Full Length Research Paper

In vitro antibacterial activities of the methanol extract of *Ziziphus mucronata* Willd. subsp. *mucronata* Willd.

O. O. Olajuyigbe and A. J. Afolayan*

Phytomedicine Research Centre, Department of Botany, University of Fort Hare, Alice, 5700, South Africa.

The antibacterial activity of crude methanolic extract of *Ziziphus mucronata* Willd. subsp. *mucronata* Willd. against both gram-positive and gram-negative bacteria were investigated using agar and macrobroth dilution methods. The extract had effective activities against both Gram-positive and Gram-negative bacteria. The agar dilution method indicated that the organisms were susceptible at concentrations ranging between 0.5 and 2.5 mg/ml. While the minimum inhibitory concentration (MIC) values ranged between 0.0391 and 0.625 mg/ml, the minimum bactericidal concentration (MBC) values range between 0.3125 and 1.25 mg/ml. Generally, the Gram-negative bacteria were more susceptible to the extract than the Gram-positive bacteria. The MIC$_{index}$ indicated that the extract was bactericidal at higher concentrations and bacteriostatic at lower concentrations. Our study revealed the broad-spectrum potential of the plant as well as established its ethnobotanical relevance in the traditional treatment of diarrhoeae and dysentery.

Key words: *Ziziphus mucronata*, methanol extract, antibacterial assay, macrobroth dilution.

INTRODUCTION

Herbal medicine, being used by all cultures throughout history, is the use of plants for their therapeutic value (Duke, 2002). According to Farnsworth (1984), a number of herbal plants and their compounds have been used, and have served as models for modern medicine. Apart from 30 to 40% of plants used in today's conventional drugs, other plants are used as herbal supplements, botanicals and teas (Kirby, 1996; Hostetmann and Marston, 2002). World Health Organization (WHO) recognized that 74% of the 119 plant-derived pharmaceutical medicines used in modern medicine correlated directly with their traditional uses as plant medicines by native cultures (Schulz et al., 2001). According to Lopez et al. (2001) and Karaman et al. (2001), these plants contain numerous biological active compounds. Many of these compounds have been shown to have antimicrobial activity acting individually or in combination on the human body to prevent health disorders (Palombo and Semple, 2001; van Wyk and Wink, 2004). Medicinal plants have been used for centuries as remedies for human diseases because

they contain components of therapeutic values (Nostro et al., 2000). They have been shown to have genuine utility and long been utilized as source of therapeutic agents worldwide. About 80% of the world population depend on it as sources of their primary health care (WHO, 1993) and have increasingly been used to treat many diseases (Basile et al., 1999). The scientific studies available on a good number of medicinal plants indicate that promising phytochemicals can be developed for many human health problems (Gupta, 1994; Dahiru et al., 2005). They are also a possible source for new potent antibiotics to which pathogenic strains are not resistant (Mohanasundari et al., 2007).

The genus *Ziziphus* found in desert areas (Jawanda and Bal, 1978) belongs to the Rhamnaceae family. The members of the taxon are known to be drought tolerant and very resistant to heat (Paroda and Mal, 1989). *Ziziphus* species are important versatile fruit trees in many arid countries and are planted as hedges to protect livestocks from predators (Cherry, 1985). *Ziziphus mucronata* Willd. subsp. *mucronata* Willd., also known as buffalo thorn, is a small to medium-sized tree, with a spreading canopy. It is distributed throughout the summer rainfall areas of sub-Saharan Africa, extending from South Africa northwards to Ethiopia. Its bark and roots are used medicinally for the treatment of various

---
*Corresponding author. E-mail: aafolayan@ufh.ac.za. Fax: +27866282295.

ailments, including rheumatism, gastrointestinal complaints, and snake bites (Tas et al., 1991). Warm bark infusions are used to relieve body pains, as expectorants for cough, against respiratory infections and for chest problems. The root infusions are used for treating gonorrhea, diarrhoea and dysentery. Decoctions of roots and leaves are applied externally to ooze boils, treat sores and glandular swellings (Venter and Venter, 2002; Amusan et al., 2005). While the fruits are sometimes sucked by small children, some rural dwellers believe that the tree serves as a protection against lightning and others cultivate this species to mark burial sites.

