ELSEVIER

Journal of Ethnopharmacology 76 (2001) 87–91

Journal of ETHNO-PHARMACOLOGY

www.elsevier.com/locate/jethpharm

Short communication

Purification and identification of active antibacterial components in *Carpobrotus edulis* L.

Elmarie van der Watt, Johan C. Pretorius *

Department of Agronomy, Faculty of Agriculture, University of the Orange Free State, Bloemfontein 9300, South Africa

Received 18 May 2000; received in revised form 30 November 2000; accepted 12 January 2001

Abstract

Very little is known about the chemical composition of *Carpobrotus edulis*, also known as Hotnotsfig or sourfig. However, some claims have been made in the past by traditional healers, regarding its usage as a medicinal plant. In this investigation it was initially illustrated that a crude methanolic extract of the plant exhibits strong anti-bacterial activity. Subsequently, the crude extract was fractionated by means of liquid-liquid chromatography, tannins removed by means of LH20 column chromatography and bioactive fractions with antibacterial properties isolated by means of preparative thin layer chromatography. Five bioactive compounds, individually or collectively responsible for the antibacterial property of *C. edulis*, were purified from an active ethyl acetate fraction. These compounds were initially identified as flavanoids using standard fingerprinting methods and eventually identified as rutin, neohesperidin, hyperoside, cactichin and ferulic acid using flavanoid standards. A sixth flavanoid with antibacterial activity was also purified but could not be identified in this way. The latter is currently isolated in larger volume for identification through nuclear magnetic resonance spectroscopy.

Keywords: Carpobrotus edulis; Antibacterial properties; Flavanoids

1. Introduction

In Asia, Latin America and Africa the extensive use of natural plants as primary health remedies, due to their pharmacological properties, is quite common (Conco, 1991). In recent years pharmaceutical companies have spent a lot of time and money in developing natural products extracted from plants, to produce more cost effective remedies that are affordable to the population (Farnsworth, 1994). Limitations of synthesized compounds in the treatment of chronic diseases and the potential of plant-based medicine as a more effective and cheaper alternative, was probably responsible for the fast growing industry of herbal medicine (Rojas et al., 1992). However, due to over exploitation, some traditionally used plants are disappearing and the sustainable usage of natural resources is currently questioned by ecologists (Nigg and Seigler, 1992; Cragg et al., 1993). It is therefore important to conduct thorough investigations of as many traditionally used medicinal plants as possible before they are eradicated (Mammen, 1996).

When choosing a natural plant for investigating its medicinal and agrochemical potential it would, according to Baker et al. (1995), be worthwhile to consider the following criteria:

1. evidence suggesting the traditional usage of the plant by the native population;
2. the purpose for which it is used;
3. the abundance of the specific plant species in nature; and
4. the sustainable utilization of the plant.

Abbreviations: MIC, minimum inhibitory concentration; L., Linnaeus; DMSO, dimethylsulphoxide; Uv., ultra-violet light; Q-TLC, qualitative thin layer chromatography; P-TLC, preparative thin layer chromatography; PGA, plate count agar NB, nutrient broth.

* Corresponding author. Tel.: +27-5140-12217 Fax. +27-5140-12217.

E-mail address: seefpret@landbou.uovs.ac.za (J.C. Pretorius).

0378-8741/01/$ - see front matter
PII: S0378-8741(01)00197-0

One of these plant families that measure up to the criteria set by Baker et al. (1995), and needs special consideration, is the family Aizoaceae. Besides the role that many. species in this family play as soil stabilizers, their leaves are traditionally used as fodder while extracts from these plants are used as preservatives, as a remedy against throat infections and in soap-making (Smith et al., 1998). Moreover, the Aizoaceae is considered as one of southern Africa's most diverse and abundant plant families but also the least studied in terms of its medicinal potential.

The species *Carpobrotus edulis*, traditionally utilized for its medicinal properties (criterium 1; Baker et al., 1995), was selected for this study. Because of its abundance (criterium 3; Baker et al., 1995), the leafjuice from *C. edulis* is more widely utilized than that of *C. acinacformis* or *C. delicious* as a traditional remedy (criterium 2; Baker et al., 1995) for a wide range of fungal and bacterial infections (Smith et al., 1998) and treatment of sinusitis, diarrhea, infantile eczema, tuberculosis and other internal chest conditions (Van Wyk, 1997). According to Roberts (1995) the leafjuice is also effective in soothing itching caused by spider and tick bites. The leaves also contain an astringent antiseptic juice which can be taken orally for treating sore throat and mouth infections (Rood, 1994). At the coastal regions of South Africa it is best known for its use as a soothing remedy for blue bottle stings but also for treating wounds and burns (Roberts, 1995).

