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Antimicrobial Activity Screening of *Philenoptera violacea (*Klotzsch*) schrire* and *Xanthocercis zambesiaca (Baker)* Dumaz-Le-Grand

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Abstract

Microorganism involvement in cancer has been identified for over a century, and different types of bacteria have been associated with carcinogenesis. *Philenoptera violacea (Klotzsch) Schrire* and *Xanthocercis zambesiaca (Baker) Dumaz-le-Grand* plant extracts are traditionally used by South African traditional healers for the treatment of inflammation related disorders; however, their efficacy has not been determined. The aim of this study was to investigate the antimicrobial activity of the mixture of leaves, flower & twig of each plant extract respectively. Antimicrobial activity was determined using *p*-Iodonitrotetrazolium chloride (INT) assay on gram-positive bacterium: *Staphylococcus aureus*, *S. epidermidis*, *S. saprohyticus*, *Bacillus subtilis,* and gram-negative bacterium: *Enterobacter cloacae*, *Escherichia coli, Pseudomonas aeruginosa*. Results of antimicrobial activity revealed that both plant extracts had no antimicrobial activity against selected micro-organisms. Thus, we couldn't support or confirm the antimicrobial activity potential of these plants as reported by traditional healers, however, factors that might have contributed to these results were not excluded.

Key words: *Philenoptera violacea*, *Xanthocercis zambesiaca*, Antimicrobial activity, *p*-Iodonitrotetrazolium chloride (INT) assay, cancer

INTRODUCTION

African populations are faced with a challenge of chronic diseases emergence whose treatment and follow-up creates a major economic problem for them[1]. Of all the alternative modalities, herbal medicine is probably the most popular and the most ubiquitous[2] as it is easily accessible and less expensive. The World Health Organization has described traditional herbal medicine as one of the surest means to achieve total health care coverage of the world's population[3]. In traditional herbal practice in Africa, indigenous medicinal plants have been employed in the treatment of several important infections[4]. Interest in medicinal plant research has escalated, with the aim of identifying alternative antimicrobial therapies to overcome resistance[5].

Traditional medicinal practices are used to treat a variety of diseases including skin disorders, tuberculosis, urinary tract infections and gastrointestinal disorders etc[6]. Microorganism involvement in cancer has been identified for over a century, and different types of bacteria have been associated with carcinogenesis. *Staphylococcus aureus* is considered as the first bacterium to be described as a cancer-producing agent, and some authors have attempted to associate it with breast cancer [7,8]. Gram-negative pathogens, such as *Escherichia coli* have been identified in the prostate, and their toxins are responsible for the inflammatory response. Inflammation should be considered a new domain in basic and clinical research in patients with prostate cancer and benign prostatic hyperplasia (BPH). Bacterial infections, urine reflux, dietary factors, hormones, and autoimmune response have been considered to cause inflammation in the prostate[9].

From a pathophysiologic point of view, tissue damage associated with inflammatory response and subsequent chronic tissue healing may result in the development of BPH nodules and proliferative inflammatory atrophy (PIA). BPH, benefit from antimicrobial and anti-inflammatory treatment. Moreover, the inflammatory microenvironment favours the survival and proliferation of neoplastic cells [10,11], thus indicating that the modulation of factors fuelling chronic inflammation may have anticancer effects. Therefore this study was performed with an aim of determining the antimicrobial activities of *Philenoptera violacea (Klotzsch) Schrire* and *Xanthocercis zambesiaca (Baker) Dumaz-le-Grand (*to be referred to as *P.violacea* and *X.zambesiaca* in this research report).

METHODOLOGY

Plant material

The plant materials, *P.violacea* and *X.zambesiaca* were authenticated, collected and extracted by scientists at the University of Free State in Bloemfontein, South Africa.

These two plants were randomly chosen from 60 plants belonging to the *Fabaceae* family which are used by South African traditional healers for the treatment of different health ailments. Twigs, leaves and flowers of *P.violacea* and *X.zambesiaca* were collected and mixed respectively.The collected materials were dried at room temperature and pulverized by mechanical mills and weighed. It was then stored in a cool place until analysis.