Although, the antimicrobial activity of some members of the *Ziziphus* genus has been reported in literatures (Sarfaraz et al., 2002; Adamu et al., 2006; Abalaka et al., 2010), there is a dearth of such information on *Z. mucronata* since the pharmacological importance of the plant has not been scientifically documented. For example, the root extract of *Z. mauritiana* was reported to be antimycobacterial (Taylor et al., 1995) while *Z. spina-christi* has been reported to have activity against bacteria and fungi (Shahat et al., 2001) including some other resistant pathogens (Nazif et al., 2002). Although, *Z. mucronata* is a South African plant used in treating infections, there is lack of scientific studies on the pharmacological importance of this plant especially antimicrobial study. The aim of this study was to investigate the antibacterial potential of the methanolic extract of the bark of *Z. mucronata* subsp. *mucronata* in order to validate its ethnomedical use in the treatment of infectious diarrhoea and dysentery.

MATERIALS AND METHODS

Plant collection and identification

The bark materials of *Z. mucronata subsp. mucronata* were collected from the plant growing within the University of Fort Hare campus in Alice, South Africa. The plant was authenticated in the Department of Botany and a voucher specimen was prepared and deposited in the Griffen Herbarium of the University. The bark samples were air-dried at room temperature and pulverized using a milling machine before extraction. Portions of about 100 g each of the pulverized samples were extracted with methanol for 48 h. The extraction was repeated for another two consecutive periods. The extracts were filtered with Whatman No. 1 filter paper and evaporated to dryness under reduced pressure at a maximum temperature of 40°C using a rotary evaporator. The extract was redissolved in methanol to the required concentrations for the bioassay analysis. The corresponding concentration was expressed in term of mg of extract per ml of solvent (mg/ml).

Test organisms

Ten bacteria used in this study included five Gram-positive (*Staphylococcus aureus* (ATCC 6538), *Streptococcus faecalis* (ATCC 29212), *Micrococcus luteus, Bacillus subtilits* KZN, *Staphylococcus aureus* (OK2a)) and five Gram-negative bacteria (*Escherichia coli* (ATCC 8739), *Klebsiella pneumonia* (ATCC

4352), *Proteus vulgaris* (CSIR 0030), *Shigella flexneri* KZN, *Shigella sonnei* (ATCC 29930). These organisms were obtained from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa. The organisms were maintained in nutrient broth and nutrient agar (Biolab) while Mueller Hinton II Agar (Biolab) was used for susceptibility, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assay.

Antibacterial assay

The antibacterial screening was essentially by the agar dilution method described by Afolayan and Meyer (1997). The dried plant extracts were dissolved in methanol to final concentration of 50 mg/ml and sterilized by filtration through 0.45 μm millipore filters (Schleicher and Schuell, Microscience, Dassel, Germany). This was used to prepare dilutions of the extract in molten Mueller Hinton agar maintained in a water bath at 50°C to concentrations ranging between 0.1 and 10 mg/ml. The inoculum of each test strain was standardized at 5×10^6 cfu/ml using McFarland Nephe-lometer standard (NCCLS, 1993). The organisms were streaked in radial patterns on the agar plates. Two nutrient agar plates containing 5% methanol representing the final methanol concentration in the test plates without the extract served as positive controls. Another two blank plates containing only nutrient agar served as negative controls (Afolayan and Meyer, 1997). Plates were incubated under aerobic conditions at 37°C for 24 h. Each test was done in triplicate and lack of visible growth on the test plates was used to indicate the inhibitory activity of the extracts.

