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THE ANTIBACTERIAL ACTIVITY OF C*LAUSENA ANISATA* HOOK, A SOUTH AFRICAN MEDICINAL PLANT

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Abstract

Background: *Clausena anisata* Hook also known as *Iperepesi* in Xhosa language is a medicinal plant used traditionally for the treatment of various ailments and some opportunistic infections associated with tuberculosis (TB). Patients in South Africa based on the phytotherapeutic information on this species in the Eastern Cape, use this medicinal plant. Hence, the antibacterial activity of various solvent extracts of the leaves and barks were respectively, evaluated using selected bacterial strains.
Method: The leaves and stem bark were tested against 10 selected strains of Gram - positive and Gram - negative bacteria through the agar dilution method. Acetone, dichloromethane and water extracts were used for the extraction. MIC was determined at different concentrations (0.1mg/ml, 0.5mg/ml, 1mg/ml and 5mg/ml) and the results obtained were compared to that of standard antibiotics.
Result: The acetone extract of the leaves were more active against both Gram-positive and Gram –negative bacteria with MIC ranging from 0.1 mg/ml - 0.5 mg / ml. The dichloromethane extract of the bark showed appreciable activities against *Staphylococcus aureus* (ATCC 6538) (MIC: 0.1mg /ml) *Escherichia coli* and, *Streptococcus pyogenes* with an MIC of 5mg/ml respectively. On the other hand, the aqueous extract of the leaves showed no activity against the tested organisms with the exception of the aqueous bark extract which inhibited *Staphylococcus aureus* (MIC: 0.5mg/ml) and *Pseudomonas aeruginosa* (MIC: 5mg/ml).
Conclusion: This study confirmed the antibacterial activities of acetone extract of the leaves of *Clausena anisata*. The capability of this extract to inhibit both Gram positive and negative bacteria is an indication that the extract is a potential broad spectrum antibacterial. The result of this study further justified its indigenous use for the treatment of bacteria commonly associated with TB especially among the people of Nkonkobe Municipality.

Key words: *Clausena anisata;* tuberculosis; antibacterial activity; herbal medicine

Abbreviations: DCM = dichlormethane**; ACT =**acetone**; MIC =**minimum inhibitory concentration; **na:** not investigated beyond 5mg/ml**; Cipro**: Ciprofloxacin; **Amox**: Amoxicillin

Introduction

Medicinal plants constitutes the richest bio-resource of drugs in traditional medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Ncube et al; 2008,). The incidence of microbial infections has increased dramatically in the past 20 years given the increase in the number of people whose immune systems are compromised by AIDS, aging, organ transplant and cancer therapy (Lawal et al; 2012). Accordingly, increases in morbidity and mortality rates resulting from microbial infections have been regarded as a major problem (Tatli and Akdemir, 2005).

Bacteria pathogens are associated with significant proportion of morbidity and mortality rates. For instance, Mycobacterium tuberculosis infects more than two billion people in the world, resulting in about 2–3 million deaths annually. Tuberculosis (TB) causes more than 25% of avoidable adult deaths in developing countries (Pio, 1998, Small and Fujiwara, 2000) Globally, many people use plant remedies in treating various respiratory illnesses including TB (Green et al; 2010, Mohammed et al; 2011). Although, epidemiology data emphasizes the burden of respiratory diseases, at both national and global levels (including TB), the frequent use of medicinal plants to treat tuberculosis and other associated infections requires scientific validation.

The indigenous people of South Africa, for centuries, have relied on herbal medicine for all aspects of their primary health care. It is estimated that about 12 - 15 million South Africans still relies heavily on traditional remedies from at least 700 indigenous plant species (Meyer and Afolayan, 1995, Grierson and Afolayan, 1999). While many rural communities now have access to clinics, yet, to a large extent, the rural dwellers still consult herbal practitioners because of their belief in the holistic nature of treatment plan and cost effectiveness of such treatment in relation to orthodox medicines (Lawal et al; 2014). Previous findings revealed that the choice of plant for bioactive studies relied on available ethno-medicinal information with a higher potential yield for antimicrobial and medicinal property other than random selection of plant (Grierson and Afolayan, 1999). Consequently, this study reports the antibacterial activities of Clausena anisata leaf and bark extracts on some bacteria strains as a way of validating its relevance in folkloric applications within Eastern Cape Province of South Africa (Lawal et al, 2014).