Sterilization

Petri dishes, beakers, McCartney bottles, pipette, test tubes, filter papers and other metal apparatus such as spatula and forceps were sterilized using hot air oven at a temperature of 160^0C for 1 hour. The wire loops were sterilized by heating on the blue flame of the Bunsen burner until red-hot and allowed to cool and 70% alcohol was used to swab/clean the work bench area to prevent contamination. The process was carried out aseptically.

Extraction method

Plant extracts were dissolved in DMSO at stock concentrations of 100 mg/mL. Working concentrations of 4 mg/mL were prepared in MH broth.

Microorganisms, growth conditions and media

Staphylococcus aureus, *S. epidermidis*, *S. saprohyticus*, *Bacillus subtilis*, *Enterobacter cloacae*, *Escherichia coli* and *Pseudomonas aeruginosa* (clinical strains) were grown in Mueller-Hinton (MH) broth (Merck). One microorganism colony was inoculated in the broth and allowed to grow for 16 h (log growth phase) at 37^0C in an orbital shaker (150 rpm).

Control drugs: Imipenem monohydrate (Sigma) and ampicillin sodium salt (Calbiochem)/doxycycline hyclate (Sigma) were used as positive controls against Gram-negative bacteria and Gram-positive bacteria, respectively. Antibiotics were dissolved in MH broth at stock concentrations of 2 mg/mL and filter sterilized (0.2 µM filter). Working concentrations of 64 µg/mL imipenem and 16 µg/mL ampicillin and doxycyline were prepared in MH broth.

Microbroth dilution method

MH broth (50 µL) was added to all test wells (i.e. plant extracts and antibiotics), accept to the highest plant extract and antibiotic concentration wells to which 100 µL of the working concentrations were added. Serial dilutions were prepared for the plant extracts (2 mg/mL to 125 µg/mL) and antibiotic (32-0.125 µg/mL and 8-0.016 µg/mL for imipenem and ampicillin/doxycycline, respectively). The cultures were assessed and adjusted to a 0.5 McFarland standard (absorbance at 600 nm = 0.08-0.1; equivalent to ~$1.5x10^8$ cells/mL) and 50 µL added to each test well. The following controls were prepared: (i) antibiotic/medium control (50 µL MH broth + 50 µL of highest antibiotic/fluconazole concentration); (ii) plant extract colour control (50 µL MH broth + 50 µL of highest plant extract concentration); (iii) 2% DMSO control (50 µL MH broth + 50 µL 4% DMSO); and (iv) microorganism control (50 µL MH broth + 50 µL microorganism). Plates were sealed with microplate sealing tape and incubated at 37^0C for 24 h.

p-Iodonitrotetrazolium chloride (INT) assay

After the incubation period, the absorbance of wells was read at 600 nm. 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT, Sigma) was prepared at a working concentration of 0.2 mg/mL in distilled water and filtered (0.2 µM filter). INT (20 µL) was added to each well and the plates were further incubated for 30-60 min at 37^0C until there was a colour change. The absorbance of wells was read at 600 nm. Viable cells reduce the yellow dye to a pink/purple colour, whereas no colour change indicated inhibition of bacterial growth.

Statistical analysis

Tests were carried out in triplicates. MIC of test samples was compared with that of positive and negative controls. The mean values were calculated from the triplicate values. Absorbance values were expressed as the Mean ± SD (n=3).

RESULTS

	1	2	3	4	5	6	7	8	9	10	11	12
A	control 1	control 3	B 2	B 1	B 0.5	B 0.25	B 0.125	C 2	C 1	C 0.5	C 0.25	C 0.125
B												
C												
D												
E	control 2	control 4	control 5	AB 32	AB 16	AB 8	AB 4	AB 2	AB 1	AB 0.5	AB 0.25	AB 0.125
F												
G												
H												

Table 1: Microplates template

control 1 = 50 uL medium and 50 uL 32 ug/mL antibiotic, control 2 = 50 uL medium and 50 uL 2 mg/mL extract B; no microorganism (colour control), control 3 = 50 uL medium and 50 uL 2mg/mL extract C; no microorganism (colour control), control 4 = 50 uL 2% DMSO and 50 uL microorganism, control 5 = 50 uL medium and 50 uL microorganism. AB = Positive control; B = *P. violacea* plant extracts*;* C= *X. zambesiaca* plant extracts