Determination of minimum inhibitory concentration (MIC)

The *in vitro* antibacterial activity of the extract and minimum inhibitory concentration was determined by the macrobroth dilution methods (NCCLS, 1993). Nutrient broth medium was used to prepare different concentrations ranging from 0.0195 to 10 mg/ml by serial dilutions. Each prepared concentration in tubes was inoculated with 100 μl of each of the 10^6 cfu/ml bacterial strain. Blank nutrient broth was used as negative control. The tubes were incubated aerobically at 37°C for 18 to 24 h. The first tube in the series with no sign of visible growth was taken as the MIC.

Determination of minimum bactericidal concentration (MBC)

For the determination of the MBC, one standard loopful of culture was taken from each of the first three broth tubes that showed no growth in the MIC tubes and inoculated on fresh nutrient agar plates. After incubation for 24 h, the least concentration of the extracts that showed no colony formation on the agar was taken as the MBC. In order to elucidate whether the observed antibacterial effects were bactericidal or bacteriostatic, MIC$_{index}$ (MBC/MIC) ratios were calculated against each test strain. MBC/MIC ratios greater than 1 was considered bacteriostatic while other MBC/MIC ratios were considered bactericidal (Shanmughapriya et al., 2008).

RESULTS AND DISCUSSION

The result of the antibacterial assay indicated that methanol extract of *Z. mucronata* subsp. *mucronata* showed good antibacterial activity against both Gram-positive and Gram-negative bacteria by agar dilution method (Table 1). The result obtained from macrobroth

Table 1. Antibacterial activity of methanolic extract of the bark of *Ziziphus mucronata* subsp. *mucronata* by agar dilution method.

Organisms	Gram +/- ve	MIC (mg/ml)
Escherichia coli (ATTC 8739)	-	1
Proteus vulgaris (CSIR 0030)	-	1
Klebsiella pneumoniae (ATCC 4352)	-	0.5
Shigella flexneri (KZN)	-	2.5
Shigella sonnei (ATCC 29930)	-	1
Staphylococcus aureus (ATCC 6538)	+	2.5
Streptococcus faecalis (ATCC 29212)	+	0.5
Bacillus subtilits (KZN)	+	2.5
Micrococcus luteus	+	2.5
Staphylococcus aureus (OK2a)	+	2.5

Table 2. Antibacterial activity of methanolic extract of the bark of *Ziziphus mucronata* subsp. *mucronata* by macrobroth dilution method.

Organisms	MIC (mg/ml)	MBC (mg/ml)	MIC_{index}
Escherichia coli (ATTC 8739)	0.1562	0.6250	4
Proteus vulgaris (CSIR 0030)	0.0391	0.6250	16
Klebsiella pneumoniae (ATCC 4352)	0.3125	0.625	2
Shigella flexneri (KZN)	0.3125	0.3125	1
Shigella sonnei (ATCC 29930)	0.1562	0.6250	4
Staphylococcus aureus (ATCC 6538)	0.6250	1.2500	2
Streptococcus faecalis (ATCC 29212)	0.6250	1.2500	2
Bacillus subtilits (KZN)	0.6250	1.2500	2
Micrococcus luteus	0.1563	0.1563	1
Staphylococcus aureus (OK2a)	0.3125	0.3125	1

assay indicated that this extract exerted a high degree of inhibitory effect on these test organisms (Table 2).

However, the MIC and MBC values varied from one organism to the other. Generally, the MBC values were higher than the MIC values. The differences in the values of MICs and MBCs suggested a selective antibacterial activity of the extract. The susceptibility of all these bacteria to the methanol extract of *Z. mucronata* subsp. *mucronata* in both agar and macrobroth dilution methods were complementary. The extract exhibited the highest MIC against *Proteus vulgaris* and highest MBC on *Micrococcus luteus*. Comparatively, the Gram-negative bacteria were more susceptible to the extract than the Gram-positive strains. This is contrary to the general reports of many workers who have reported that plant extracts are more active against Gram-positive than Gram-negative bacteria (Jigna and Sumitra, 2006; Sofidiya et al., 2009; Afolayan and Ashafa, 2009). The difference in sensitivity between Gram-positive and Gram-negative bacteria to the plant extract could be ascribed to the anatomical or structural differences between these microorganisms. Gram-positive bacteria lack the normal outer membrane and the cell wall is usually much thicker than that of Gram-negative species (Archibald et al., 1993; Ghuysen and Hakenbeck, 1994).

