) and allowed to grow for 16 hours (log phase) at 37˚C.

Gentamicin sulfate and vancomycin hydrochloride (Sigma, USA) were used as positive controls against *K. pneumoniae* and *S. aureus*, respectively. Antibiotics were dissolved in double distilled water at stock concentrations of 2 mg/mL and filter sterilized (0.2 µm filter). Working concentrations of the antibiotics were prepared in MH broth, depending on the minimum inhibitory concentration (MIC) value.

Fifty µL of MH broth was added to the wells of a sterile 96 well plate and 50 µL of test extracts were added to relevant wells. This was followed by a serial dilution of extracts to achieve the concentration range as indicated in Figures 1 and 2. The cultures were assessed and adjusted to a 0.5 McFarland standard [1.175 % $BaCl_2$ and 1 % H_2SO_4 with absorbance 600nm = 0.08-0.1 to achieve ±1.5 x 10^8 cells/mL]. Fifty µL aliquots of the relevant bacteria was added to each test well. Plates were sealed and incubated at 37˚C for 24 hours.

p-Iodonitrotetrazolium chloride (INT) was prepared at a working concentration of 0.2 mg/mL in ddH_2O and filter sterilized (0.2 µm filter). Fifty µL INT was added to each well and the plates were further incubated for 30 – 60 minutes at 37˚C until a colour change was observed (yellow to pink/purple to indicate the reduction of the dye by viable bacteria). No colour change indicated the inhibition of bacterial growth. Absorbance (abs) was measured at 600 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, USA).

Percentage inhibition was defined as: Percentage inhibition = 1 – (test well abs / mean abs triplicate bacteria only well) x 100

Results

Determination of antibacterial activity of extracts:

The antibacterial activity of plant extracts was performed in triplicate using the INT assay and a representative of a Gram-positive and Gram-negative bacteria. Figures 2 and 3 show the results of this assay, Gentamicin was used as a positive control for this study.

Discussion

Effective separation of the active compounds purely depends on the type of solvent used in the extraction process[4]. Screening of the extract 6 (ethanoic extract) of *Pappea capensis* extracts on *Staphylococcus aureus* (fig. 2) showed good antimicrobial activity with 90% bacterial death at 2mg/ml, followed by methanolic extract (no8) which showed 50% bacterial death at 2mg/ml. There was no antimicrobial activity at low concentrations of water extract except for 2mg/ml. The ethanoic extraction (no6) shows antimicrobial activity against *Klebsiella pneumoniae* with more than 50% bacterial death achieved at 2mg/ml. Similar antimicrobial activity was seen for water extract (no 7) and methanolic extract (8). Fifty percent killing of *K. pneumoniae* will only be achieved by water extract of >2mg/ml and methanolic exceeded the 50% bacterial killing with 2mg/ml. The antibacterial activity showed interesting results with water extract (no 7) indicating Gram specificity, only inhibiting growth of *K. pneumoniae* but not *S. aureus*. This correlates to the results of the study done on Jordanian plants, even though it was the different plants to *Pappea capensis*[2]. It's quite interesting because generally it is expected for the plants extract to be active against Gram positive bacteria as compared to the Gram negatives[12]. The ethanoic extract and methanolic extract results correlates with the research done on Yemen and Jordanian plants where they were found to be active

against both Gram positive and Gram negative organisms even though they are different[2,13]. Moreover, methanolic extract (no8) showed less % bacterial cell death against *S. aureus* than *K.* pneumoniae at the lower concentrations. The strength of all extracts was low compared to both controls with an MICs of 2ug/ml to obtain 50% bacterial cell death.

Conclusion

The antimicrobial screening showed that different extraction methods isolated active compound solvent types. Ethanoic and methanolic extracts showed to have active compounds against Gram positive and negative organisms. Both extracts (ethanoic and methanolic) indicated that potential antimicrobial compounds were in the high polarity fraction. Water is the most used solvent by traditional healers to make medicinal mixtures for their patients. However, in this study ethanoic and methanolic extracts produced more antimicrobial activity than the water extract, and this was also reported by other studies[13,14]. The active antimicrobial compounds found in this study need further investigation by purified extracts in order to identify the active compounds associated with antimicrobial activity observed on the three extracts.

Acknowledgments

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Figure 1. Pappea capensis tree