Methodology

Study area

The study area falls within Nkonkobe Municipality latitude 30^{o} 00 to 34^{o}15’S and longitudes 22^{o} 45’ to 30^{o} 15’E. It is bounded by the sea in the east and the drier Karoo (semi-desert vegetation) in the west. The elevation ranges from sea-level to approximately 2200m in the north and the vegetation is veld type, known as the Eastern Cape thorn veld (Masika and Afolayan, 2003). The study areas were: Hala, Upper-Ncera, Sheshegu and Gquamashe.

Plant collection

The barks and the leaves of *C. anisata* were collected in April 2013 at Hala and upper Ncera villages within Nkonkobe Municipality,

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Eastern Cape Province of South Africa. The plant was identified at the Griffen herbarium, Department of Botany, University of Fort Hare, and a voucher specimen (LAWMED 01) was deposited in the Griffen herbarium of the University.

Plant materials and preparation of extracts

C. anisata bark was washed and dried in an oven (Stainless steel PA Cuthbert and Co., Modderfontein) overnight at 30°C. The leaves were air - dried at room temperature; the dried samples were ground which was later used for preparing solvent extracts. 50g each of the bark and leaves were soaked in 500mL of distilled water and then shaken horizontally for 24hr using a linear shaker (Labotec Scientific Orbital Shaker, SA). The resulting mixture was filtered using Whatman qualitative filter paper (Sigma-Aldrich, Germany). The filtrate was then freeze dried. The organic solvent extraction of the plant was done with acetone and dichloromethane respectively. The respective solvents was shaken horizontally for 24 hr using a linear shaker (Labotec Scientific Orbital Shaker, SA), the extract were filtered using a Buchner funnel and Whatman qualitative filter paper (Sigma-Aldrich, Germany) and each filtrate was concentrated to dryness under reduced pressure at 40^{o}C using a rotary evaporator. A stock solution of 50 mg/ml of the extract was prepared, from the dry extracts, and stored in the fridge at about 4°C for future use. Each extract were re-dissolved in their respective solvent to the required concentration for the bioassay.

Bacterial and media

The bacteria used in this study were chosen based on the causative organisms of TB and its opportunistic infections mentioned in the previous study (Omoruyi et al., 2012, Lawal et al., 2014). The bacteria use were *Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 10702, *Enterococus faecalis* ATCC 29212, *Listeria monocytogenes*, *Salmonella typhimurium* ATCC 13311, *Serratia marcescens* ATCC 9986, *Streptococus pyogenes*, *Pseudomonas aeruginosa* ATCC 19582, *Escherichia coli* ATCC 19582 and *Shigella flexneri* (KZN). The test organisms were obtained from the Department of Biochemistry and Microbiology, University of Fort Hare. Nutrient agar was suspended in demineralised water, boiled while stirring until completely dissolved and was autoclaved at 121^{o}C for 15 minutes. The agar plates containing the extracts were prepared to give four different concentrations (0.1mg/ml, 0.5mg/ml, 1mg/ml and 5mg/ml).

Preparation of inocula

All test bacteria were maintained on nutrient agar and to prepare inoculum for the agar dilution procedure, the test micro-organisms were recovered in sterile nutrient broth and incubated overnight at 37 °C. Prior to streaking the inoculum onto the agar extract plates, 24 hours old culture was diluted 1:100 v/v in fresh sterile nutrient broth. The colony suspension method according to EUCAST (2003) was used for preparation of the inoculum. The test organism was cultured in nutrient agar overnight. Identical colonies from the culture were suspended in sterile saline. The suspension was adjusted with saline to give an optical density of 0.1 at 600nm. The adjusted inoculum was then diluted 1:100 in broth to give an approximate inoculum of 5×10^{5} cfu/ml as compared with Mcfarland standard (Cheesebrough, 2002, Olajuyigbe and Afolayan, 2012).

Bioassay

Each of the ten bacterial species (five Gram positive and five Gram negative) was maintained on nutrient agar plate (Oxoid) and was recovered for testing by growth in nutrient broth (Oxoid) for 24 hr. Before streaking, each culture was diluted 1:100 with fresh sterile nutrient broth

Antibacterial activity of the plant extracts and Minimum Inhibitory Concentration (MIC)

The antibacterial activity of the leaves and bark extracts was assaying using agar dilution method with a little modification following the procedures of Afolayan and Meyer (1997), Olajuyigbe and Afolayan, (2012). Agar was prepared according to the manufacturer's instructions and placed in a water bath at 50 °C. The extract stock solution (50mg/ml) was filtered through a 0.22 µm filter then incorporated in the molten agar at different volumes to obtain a range of concentrations between 0.1 and 5 mg/ml.