The Gram-positive cell wall is made up of multiple layers of peptidoglycan, a polymer comprising repeating subunits of N-acetylglucosamine (NAG) and N-acetylmuranic acid (NAM) which are attached by a β(1-4) linkage, and a small string of four amino acids (L-alanine, D-glutamic acid, diaminopimelic or lysine, and D-alanine) that extend from NAM. The subunits are covalently attached by peptide glycine interbridges formed between the amino acid chains. Physiologically, the peptidoglycan in the cell wall gives shape, rigidity and protects the cell, but remains porous allowing certain biomolecules to translocate. The Gram-negative cell wall is structurally more complex than the Gram-positive cell wall. It comprises the peptidoglycan biopolymers which are only a few layers when compared to several hundreds present in Gram-positive bacteria. Gram-negative bacteria possess inner and outer cell membranes unlike the Gram-positive bacteria. The amino acid side chains of the peptidoglycan subunits are not connected by peptide interbridges but are directly covalently bonded to each other and are suspended in the periplasmic space

(Nelson et al., 2009). The cell wall in Gram-positive bacteria does not restrict the penetration of antimicrobials (Lambert, 2002). In Gram-negative bacteria, the outer membrane together with a set of multi-drug resistance pumps are effective barriers to antimicrobial compounds (Tegos et al., 2002). The extract of *Z. mucronata* could have promoted a local disturbance and the alteration of the physicochemical properties of the outer membrane, the membrane proteins and porin pathways to cause an increase in membrane permeability and the inflow of the extract (Chapple et al., 2004; Lohner and Blondelle, 2005). These events probably allowed sufficient amount of the extract to adsorb, diffuse, penetrate and interact with the target sites thereby preventing the active mechanism of resistance in the Gram-negative bacteria. This property of the extract is particularly noteworthy as it might be an indication of its broad spectrum antibacterial capability.

While Reuben et al. (2008) once stated that the MIC and MBC are often close or hooked values, El-Mahmood (2009), however, reported that the MBC values can either be the same or higher than the corresponding MIC values. Using turbidity as a measure of growth in this study, the MBC values appeared to be more reliable than the MIC values because it indicated that the extract is bactericidal. The MIC$_{index}$ however, and expectedly, suggested that the plant extract was bacteriostatic at lower concentration and bactericidal at higher concentration. This observation is of potential importance in the health care delivery system. It showed that the plant could be used as an alternative to orthodox antibiotics in the treatment of infectious diseases caused by these microbes.

CONCLUSION

In conclusion, the activity of the extract against both Gram-negative and Gram-positive bacteria has testified to its broad spectrum antimicrobial activity. The results of this study have further revealed the great therapeutic potentials of *Z. mucronata* subsp. *mucronata* and justified its ethnobotanical use in the treatment of diarrhoeae and dysentery in this part of the world.

ACKNOWLEDGEMENT

REFERENCES

Abalaka ME, Daniyan SY, Mann A (2010). Evaluation of the antimicrobial activities of two *Ziziphus* species (*Ziziphus mauritiana* L. and *Ziziphus spinachristi* L.) on some microbial pathogens. Afr. J. Pharm. Pharmacol., 4(4): 135-139.

Adamu HM, Abayeh OJ, Ibok NU, Kafu SE (2006). Antifungal activity of extracts of some Cassia, Detarium and Ziziphus species against dermatophytes. Nat. Prod. Rad., 5(5): 357-360.

Afolayan AJ, Ashafa AOT (2009). Chemical composition and antimicrobial activity of the essential oil from *Chrysocoma ciliate* L. leaves. J. Med. Plants Res., 3(5): 390-394.