C. edulis (sour fig) is an undemanding and fast growing succulent distributed in many parts of the world. According to Watt and Breyer-Brandwijk (1962) it flourishes on sandy soils and dunes along coastal areas. However, in South Africa it is found in all provinces on virtually all soil types. Non-destructive utilization by traditional healers of mainly the leaves contributes to its sustainable exploitation (criterium 4; Baker et al., 1995). Moreover, it can be grown from both seeds and cuttings (Wisura and Glen, 1993) and can easily be established.

However, very little is known about the active substances responsible for its healing properties and antibacterial agents have not previously been isolated. According to Eloff (1998), the possibility exists that natural antimicrobial components in plants can inhibit the growth of bacteria by means of unknown mechanisms other than that of known antibiotics, and for this reason the search for new antibiotics must continue. In this study the antibacterial activity of the crude extract was comfirmed. Subsequently, the crude extract was fractionated and only the bioactive compounds in the fractions which tested positively for antibacterial activity were flirther purified and identified.

2. Materials and methods

2.1. Materials

2.1.1. Plant material

Carpobrotus edulis L. plants were collected in Bloemfontein, South Africa (29° 06′ 58″ South; 26° 11′ 08″ East) and authenticated by the National Museum (Bloemfontein) where a voucher specimen is kept. The freshly collected leaf material was dried in an oven at 60°C for 7 days, ground to a fine powder and stored in Scott Duran airtight bottles at 4°C.

2.1.2. Other materials and chemicals

Aluminum thin layer chromatography plates (silica gel 60 F 254; 20 × 20 cm) were purchased from Merck (Germany) and preparative thin layer chromatography glass plates (silica + indicator, 1 mm, G 1510/LS 254; 20 × 20 cm) from Schleicher and Schuell (Germany) or Sigma (Germany). Most of the chemicals used were supplied by Merck (Germany) and were of the purest grade available. PDA (potato dextrose agar), PCA (plate count agar) and nutrient broth as well as the packing material for column chromatography (Kieselgel 60; particle size 0.063–0.200 mm; 70–230 mesh ASTM) were purchased from Merck (Germany).

2.2. Methods

2.2.1. Preparation of ethanolic and methanolic crude extracts

Six hundred millilitres absolute ethanol, containing 10 mM ascorbic acid, were added to 200 g of the dry plant material, stirred for an 1 h to remove all low molecular weight phenolics and centrifuged at 15 000 r.p.m. for 10 min at room temperature (Hagerman, 1998). The ethanolic supernatant was decanted and stored for later use.

The pellet was subsequently extracted four times with 150 ml methanol, containing 10 mM ascorbic acid, and filtered under vacuum through a Schleicher and Schuell 595 filter paper. After each extraction, which continued for one hour, the samples were again centrifuged at 15 000 r.p.m. for 10 min and the tannin-containing methanolic supernatant decanted. An equal volume of 0.05 M sodium acetate, (pH = 4), was subsequently added to the methanolic extract. The methanol was completely removed by evaporation at 30°C using a Buchi Rotavapor (Bibby Sterilin LTD, England) and the pooled methanolic extract further fractionated.

2.2.2. Fractionation of the methanolic extract by means of liquid–liquid chromatography and removal of tannins

The tannin-containing methanolic extract was fractionated three times with ethyl acetate in a 1:1 (v/v) ratio on a mechanical shaker. The lower aqueous phase

and the top ethyl acetate phase were retained and screened for possible antibacterial activity. The aqueous phase was evaporated under vacuum at 30°C to a volume of about 20 ml after which absolute ethanol was added to a final concentration of 80% ethanol. The latter ethanolic aqueous phase was subsequently applied to 20 g Sephadex LH 20, in a coarse sintered glass fimnel, previously equilibrated in 80% ethanol. Using very gentle suction (1 atmosphere), the liquid was slowly removed from the Sephadex beads under vacuum while stirring gently with a glass rod.

Tannins adsorbed to the Sephadex LH 20 beads (Hagerman, 1998) and all compounds, except the tannins, were removed by washing gently with 95% ethanol and the filtrate collected. Tannins were subsequently removed by washing the Sephadex with 50% acetone. All filtrates were evaporated to dryness at 30°C and tested for antibacterial activity. Only the bioactive fraction was flirther purified.

2.2.3. Isolation and purification of active substances in the bioactive ethyl acetate fraction

Twenty five milligrams of the bioactive tannin-free ethyl acetate fraction was further fractionated by means of preparative thin layer chromatography (P-TLC; 20×20 cm glass plates covered with Silica 60, F254; 0.5 mm) using ethyl acetate:methanol:water (100:13.5:10) as the mobile phase. After developing the plates, the positions of different compounds were determined by fluorescence under UV-light (254 and 365 nm).

