Journal of Applied Biological Sciences 6 (1): 37-40, 2012
ISSN: 1307-1130, E-ISSN: 2146-0108, www.nobel.gen.tr

The Hydro-alcoholic Extract of Leaves of *Eucalyptus camaldulensis* Dehnh. has Antibacterial Activity on Multi-drug Resistant Bacteria Isolates

Nima H. JAZANI[1] Peyman MIKAILI[2*] Jalal SHAYEGH[3]
Negar HAGHIGHI[4] Negar AGHAMOHAMMADI[4] Minoo ZARTOSHTI[5]

[1] Center for food sciences and nutrition, Urmia University of Medical Sciences, Urmia, IRAN

[2] Department of Pharmacology, Urmia University of Medical Sciences, Urmia, IRAN

[3] Department of Veterinary Medicine, Faculty of Agriculture and Veterinary Medicine, Shabestar branch, Islamic Azad University, Shabestar, IRAN

[4] Students Research Committee, Urmia University of Medical Sciences, Urmia, IRAN

[5] Department of Microbiology, Immunology and Genetics, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, IRAN

[*]Corresponding Author
e-mail: peyman_mikaili@yahoo.com

Received : September 24, 2011
Accepted : October 30, 2011

Abstract

Eucalyptus camaldulensis Dehnh. is regarded to possess various medicinal properties such as anesthetic, antiseptic, astringent and anti diabetic, but there are few studies on its antibacterial effects. In the present study antibacterial activity of *E. camaldulensis* on 100 Multi Drug Resistant isolates of *S. aureus*, *A. baumannii*, *P. aeruginosa*, *K. pneumoniae* and *E. coli* were evaluated. *E. camaldulensis* samples were collected from the fields of Urmia in Northwestern Iran. Plants were cut, and powder was prepared. Powdered plants were extracted by maceration at room temperature for 72 hours. Bacterial isolates were collected from clinical specimens of different wards of educational hospitals in Urmia, Iran during a 12-month period. The susceptibility of isolates to *E. camaldulensis* extracts was determined using a broth microdilution method. Considering to the wide application of ciprofloxacin in treatment of bacterial nosocomial infections, the antibacterial effects of ciprofloxacin on isolates was also determined. All the multi-drug resistant bacterial isolates were sensitive to different concentrations of *E. camaldulensis* hydro-alcoholic extract. The most sensitive bacterial isolates to *E. camaldulensis* extracts were *P. aeruginosa* isolates, although 69% of isolates were resistant to ciprofloxacin. Results demonstrated that this herbal drug may represent a new source of antimicrobial agent for controlling the hospital acquired infections. However, studies that are more adequate must be carried out to verify the possibility of using it for fighting these bacteria in human infections.

Keywords: herbal medicine, ciprofloxacin, resistant bacteria, antimicrobials, hospital-acquired infections

INTRODUCTION

Eucalyptus camaldulensis is a large-scale evergreen tree 24-50 meters high, with a stout, short and crooked trunk. It is almost 2 meters in diameter. It has various types of leaves; some are drooping and narrowly lanceolate. The leaf sizes are 8–22 cm in length and 1–2 cm in width. They often are curved or sickle-shaped, tapering to long point, short-pointed at base, entire glabrous. The color of leaves also is different. They differ from dull pale green on both surfaces to grayish in some trees (Little, 1983).

Watt and Breyer's chemical experiments demonstrated *E. camaldulensis* leaves contain 5–11% tannin. The fruits and also the leaves of the plant contain flavonoids and sterols. The bark contains 2.5–16% tannin, the wood 2–14% (Watt and Breyer-Brandwijk, 1962).

E. camaldulensis (known as red gum) has been used in the folk medicine worldwide.

It has been regarded to possess various medicinal properties such as: anesthetic, antiseptic, astringent, and it has been used to treat colds, colic, coughs, diarrhea, dysentery, hemorrhage, laryngalgia, laryngitis, pharyngitis, sore throat, spasm, trachealgia, and wounds among people for many years (Duke and Wain, 1981).

Despite its wide spectrum usage in medicine, there are a few studies on its antibacterial effects. Resistant Gram-positive pathogens, such as *Staphylococcus aureus*, have become a serious problem in the medical community. *S. aureus* is an organism with several virulent factors and resistance mechanisms. It is also a significant cause of a wide range of infectious diseases in human. *S. aureus* often causes life-threatening deep seated infections like bacteremia, endocarditis and pneumonia (Kanafani and Fowler 2006).