Concentration	B	C	AB
2 mg/ml	0.724 ±0.01	0.722 ±0.01	0.056 ±0.01
1 mg/ml	0.641 ±0.03	0.754 ±0.05	0.074 ±0.01
0.5 mg/ml	0.691 ±0.05	0.735 ±0.02	0.050 ±0.01
0.25 mg/ml	0.574 ±0.04	0.677 ±0.07	0.043 ±0.01
0.125 mg/ml	0.514 ±0.06	0.700 ±0.02	0.047 ±0.01

Table 2: OD600 nm readings after INT was added

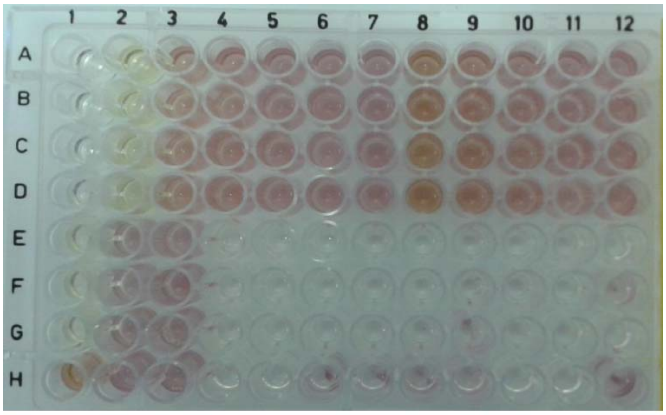

Figure 1: Photos of the INT plates

Microorganism	Positive control	MIC
Bacillus subtilis	Imipenem	4-8 µg/mL
Enterobacter cloacae	Imipenem	16 µg/mL
Escherichia coli	Imipenem	16-32 µg/mL
Pseudomonas aeruginosa	Imipenem	32 µg/mL
Staphylococcus aureus	Ampicillin/doxycycline	0.5-1 µg/mL
Staphylococcus epidermidis	Ampicillin/doxycycline	0.125-2 µg/mL
Staphylococcus saprophyticus	Ampicillin/doxycycline	1-2 µg/mL

Table 3. MIC values of antibiotics used

Table 1 illustrates the setup of the microplates used for this assay. Absorbance readings of 96-well plates were taken at a wavelength of 600 nm before and after INT addition. The absorbance after INT was added was greater (>1.00) for both plant extracts when compared to that of the positive control (table 2) as a result of microbial growth even at the highest concentration of 2 mg/ml. Therefore, MIC couldn't be determined and the percentage inhibition for the plant extracts was also not calculated as there was no observable inhibition of microbial growth (figure 1). For the Gram-positive bacteria (i.e. *S. aureus*, *S. epidermidis* and *S. saprophyticus*) imipenem was initially used as a positive control, but it caused complete inhibition even at the lowest concentration (0.125 µg/mL). Ampicillin and doxycyline were used to determine MIC values for the Gram-positive bacteria (Table 3).

DISCUSSION

Natural products have played a vital role in the discovery of antimicrobial drugs, with the drug either being completely derived from the natural product, or serving as a lead for novel drug discovery. Plants synthesise a diverse array of compounds (secondary metabolites) which play a key role in the natural defence mechanism. These aromatic compounds have been found to be useful antimicrobial phytochemicals and, as a result, these compounds are now divided into different chemical categories: phenolics, terpenoids and essential oils, alkaloids, lectins and polypeptides, as well as polyacetylenes[12]. Fluoroquinolones were presented as a totally synthetic, significant class of antibiotics in the 1990's [13]. Some examples of naturally occurring antimicrobials which are used currently include drug classes such as the penicillins and cephalosporins (β-lactam being the empirically active component). An increase in the isolation and identification of antimicrobial compounds may contribute greatly to the success in antibiotic discovery.