Compounds were separately removed by scraping off the silica and washing five times with methanol or ethanol according to the *RF*-values. Compounds with *RF*-values exceeding 0.5 were removed by eluting with ethanol and those with *RF*-values lower than 0.5 were eluted with methanol (Ouellette, 1992). Pureness of the compounds was tested by means of qualitative thin layer chromatography (Q-TLC) using different mobile phases. Best separations were achieved with ethyl acetate:methanol:water (100:13.5:10) and ethyl acetate:formic acid:acetic acid:water (100:11:11:26). Seventeen fractions were recovered of which three showed antibacterial activity. From these six compounds were purified.

2.2.4. Identification ofactive substances using standards

The group of chemicals to which the six purified compounds belonged were initially identified by using standard staining procedures (Wagner and Bladt, 1996) and subsequently identified by means of TLC using commercially available standards. Antibacterial activity was confirmed for each of the purified compounds and tested against 11 known human pathogenic bacteria.

2.2.5. Antimicrobial activity

Antibacterial activity was qualitatively evaluated by means of the agar plate difflision assay technique (Rios et al., 1988). Plate count agar (PGA) was used for bacterial cultures of the test organism *Moraxella cattharalis* and stock cultures of human pathogenic bacteria were maintained on nutrient agar (NA) slants at 4°C. Agar plates containing 1 ml (106 bacteria/ml) of an overnight broth culture were prepared.

The crude extract and semi-purified fractions were assayed as aqueous suspensions in 10% DMSO at a concentration of 50 mg cm^{-3} by transferring a maximum of 40 μl into 5 mm holes made in the agar with a sterile cork borer. Pure compounds were tested at 10 mg/ml. A solution of 10% (v/v) DMSO was used as the negative control, while three positive controls (10 μg ampicillin, 30 μg chloroamphenicol and 10 units penicillin) were also used. Plates were incubated at 35°C for 24 h. Inhibition zones were measured and used as a qualitative indicator for antibacterial activity.

3. Results

The antibacterial activity of a methanolic crude extract of *C. edulis* against the gram-negative bacteria *M. catharralis*, at a concentration of 50 mg cm^{-3}, was confirmed (results not shown). The latter strain was selected as a test organism as it prooved to be the most sensitive of all organisms tested previously in our laboratory and is currently used in routine preliminary screening procedures. No activity was detected in the ethanolic crude extract.

Of the liquid-liquid fractions obtained from the crude extract, only the ethyl acetate and acetone fraction (tannins), were active. The tannin fraction was ignored as most pharmaceutical companies are not interested in tannins because of its side effects in humans. From the ethyl acetate fraction seventeen semi-purified fractions were isolated. Of these only three fractions were active against the gram-negative test organism *M. catharralis*, namely fractions 4, 5 and 6. These three fractions were turther purified, six compounds isolated and their antibacterial activities confirmed against the test organism *M. catharralis* (results not shown).

Using standard colouring reagents (Wagner and Bladt, 1996), UV-light fluorescence (254 and 365 μm) and known standards, five of the six compounds were identified as flavanoids namely rutin, neohesperidin, hyperoside, cactichin and ferulic acid. The sixth compound, also a flavanoid, remained unidentified.

Subsequently, the antibacterial activities of the six purified compounds were tested against eleven human pathogens (Table 1). All six compounds showed significant antibacterial activity against *M. catharralis* (gram negative) and two of the gram positive bacteria,

Staphylococcus epidermidis and *Staphylococcus aureus*. Negative results against gram negative bacteria were foreseen as this group of bacteria shows high resistance to antibiotics (Singleton, 1997) but, Neohespiridin and hyperoside were also active against *Pseudomonas aeruginosa* (gram negative).

Ferulic acid was the only compound with activity against *Bacillus subtilis* and *Streptococcus pneumoniae* (both gram positive). Not one of the purified compounds was active against the gram negative bacteria *Haemophilus influenzae*, *Eschericha coli*, *Klebsiella pneumoniae*, or *Acinetobacter baumanii*, as well as the gram positive *Streptococcus pyogenes*. However, the semi-purified tannin fraction showed some activity against the first two organisms in the latter group as well as against *S. pyogenes*.

4. Discussion

A very high tannin content was observed in the leaves of *C. edulis*. The tannin content of plants fluctuate during the year (Ewing and Fife, 1972; Jansman, 1993) and can be as low as 0.5% at the end of the growing season or as high as 5% during the growing season (Harborne, 1991). As a high tannin content in plant extracts obstructs the TLC-separation of other compounds (Hagerman, 1998), tannins were removed from the crude extract prior to separating and purifying other compounds.

Tannins have diverse effects on biological systems because they are potential metal ion chelators, protein precipitating agents and biological antioxidants (Freudenberg and Weinges, 1962; Hagerman et al., 1997). Moreover, tannins cause side effects in animals and man (Ihlenfeldt, 1994), and pharmaceutical companies do not place a high premium on tannins as potential primary health agents (Borris, 1996).