Acinetobacter baumannii is a gram-negative opportunistic *bacillus*. It is found in many hospital environments and can be colonized in human body in the hospital environments. The combination of its environmental colonization and its very high resistance to antimicrobials renders it as a successful nosocomial pathogen. The multiple-drug resistant (MDR) strains of *A. baumannii* often spread to cause outbreaks throughout hospital wards. *A. baumannii* causes a wide range of clinical complications, such as pneumonia, septicemia, urinary tract infection, wound infection, and meningitis, especially in immunocompromised patients (Nordmann, 2004).

Pseudomonas aeruginosa is an opportunistic pathogen found as a part of the normal flora of the human skin (Larson and Ramphal, 2002). In immunocompromised hosts, *P. aeruginosa* can colonize and infect the burn and wound sites. It can be rapidly disseminated from the wounds into other organs via the bloodstream and can produce severe infections such as endotoxic shock (Dale et al., 2004). Antibiotics are generally ineffective against most serious infections especially burn wound infections due to *P. aeruginosa*. Additionally, the treatment of these infections is frequently complicated by antibiotic resistance, a problem that is increasing in the recent years.

K. pneumoniae are a group of gram negative rods and they can cause different kinds of infections especially in a hospital setting. They are resistant to numerous antibiotics. Their resistance to antibiotics restricts the choice of antibiotics for therapy (Keynan and Rubinstein, 2007).

Hospital acquired urinary tract infections account for 35-45% of the nosocomial infections (Kamat et al, 2009). *Escherichia coli* is the main agent of this type of diseases. Antibiotic therapy is the gold standard for treatment of such infections; however, long-term therapy may result in many side effects and cause selection of resistant bacteria. Therefore, we need new treatments that could replace antibiotic therapy (Jazani et al 2007).

In respect of high resistance of nosocomial isolates of mentioned bacteria to antimicrobials, introducing the new antimicrobial agents against these kind of microorganisms is one of the most important goals in treatment of such infections (Perez et al., 2007).

In this study we evaluated the antibacterial activity of the hydro alcoholic extract of *Eucalyptus camaldulensis* Dehnh. on 100 multi-drug resistant isolates of *S. aureus*, *A. baumannii*, *P. aeruginosa, K. pneumoniae* and *E. coli*.

MATERIALS AND METHODS

Extract preparation: Eucalyptus samples were collected from the fields in the vicinity of Salmas, a city in northwestern Iran, and the identities were confirmed by a botanist. Plants were cut, chopped and dried and powder was prepared. Powdered plants were extracted by maceration at room temperature for 72 hours. The hydroalcoholic extracts were combined and concentrated to yield a dried powder. This hydroalcoholic extract was kept in refrigerator for all experiments (Garjani et al, 2009).

Bacterial strains and culture media: A total of 100 isolates of *S. aureus*, *A. baumannii*, *P. aeruginosa, K. pneumoniae* and *E. coli* (20 isolates from each species) were collected from clinical specimens of different wards of educational hospitals in Urmia, Iran, during a 12 months period between April 2006-2007. The isolates were further processed by the standard methods to identify as the *S. aureus*, *A. baumannii*, *P. aeruginosa, K. pneumoniae* and *E. coli* isolates (Baron and Finegold, 1990). The susceptibilities of the isolates to different antibiotics were tested using agar disk diffusion method and multidrug resistant isolates were selected for further experiments. Isolated bacteria were maintained for long storage on skimmed milk medium (BBL) by adding 10% glycerol in -60°C, cultures were maintained for daily use on Nutrient agar (BBL) slants on 4°C. The Muller Hinton Agar (MHA) and Muller Hinton Broth (MHB) media (Pronadisa) were used for detection of antibiotic resistance of isolates. *Acinetobacter calcuaceticus* PTCC 1318, *Enterococcus faecalis* ATCC29212, P. aeruginosa ATCC27853, *P. aeruginosa* PAO1, *E. coli* ATCC25922, *K. pneumoniae* ATCC10031, and *Staphylococcus aureus* ATCC25923 have been used as reference strains.