The agar-extract was poured into sterile Petri dishes and allowed to cool. The controls were: one containing only nutrient agar, another containing nutrient agar and 10 % each of solvent of extractions (negative controls) and a third set of plates containing 0.05mg/ml each of amoxicillin and ciprofloxacin which serves as positive controls. 100µl of the standardized bacterial cultures were radially streaked onto the solidified agar-extract plates. Plates were incubated aerobically at 37^{0}C for 24 hrs. Each test was done in triplicates. Wide-ranging inhibition of bacterial growth was expected for an extract to be confirmed active and the concentration at which there was no visible growth of the organism on the agar plates was considered the minimum inhibitory concentration (MIC) of the extract.

Results and discussion

The antibacterial effects of the acetone, water and dichloromethane extracts of the leaf and bark of *C. anisata* against the ten bacteria are presented in table 1. Among the six plant extracts tested against the bacteria, the acetone (act) leaf extract and the dichloromethane (dcm) bark extract were the most active with the MIC varying from 0.1 to 5mg/ml (Table 1). These extracts showed a wide spectrum of activity based on the numbers of bacteria inhibited. The acetone bark extract was active against five of the bacteria, while the dichloromethane extract of the leaf was active against two of the bacteria. None of the aqueous leaf extract was active against any of the bacteria, but the aqueous extract of the bark showed a little activity against *Staphylococcus aureus* (1mg/ml) and *Pseudomonas aeruginosa* (5mg/ml). The inhibitory activity of the active plant part and medium of extraction based on the overall MIC was in order: *C anisata* (act) leaf extract > dcm bark extract > act bark extract > dcm leaf extract > aqueous bark extract (Table 1). The highest activity against the tested bacteria was recorded with the act leaf extract with the MIC of 0.1mg/ml, 0.1mg/ml, and 0.5mg/ml against *Streptococus pyogenes*, *Staphylococcus aureus* and *Bacillus cereus* respectively (Table 1).

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Table 1: Antibacterial activities (MIC) of the leaves and bark extract of *C. anisata*

		Acetone		**DCM**		**Water**			
Bacteria species	Gram +/-	Leaf	Bark	Leaf	Bark	Leaf	Bark	Cipro[a]	Amox[b]
Streptococcus pyogenes	+	0.1	5	na	5	na	Na	<1	<1
Staphyllococcus aureus (ATCC 6538)	+	0.1	na	1	1	na	0.5	<1	<1
Bacillus cereus (ATCC 10702)	+	0.5	na	na	na	na	Na	<1	<1
Enterococcus faecalis (ATCC 29212)	+	1	na	na	na	na	Na	<1	<1
Listeria monocytogenes	+	1	5	na	5	na	Na	<1	<1
Salmonella typhimurium(ATCC 13311)	–	na	na	na	5	na	Na	<1	<1
Serratia marcescens (ATCC 9986)	–	na	na	na	5	na	Na	<1	<1
Pseudomonas aeruginosa (ATCC 19582)	–	1	1	5	1	na	5	<1	<1
Escherichia coli (ATCC 8739)	–	5	5	na	5	na	Na	<1	<1
Shighella flexnerii (KZN)	–	1	5	na	5	na	Na	<1	<1

Key: na: not investigated beyond 5mg/ml**; DCM:** dichloromethane, **Cipro**: Ciprofloxacin; **Amox**: Amoxicillin

The lowest activity was obtained with the DCM leaf extract and aqueous bark extract of *C. anisata* with the MIC of 5mg/ml each against *Pseudomonas aeruginosa.* The most susceptible bacteria based on the MIC of 0.1mg/ml and 0.5mg/ml were *Streptococus pyogenes, Staphylococcus aureus, Escherichia coli* and *Bacillus cereus* while *Salmonella typhimurium* and *Serratia marcescens* were the most tolerant bacteria to some of the extract except dcm bark extract that inhibited their growth at the highest concentration (5mg/ml) used (Table 1). This supports the resistance of the Gram-negative bacteria on various extract which has also been reported by some researchers (Afolayan et al, 2007). The varying concentrations between 5 and 0.1mg/ml of the plant extracts were examined in order to determine the MICs. The MIC of the plant extracts against 10 bacteria is presented in table 1. The lowest MIC was obtained with the acetone leaf extract (0.1mg/ml against *Streptococus pyogenes* and *staphylococcus aureus,* and also 0.5mg/ml against *Bacillus cereus* and *Escherichia coli*) and the dcm extract of the bark (0.5mg/ml against *Staphylococcus aureus*). The acetone extract of the leaf and bark of *C. anisata* was inactive against *Salmonella typhimurium* and *Serratia marcescens* at the concentrations tested. Therefore, acetone leaf extracts exhibited potent antibacterial activities with the broad spectrum antibacterial properties.