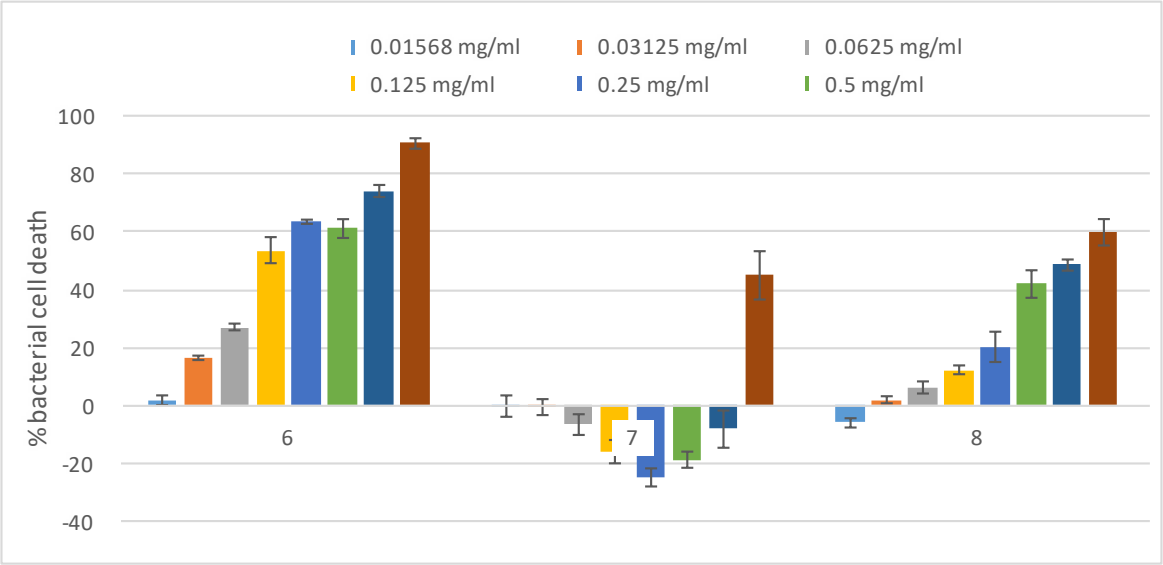

Figure 2. Screening for antibacterial activity of six extracts (as indicated) against *S. aureus* for a concentration range of 0.0156 mg/ml – 2 mg/ml. Vancomycin was used as a positive control at its MIC of 2 µg/ml. Error bars indicate standard deviation of quadruplicate values done as a single experiment.

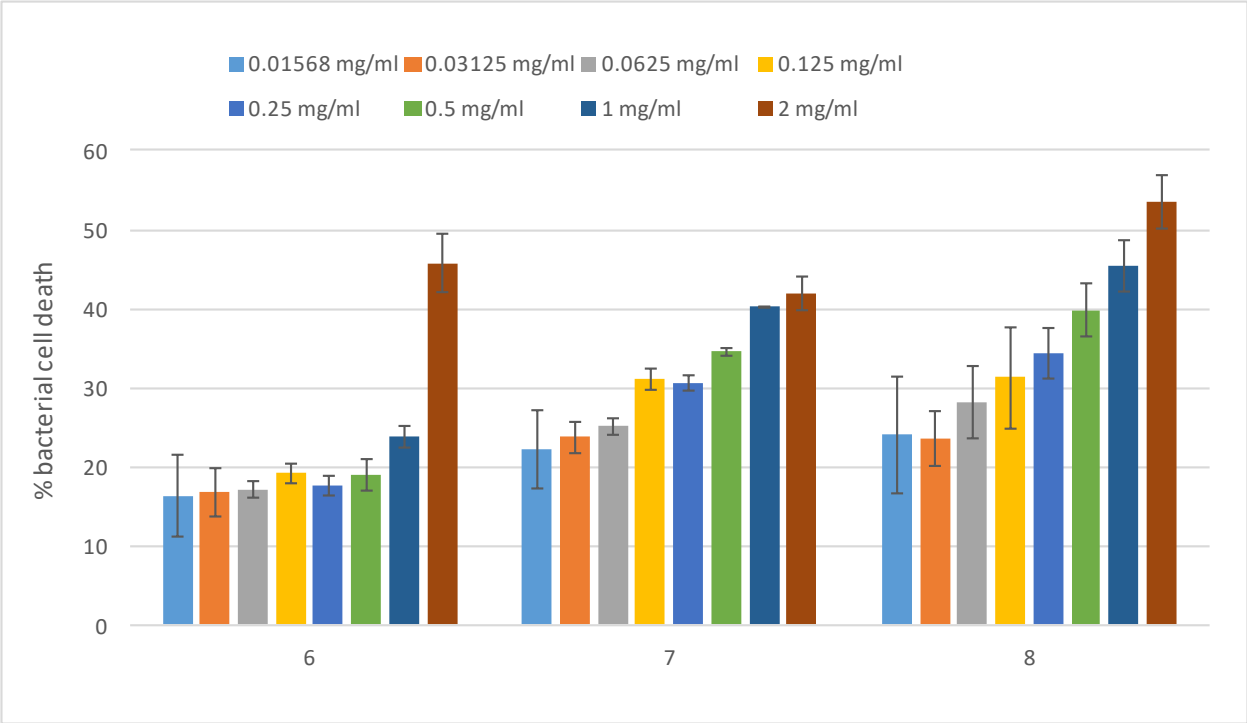

Figure 3: Screening for anti-microbial activity of nine extracts against *K. pneumoniae* for a concentration range of 0.0156 mg/ml – 2 mg/ml. Gentamicin was used as a positive control at its MIC of 2 µg/ml. Error bars indicate standard deviation of quadruplicate values done as a single experiment.