Afolayan AJ, Meyer JJM (1997). The antimicrobial activity of 3, 5, 7-trihydroxyflavone isolated from the shoot of *Helichrysum aureonitens.* J. Ethnopharmacol., 57(3): 177-181.

Amusan OO, Sukati NA, Hlophe FG (2005). Herbal remedies from Shiselweni Region of Swaziland. Phytotherapeutics, Rec. Prog. Med. Plants, 10: 451-471.

Archibald AR, Hancock IC, Harwood CR (1993). In *Bacillus subtilis* and other Gram-positive Bacteria: Physiology and Molecular Genetics (ed. Sonenshein, A. L.), A.S.M., pp. 381-410

Basile A, Giordano S, Lopez-saez JA, Cobianchi RC (1999). Antibacterial activity of pure flavonoids isolated from mosses. Phytochem., 52: 1479-1482.

Chapple DS, Hussain R, Joannou CL, Hancock RE, Odell E, Evans RW, Siligardi G (2004). Structure and association of human lactoferrin peptides with *Escherichia coli* lipopolysaccharide. Antimicrob. Agents Chemother., 48: 2190-2198.

Cherry M (1985). The needs of the people. *In* Plants for Arid Lands. Eds. G.E. Wickens, J.R. Goodin, and D.V. Field. Unwin Hyman Ltd., London, pp. 1-8.

Dahiru D, William ET, Nadroi MS (2005). Protective effects of Ziziphus mauritiana leaf extract on carbon tetrachloride-induced liver injury. Afr. J. Biotech., 4(10): 1177-1179.

Duke JA (2002). Handbook of medicinal herbs. Maryland, USA.

El-Mahmood AM (2009). Antibacterial activity of crude extracts of *Euphorbia hirta* against some bacteria associated with enteric infections. J. Med. Plants Res., 3: 498-505.

Farnsworth NR (1984). Recovery and susceptibility testing of Mycobacterium tuberculosis from extra pulmonary specimens by the BACTEC radiometric method. J. Clin. Microbiol., 19(5): 720-721

Ghuysen JM, Hakenbeck R (1994). Bacterial Cell Wall 1–606 (Elsevier Science, Amsterdam (eds)).

Gupta SS (1994). Prospects and perspectives on natural plant products in medicine. Ind. J. Pharmacol., 26: 1-2.

Hostetmann K, Martson A (2002). Twenty years of research into medicinal plants: results and perspectives. Phytochem. Rev., 1: 275-285.

Jawanda JS, Bal JS (1978). The ber, highly paying and rich in food value. Indian Hortic, Oct–Dec, pp. 19-21.

Jigna P, Sumitra C (2006). *In vitro* antimicrobial activities of extracts of *Launaea procumbns* Roxb. (Labiateae), *Vitis vinifera* L. (Vitaceae) and *Cyperus rotundus* L. (Cyperaceae). Afr.J. Biomed. Res., 9(2): 89-93.

Karaman S, Digrak M, Ravid V, Iclim A (2001). Antibacterial and antifungal activity of the essential oils of Thymus revolutus Celak from Turkey. J. Ethnopharmacol., 76: 183-186.

Kirby GC (1996). Medicinal plants and the control of protozoal disease, with particular reference to malaria. Trans. R. Soc. Trop. Med. Hyg., 90(6): 605-609.

Lambert PA (2002). Cellular impermeability and uptake of biocides and antibiotics in Gram-positive bacteria and mycobacteria. J. Appl. Microbiol., 92 Suppl.: 46S-54S.

Lohner K, Blondelle SE (2005). Molecular mechanisms of membrane perturbation by antimicrobial peptides and the use of biophysical studies in the design of novel peptide antibiotics, Comb. Chem. High Through put. *S*creen, 8: 241-256.

Lopez A, Hudson JB, Towers GHN (2001). Antiviral and antimicrobial activities of Colombian medicinal plants. J. Ethnopharmacol., 77: 189-196.