Determination of antimicrobial activity of Eucalyptus extracts: The susceptibility of isolates to Eucalyptus extracts was determined using a broth microdilution method based on CLSI guidelines. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Eucalyptus extracts for isolates were determined in Muller-Hinton Broth (MHB; Oxoid) medium (Jazani et al, 2009) (Papadopoulos et al., 2006). 10 mg of Eucalyptus powder was dissolved in 1000 µL of dimetylsufoxide (DMSO, Sigma). The initial concentration of Eucalyptus powder in the first tube contained MHB was 500 µg/mL. This was used to prepare serial doubling dilutions over the range 500-3.9 µg/mL. 1.5×10^6 inoculums of the isolates were added to each concentration in MHB. A tube containing growth medium without Eucalyptus extracts and an un-inoculated tube were used as a positive and negative growth control respectively. Antibacterial activity was measured by determining MICs and MBCs. The MIC was the lowest concentration of essential oil that resulted in a clear tube. Ten microlitres from each tube was spot-inoculated onto Nutrient Agar (NA) and incubated overnight at 37 °C to determine the MBC. The highest dilution that inhibits bacterial growth on nutrient agar after overnight incubation was taken as MBC (Baron and Finegold, 1990),(Papadopoulos et al., 2006). Experiments were performed at least three times and the modal value selected.

Determination of antimicrobial activity of ciprofloxacin: Considering the wide application of ciprofloxacin in treatment of bacterial nosocomial infections, the antibacterial effects of ciprofloxacin also determined on the isolates and its effectiveness was compared with Eucalyptus extracts. Ciprofloxacin powder was kindly provided by Exir pharmaceutical company, Tehran, Iran. The pure content of active ciprofloxacin was 96% in the provided powder. For determining the sensitivity of bacterial isolates to ciprofloxacin, classic broth dilution susceptibility test were used (Sahm and Weissfeld, 2002). MIC and MBC of isolates to ciprofloxacin were also determined. The initial concentration of antibiotic in the first tube was 500µg mL^{-1}, this solution was diluted serially in 8 steps. 1.5×10^6 inoculums of the isolates were added to each concentration of ciprofloxacin in MHB. A tube containing growth medium without ciprofloxacin and an un-inoculated tube were used as a positive and negative growth control respectively. *In vitro* resistance was defined as MBC of 4 or more µg mL^{-1} for bacterial isolates (Chaudhry et al., 1999).

RESULTS

A total of 100 multi-drug resistant isolates with nosocomial origin of gram negative and gram positive bacteria were collected from clinical specimens submitted to the educational hospital clinical microbiology laboratories of selected hospitals in Urmia, Iran. The Sensitivity of bacterial isolates to Eucalyptus hydroalcoholic extract has been shown in Figure 1.Also the MIC and MBC of Eucalyptus hydroalcoholic extract against standard bacterial strains has been shown in Table 1.The Sensitivity of bacterial isolates to ciprofloxacin has been shown in Figure 2. 69 isolates (69% of all isolates) were resistant (MBC $\geq$ 4 or µg mL^{-1}) and the other isolates were sensitive to ciprofloxacin (MBC $\leq$ 4 µg mL^{-1}) (Figure 2).

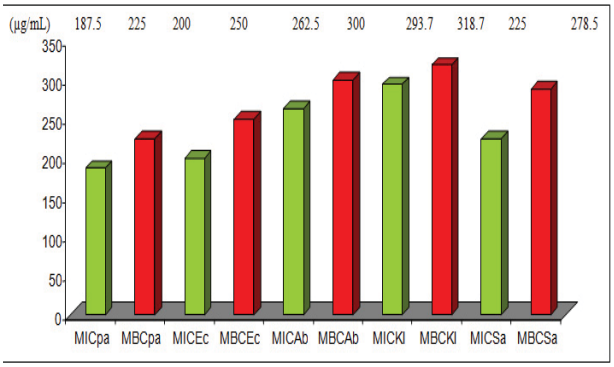

Fig.1. Antibacterial activity of Eucalyptus hydroalcoholic extract against 100 nosocomial isolates of multi drug resistant gram negative and gram positive bacteria. Pa: *Pseudomonas aeruginosa*, Ec: *E. coli*, Ab: *Acinetobacter baumannii*, Kl: *K. pneumoniae*, Sa: *Staphylococcus aureus*. MIC: Minimum Inhibitory Concentration, MBC: Minimum Bactericidal Concentration.