Herbalist uses a mixture of herbal remedies for the management or cure for several types of opportunistic bacterial infections. *C. anisata* a threatened South African shrub, is one of such plants used by the herbalist and traditional healers for the treatment/management of a wide variety of diseases such as; ulceration of lungs, excessive cough, tuberculosis skin infections, ulcers, sores and other bacterial infections (Hamza et al., 2006, Lawal et al., 2014).

The acetone extract of the leaf showed a potent antibacterial effect as demonstrated on both Gram- positive and Gram-negative bacteria with lowest concentration of the extract (0.1mg /ml). This could be attributed to the ability of the solvent to extract both polar and non-polar compounds seen as responsible for the growth of the bacteria pathogen, which is in support of the previous report by Masika and Afolayan (2002) and Lewu et al., (2006) on the choice of solvent for antibacterial screening. Similar antibacterial study have been reported in other countries for instance India and Ghana on *C. anisata* and also in Kwa-Zulu Natal Province of South Africa, for its antimicrobial potential (Parekh et al., 2005, York et al; 2012 Nicholas et al., 2014) this could justify its folkloric uses in Eastern Cape for respiratory infections including TB as previously reported (Lawal et al., 2014). However, the acetone bark extracts demonstrated inhibition on limited organisms with high MIC of 5mg/ml. The aqueous extract of the leaf showed no inhibition against all tested bacteria but its bark extracts showed inhibition against *Staphylococcus aureus* and *Pseudomonas aeruginosa* 0.5mg/ ml and 5mg/ ml respectively. The activities of water extract have been previously reported by (Afolayan et al, 2007, Lewu et al., 2005) which corroborate with this investigation using the same medium of extraction. Hence acetone could be a better solvent for bioactive constituents for the extraction of bioactive constituents from *C. anisata.*

Bacillus cereus, Streptococus pyogenes and *Pseudomonas aeruginosa* have been reported to be associated with respiratory infections and also the cause of chronic lung infections in cystic fibrosis infection, a leading cause of morbidity and mortality (Viljoen et al., 2004, Tamishi et al ; 2008). Overall antibacterial activities investigated with the extracts of the leaves and bark of *C. anisata*, the acetone leaves extracts showed the highest inhibitory properties as compared with the reference drugs (Amoxicillin and Ciprofloxacin). The inhibitory activities of *C. anisata* signify its broad spectrum antibacterial potency with a promising source of antibacterial drug development. In order to consider the crude extract as active, it should have an MIC of 0.1mg/ml or less (Aligiannis et al, 2001). In accordance with the above criterion acetone leaves extract of *C. anisata* can be considered noteworthy for further investigation on the isolation of the bioactive compound and its safety.

Generally, it was noted in this study that Gram-negative bacteria are more resistant to the plant extract than the Gram positive strains of the bacteria. This is a confirmation to the previous study on the reactions of bacteria to the crude extract (Afolayan et al, 2007).

Conclusion

The results of this study showed the activity of the acetone leaf extract of *Clausena anisata* against the tested bacteria with the least

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MIC. This signifies the potency of this South African medicinal shrub and could be a lead to a promising drug for opportunistic bacteria infection management. Hence, this study supports the folkloric use of *C. anisata* for the treatment of respiratory infections and other bacteria diseases. This study recommends the use of leaves in reference to the stem bark because it will reduce the pressure on the barks for herbal preparation which in-turn will protect the unsustainable harvest of the bark and also prevent the shrub from been endangered.

Competing interest: We declare no competing interest

Authors Contributions

I. O was responsible for the collection of plant materials within the study areas, carried out all the experiments, performed data analysis and drafted the manuscript. AJ designed the study, coordinated plant material storage, supervised the laboratory experiments and made substantial contribution to revise the manuscript critically. DS edited the manuscript. We all read and approved the final manuscript.

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