Mohanasundari C, Natarajan D, Srinivasan K, Umamaheswari SA, Ramachandran A (2007). Antibacterial properties of *Passiflora foetida* L. – a common exotic medicinal plant. Afr. J. Biotech., 6(23): 2650-2653.

Nazif NM (2002). Pharmacognosy and Chemistry of Medicinal Plants, National Research Centre, Dokki, Cairo 12311, Egypt. Food Chem., 76(1): 77-81.

NCCLS (National Committee for Clinical Laboratory Standards) (1993).

Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically; Approved Standard, M7-A3. NCCLS, Villanova, PA.

Nelson ML, Grier MC, Barbaro SE, Ismail MY (2009). Polyfunctional antibiotics affecting bacterial membrane dynamics. Anti-Infective Agents Med. Chem., 8: 3-163.

Nostro A, Germanǒ MP, D'Angelo V, Marino A, Cannattelli MA (2000). Extraction methods and bioautography for evaluation of medicinal plants antimicrobial activity. Lett. Appl. Microbiol., 30: 379-384.

Palombo EA, Semple SJ (2001). Antibacterial activity of traditional Australian medicinal plants. J. Ethnopharmacol., 77: 151-157.

Paroda RS, Mal B (1989). New plant sources for food and industry in India. In: Wickens GE, Haq N, Day P (eds) New crops for food and industry. Chapman and Hall, London, pp. 135-149.

Reuben KD, Abdulrahman FI, Akan KC, Usman H, Sodipo OA, Egwu GO (2008). Phytochemical screening and *in vitro* antimicrobial investigation of the methanolic extract of *Croton Zambesicus* Muell ARG stem bark. Eur. J. Sci. Res., 23: 134-140.

Sarfaraz A, Ansari SH, Porchezhian E (2002). Antifungal activity of alcoholic extracts of *Ziziphus vulgaris and Acacia concinna. Hamdard Medicus.* Bait al-Hikmah, Karachi, Pakistan. 14/15: 42-45.

Schulz V, Hansel R, Tyler V (2001). Rational *Phytotherapy* 4th edition. Berlin: Springer Verlag.

Shahat AA, Pieters L, Apers S, Nazeif NM, Abdel-Azim NS, Berghe DV, Vlietinck AJ (2001). Chemical and biological investigations on *Zizyphus spina   christi*   L. Phytother. Res., 15(7): 593-597.

Shanmughapriya SA, Manilal A, Sujith S, Selvin J, Kiran GS, Natarajaseenivasan   K (2008). Antimicrobial activity of seaweeds extracts against multi-resistant pathogens. Ann. Microbiol., 58: 535-541.

Sofidiya MO, Odukoya OA, Afolayan AJ, Familoni OB (2009). Phenolic contents, antioxidant and antibacterial activities of *Hymenocardia acida*. Nat. Prod. Res., 23(2): 168-177.

Tas P, Stopper H, Koschel K, Schiffmann D (1991). Influence of carcinogenic oestrogen diethylstilboestrol on the intracellular calcium level in C6 rat glioma cells. Toxicol., *In vitro*, 5: 463-465.

Taylor RSL, Manandhar NP, Towers GHN (1995). Screening of selected medicinal plants of Nepal for antimicrobial activities. J. Ethnopharmacol., 46: 153-159.

Tegos G, Stermitz FR, Lomovskaya O, Lewis K (2002). Multidrug pump inhibitors uncover remarkable activity of plant antimicrobials. Antimicrob. Agents Chemother., 46: 3133-3141.

van Wyk BE, Wink M (2004). Medicinal Plants of the World, 1st edn. South Africa: Briza publications.

Venter F, Venter JA (2002). Making the Most of Indigenous Trees (rev. edition), Briza Publications, Pretoria, South Africa, p. 28.

WHO (1993). Regional Office for Western Pacific, Research Guidelines for Evaluating the Safety and Efficacy of Herbal Medicines. Manila, p. 2.