Table 1. The MIC and MBC of Eucalyptus hydroalcoholic extract against standard bacterial strains.

Standard Bacterial isolates	Eucalyptus hydroalcoholic extract (µg/mL)
Acinetobacter calcuaceticus PTCC 1318	MIC=62.5, MBC=125
Enterococcus faecalis ATCC29212	MIC= MBC=250
Pseudomonas aeruginosa ATCC27853	MIC= MBC=125
E.coli ATCC25922	MIC= MBC=250
Klebsiella pneumoniae ATCC10031	MIC=62.5, MBC=125
Staphylococcus aureus PTCC1112	MIC= MBC=250
Staphylococcus aureus ATCC25923	MIC= MBC=125
Pseudomonas aeruginosa PAO1	MIC= MBC=125

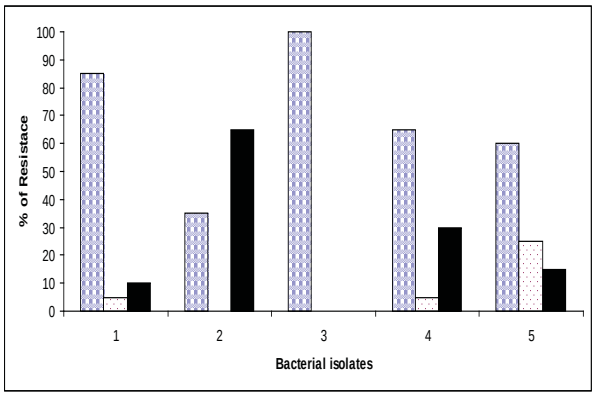

Fig.2. The rates of resistance to Ciprofloxacin for 100 clinical isolates of multi-drug resistant bacteria. Resistant (checked), Intermediate (spotted), Sensitive (black). 1: *Staphylococcus aureus*, 2: *E. coli*, 3: *Acinetobacter baumannii*, 4: *Klebsiella pneumoniae* And 5: *Pseudomonas aeruginosa*

DISCUSSION

Bacterial resistance to currently used antibiotics is becoming a concern to public health. Hence, introducing the new antimicrobial agents against these kinds of bacteria is one of the most important goals in treatment of such infections.

Antibiotics made from natural sources seem to be a great step toward this important goal so this makes the search for natural therapeutics an attractive option. However, there are limited studies on investigation of the antibacterial effects of Eucalyptus extract on multi drug resistant bacteria.

Iran is a rich source of herbal medicines, which been used as folk medicine since ancient times. Today, academic attempts are being done to modernize these potentials.

Ayepola and his team evaluated the antibacterial activity of the methanol extract, dichloromethane fraction and methanol residue at 10mg mL-1 of the leaf extracts of *Eucalyptus camaldulensis* against *K. pneumoniae, Salmonella* typhi, *Yersinia enterocolitica*, P. aeruginosa, *S. aureus* and *Bacillus subtilis* using agar diffusion method. The determination of MIC of the methanol extract and the dichloromethane fraction was done using the agar dilution method. According to the results obtained, methanole extract, dichloromethane fraction and the methanol residue represent a broad spectrum of activity. However, the petroleum ether fraction did not inhibit all test organisms.

The methanol extracts had stronger activity against *S. typhi*, *S. aureus* and *B. subtilis* (15-16mm) than *K. pneumoniae, Yersinia enterocolitica* and *P. aeruginosa* (14mm). The dichloromethane fraction exhibited higher activity against *K. pneumoniae, S. typhi*, *Y. enterocolitica* and *B. subtilis* (15–16mm) than *S. aureus* and *P. aeruginosa* (13-14mm). The methanol residue had a lower activity against all the test organisms except *K. pneumoniae* and *S. typhi*. Additionally, the antibacterial effects of gentamycin were determined on isolates and compared the effectiveness with *Eucalyptus* extracts, and the results showed that *K. pneumoniae* and *Yersinia enterocolitica* were resistant to gentamycin, which were inhibited by the extracts (Ayepola, 2008).