The efficacy of an antimicrobial agent can be estimated through the determination of the minimum inhibitory concentration (MIC), being the minimum concentration at which no microbial growth occurs after a specified exposure time to the antimicrobial agent[14]. There are numerous methods for the screening of biological extracts for potential antimicrobial activity. The microbroth dilution susceptibility method in 96 well microtiter plates has become the preferred method for drug susceptibility testing because it has small sample requirements, cost effective and a high-throughput rate[15]. The p-Iodonitrotetrazolium chloride (INT) assay is a microplate assay that determines the minimum inhibitory concentration (MIC) of biological extracts using INT dye. This dye acts as an electron acceptor and is reduced by viable bacteria to produce a coloured product. The yellow tetrazolium dye is reduced by viable microorganisms to a pink/purple colour. The MIC, for the INT assay, is defined as the lowest extract concentration that exhibits complete bacterial growth inhibition and prevents the dye from changing colour [16].

The ideal chemotherapeutic agent has a high therapeutic index with selective toxicity to cause damage to pathogens without causing similar harmful effects to its eukaryotic host[17]. This lethal damage to pathogens might be through the inhibition of cell wall synthesis, protein synthesis or nucleic acid synthesis, as well as through the disruption of the cell membrane and the inhibition of certain essential enzymes. This results in selective disruption of the specific structure and/or function essential to bacterial growth and survival[14]. However, there are factors that can influence efficacy of antimicrobial agent such as the ability of the drug to reach the target site of infection and the susceptibility of the pathogen to the particular chemotherapeutic agent. Nonpolar or lipophilic extracts are known to not easily diffuse into agar. The differences in solubility, volatility, and diffusion characteristics are among the factors that affect the antimicrobial potency of medicinal plant extracts[18].

X. zambesiaca is used by traditional healers in Limpopo (South Africa) for treatment of symptoms such as wounds, boils, purulent sores and diarrhea[19]. A study by Masoko (2013) reported good antimicrobial activity of leaves of *X. zambesiaca* with methanol, using a microplate

serial dilution technique with *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Enterococcus faecalis* as test organisms[19]. *P.violacea* is reported to be used in traditional remedies to treat gastro-intestinal problems, powdered root-bark for colds and snakebite treatment, root infusions as hookworm remedy and most part of the plant has been used to treat diarrhoea[20]. Antimicrobial activity of *P. violacea* and *X. zambesiaca* plant extracts was determined using INT assay against selected Gram positive and Gram negative bacterium that contributes to carcinogenesis. From our results, both plant extracts showed no antimicrobial activity against *S. epidermidis*, *S. saprohyticus*, *Bacillus subtilis*, *Enterobacter cloacae* and also against bacteria tested on by Masoko (2013) such as *Pseudomonas aeruginosa, Staphylococcus aureus*, *Escherichia coli*[19]. These results were not expected, as it has been reported previously that *X. zambesiaca leaves* had antimicrobial activity against Gram-negative and Gram-positive bacteria[19].

Weak microbial inhibitors are classified as those agents with MIC values greater than the ranges listed in Table 3. The sensitivity of *Staphylococcus aureus*, *S. epidermidis*, *S. saprohyticus*, *Bacillus subtilis*, *Enterobacter cloacae*, *Escherichia coli* and *Pseudomonas aeruginosa* with respect to *P. violacea* and *X. zambesiaca* plant extracts was similar, indicating poor antimicrobial activity. However, in our study, we mixed different part of the plant: leaves, twigs and flowers of the plants respectively. Thus there is a possibility of other compounds from twig and flowers suppressing the activity of active antimicrobial compounds from the leaves of our plant extracts instead of having a synergetic effect. There are also a number of factors which might contribute to these outcomes such as the area where the plants are collected and climate changes. The other factor might be the preparation of treatment by traditional healers as sometimes they prepare treatment using more than one plant and there is no systematic approach in doing that. The solvent used to extract also contributed to our results as traditional healers use other solvents such as water and different extraction method such as boiling.

CONCLUSION

To conclude, the results of this study showed that both *X. zambesiaca* and *P. violacea* plant extracts had no antimicrobial activity against *S. epidermidis, S. saprohyticus, Bacillus subtilis, E. cloacae, P. aeruginosa, S. aureus* and *E. coli*. We therefore couldn't support or confirm the antimicrobial activity potential of these plants as reported by traditional healers, however, factors that might have contributed to these results are not excluded.

CONFLICT OF INTEREST

Authors declare that there were no conflicts of interest to be disclosed.

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