Takahashi et al. also measured The antimicrobial activities of leaf extracts from 26 species of eucalyptus on *S. aureus*, *Bacillus cereus*, *Enterococcus faecalis*, *Alicyclobacillus acidoterrestris, Propionibacterium acnes, E. coli, Pseudomonas putida,Trichophyton mentagrophytes* and MRSA. For this aim, they dried 10 gram of collected eucalyptus leaves in vacuum and immersed them in 200 ml methanol–dichloromethane (1:1) at room temperature for 2 days and separated the solvents from the leaves by filtration and concentrated to give methanol–dichloromethane extracts. Finally, the extracts were used to determine of MIC. Extracts of *Eucalyptus globulus, E. maculata* and *E. viminalis* significantly inhibited the growth of *S. aureus*, MRSA, *B. cereus, Enterococcus faecalis*, *Alicyclobacillus acidoterrestris, Propionibacterium acnes,* and *Trichophyton mentagrophytes* (a fungus), but they did not show strong antibacterial activity against Gram-negative bacteria like *E. coli*, and *Pseudomonas putida*. In this study, MIC values of *Eucalyptus camaldulensis* against the used organisms in the study were as follow: *S. aureus*=63, *B. cereus*=125, *Enterococcus faecalis*=125, *Alicyclobacillus aci doterrestris*>250,*Propionibacterium acnes*=125, *E. coli*>250, *Pseudomonas putida*>250, *Trichophyton mentagrophytes*=125, and MRSA=63. They also evaluated antimicrobial compounds

isolated from *E. maculate*, and totally concluded, *Eucalyptus* spp. leaf posses various antimicrobial constituents with different spectra (Takahashi, 2004).

Other studied antibacterial activity of 39 methanolic extracts from 25 Australian native plants, against two Gram-positive (*Bacillus cereus, Bacillus subtilis*) and two Gram negative (*Aeromonas hydrophilia, Pseudomonas fluorescens*) bacterial species using the disc diffusion assay. Among these plants, they studied 3 species of Eucalyptus plant including *Eucalyptus baileyana* leaves and *Eucalyptus major* leaves and flowers. The concentration of each methanolic extract of the plant, they used for test were 14.5 (mg/ml), 28.5 (mg/ml), and 35.5 (mg/ml), respectively. The mean diameters of inhibition in the triplicate experiments on *P. fluorescens, B.cereus, B. subtilis*, for *Eucalyptus baileyana* leaves are 7.0±0, 9.3±0.3, respectively. Additionally, the results for *Eucalyptus major* leaves were as 15.3±0.3, 12.0±1.03, 10.0 ± 0; and for *Eucalyptus major* flowers as 23.3±1.2, 12.6±0.33, 13.3±0.3.

None of the plants showed antibacterial activity against *A. hydrophilia*. This study indicated further evidence of the antimicrobial activities of some Australian native plants. Also mentioned that Eucalyptus is particularly worthy of further study due to the range of bacteria it is capable of inhibiting. Therefore, like the results our study showing the most of gram positive and gram-negative bacteria, were sensitive to Eucalyptus extract in various concentrations. In the present study all multi-drug bacterial isolates were sensitive to different concentrations of *Eucalyptus* hydroalcoholic extract. The most sensitive bacterial isolates to *Eucalyptus* extracts were *P. aeruginosa* isolates (Figure 1). In addition, *K. pneumoniae* ATCC10031 and *S.aureus* ATCC25923 were the most sensitive strains among the standard isolates (Table 1), however, clinical isolates showed high resistance to ciprofloxacin (Figure 2).

In the present study, the results revealed that the *Eucalyptus* hydroalcoholic extract possessed antibacterial effect against all multi-drug resistant bacterial isolates; furthermore, confirming the popular use, the obtained results demonstrate that this herbal drug could represent a new source of antimicrobial agents, for the control of hospital acquired infections. However, studies that are more adequate must be carried out to verify the possibility of using it for fighting these bacteria in human body infections.

Acknowledgment

This study has been supported by a research grant from student's research committee of Urmia Medical Sciences University.

REFERENCES

[1] Ayepola O.O .,B.A. ADENIYI, The Antibacterial Activity of Leaf Extracts of *Eucalyptus camaldulensis* (Myrtaceae); Journal of Applied Sciences Research, 4(11): 1410-1413, 2008.

[2] Baron, E.J. And S.M. Finegold, 1990. Bailey and Scott's Diagnostic Microbiology. 8th Edn., Mosby Company, St. louis.

[3] Chaudhry, N.A., H.W. Flynn Jr., T.G. Murray, H. Tabandeh, M.O. Mello Jr. and D. Miller, 1999. Emerging ciprofloxacin-resistant *Pseudomonas aeruginosa.* Am.J.Ophthalmol., 128: 509-510.

[4] Dale, R.M.K., Schnell,G. and J.P. Wong, 2004. Therapeutic Efficacy of Nubiotics against Burn Wound Infection by *Pseudomonas aeruginosa.* Antimicrob Agents Chemother. 48: 2918–2923.

[5] Duke, J.A. and Wain, K.K. 1981. Medicinal plants of the world. Computer index with more than 85,000 entries. 3 Vols.

[6] Forbes, B., Sahm, D.F. and A. Weissfeld, 2002. Baily and Scott's Diagnostic Microbiology. Mosby, St. Louis.

[7] Garjani, A., A. Afrooziyan1, H. Nazemiyeh, M. Najafi1, A. Kharazmkia1 and N. Maleki-Dizaji, 2009. Protective effects of hydroalcoholic extract from rhizomes of Cynodon dactylon (L.) Pers. on compensated right heart failure in rats. BMC Complementary Alternative Med., 9:28.

[8] Jazani, N.H., Zartoshti, M., Shahabi, S., Yekta, Z. and S. Nateghi, 2007. Evaluation of the synergetic effect of water soluble extracts of green tea (*Camellia sinensis*) on the activity of ciprofloxacin in urinary isolated *E. coli*. J. Boil. Sci., 7: 1500-1503.

[9] Jazani, NH., Zartoshti, M., Babazadeh, H., Ali-daiee, N., Zarrin, S. and S. Hosseini, 2009. Antibacterial effects of Iranian fennel essential oil on isolates of *Acinetobacter baumannii*. Pak J Biol Sci., 12: 738-41.

[10] Kamat, U.S., A. Fereirra, D. Amonkar, D.D. Motghare and M.S. Kulkarni, 2009. Epidemiology of hospital acquired urinary tract infections in a medical college hospital in Goa. Indian J Urol., 25: 76-80.

[11] Kanafani, Z.A. And V.G. Jr., Fowler, 2006. <I>*Staphylococcus aureus*</I> infections: New challenges from an old pathogen. Enferm Infecc Microbiol Clin., 24: 182-93.

[12] Keynan, Y. and E. Rubinstein, 2007. The changing face of *Klebsiella pnuemoniae* infections in the community. Int. J.Antimicrob. Agents., 30: 385-389.

[13] Larson, L.L., and R. Ramphal, 2002. Extended-spectrum beta-lactamases. Semin Respir.Infect., 17: 189-194.

[14] Little, E.L. Jr. 1983. Common fuelwood crops: a handbook for their identification. McClain Printing Co., Parsons, WV.

[15] Liu, J., Y.C. Song, Z. Zhang, L. Wang, Z.J. Guo, W.X. Zou and R.X. Tan, 2004. *Aspergillus fumigates* CY018, an endophytic fungus in *Cynodon dactylon* as a versatile producer of new and bioactive metabolites. J. Biotechnol., 114: 279–287.

[16] Nordmann P., 2004. *Acinetobacter baumannii*, the nosocomial pathogen par excellence. Pathol Biol (Paris)., 52: 301-303.

[17] Papadopoulos, C.J., Carson, C.F., Hammer K.A. And T.V. Riley, 2006. Susceptibility of *pseudomonads* to Melaleuca alternifolia (tea tree) oil and components. J. Antimicrobial. Chemother., 58: 449-451.

[18] Perez, F., Hujer, A.M., Hujer, K.M., Decker, B.K., Rather P.N. and R.A. Bonomo, 2007. Global challenge of multidrug-resistant *Acinetobacter baumannii*, Antimicrobial. Agents Chemother., 51: 3471-3484.

[19] Takahashi T., R. Kokubo and M. Sakaino, Antimicrobial activities of eucalyptus leaf extracts and flavonoids from Eucalyptus maculate, Letters in Applied Microbiology 2004, 39, 60–64.

[20] Watt, J.M. and Breyer-Brandwijk, M.G. 1962. The medicinal and poisonous plants of southern and eastern Africa. 2nd ed. E.&S. Livingstone, Ltd., Edinburgh and London.