After removal of tannins, a bioactive ethyl acetate fraction was obtained from the remaing methanolic extract from which six compounds were isolated and five identified as the flavanoids rutin, neohesperidin, hyperoside, cactichin and ferulic acid. Flavanoids are common in leaves of plants and protect them against the damaging effects caused by UV-radiation and antimicrobial infections (Bruneton, 1995). Pharmaceutical applications of flavanoids include the lowering of vein permeability and the treatment of circulatory disorders. Different flavanoids and phenolic compounds react with free radicals to reduce the degradation of membranes by preventing the reaction between free radicals and phospholipids (Bruneton, 1995). They can also be used as antioxidants and in vitro as enzyme inhibitors (Smith et al., 1998). Flavanoids are also known to possess antibacterial properties (Hostettman et al., 1995).

Rutin and its derivatives are combined with alkaloids for the treatment of senile cerebral defects (Vlahov, 1992). It also relieves micro trauma on tissue (Smith et al., 1998). Although rutin is relatively abundant in plants, only a small number of drugs contain quantities sufficient for industrial extraction (Bruneton, 1995). Rutin can also help to improve the effectiveness of vitamin C. This bioflavonoid can also help to strengthen veins, thus preventing bleeding (Kinghorn and Balandrin, 1993).

Neohesperidin is less common than its unsaturated homologue, naringin, because most plant families accumulate its C-alkyl derivatives (Markham, 1982) and nothing is known about its antibacterial properties.

Table 1
Antibacterial activity of six purified compounds isolated from the ethyl acetate fraction as well as the semi-purified tannin fraction against known human pathogens. The pure compounds were tested at a concentration of 10 mg cm^{-3} and the tannin fraction at 50 mg cm^{-3}

Bacterium		Compound/fraction and mean inhibition zone diameter (mm)							
Gram (−)/(+)		Rutin	Unknown	Neohesperidin	Hyperoside	Cactichin	Ferulic acid	Semi-purified tannin fraction	Antibiotic control
B. subtilis	+	–	–		–	–	8	13	35[c]
S. epidermidis	+	10	10	9	12	12	9	16	33[c]
S. aureus	+	11	10	9	11	11	8	12	32[c]
S. pneumoniae	+	–	–		–	–	12	–	21[c]
S. pyogenes	+	–	–		–	–	–	10	28[b]
Mcatarrhalis	−	13	9	12	14	13	9	16	18[a]
H. influenrae	−	–	–		–	–	–	10	29[c]
E. coli	−	–	–		–	–	–	9	18[c]
K. pneumoniae	−	–	–	–	–		–	–	33[c]
P. aeruginosa	−	–	–	10	11		–	–	32[c]
A. baumanii	−	–	–	–	–		–	–	17[a]

[a] Ampicillin (10 μg disks).
[b] Penicillin (10 units).
[c] chloramphenicol (30 μg disks).

Hyperoside is quite common in a wide range of plants, contributes to the anti-inflammatory properties of these plants (Bruneton, 1995) and exhibits remarkable in vitro monoamine oxidase (MAO) inhibition. MAO is one of the two major enzymes involved in the catabolism of norepinepherine, epinephrine and dopamine within the brain.

Cactichin was described by Bruneton (1995) as a condensed form of a tannin. It can be used to protect the skin layers against fluid losses and also has a vasoconstricting effect on small veins (Jansman, 1993). It kills bacteria by directly damaging the cell membrane, confirming that catechin is an antibiotic. Other studies have confirmed that catechin shows synergetic effects when combined with known antibiotics (Shimamura, 1999).

Ferulic acid is a natural protector against ultraviolet radiation known to cause skin disorders such as cancer and also to accelerate aging of the skin. The flavanoid is commonly used for the treatment of dyspepsia, as a herbal health supplement and as a herbal antioxidant (Bruneton, 1995). No information on its antiseptic properties could be traced in literature. In this study ferulic acid was the only active flavanoid against the gram positive bacterium *B. subtilis*, known to cause the highly contagious disease anthrax.

In conclusion, this study confirmed the selective antibacterial property of a crude extract from *C. edulis*. This activity, albeit weak compared to that of the positive controls (ampicillin, chioramphenicol and penicillin), can be attributed to the presence of different flavanoids as well as tannins in *C. edulis*.

References

Baker, J.T., Borris, R.P., Carte, B., Cardell, G.A., Soejarto, D.D., Gragg, G.M., Gupta, M.P., Iwn, M.M., Madulid, D.R. and Tyler, V.E. (1995). Natural product drug discovery and development: New perspectives on international collaboration, Journal of Natural Products 58(9): 1325-1357.

Borris, R.P. (1996). Natural products research: Perspectives from a major pharmaceutical company, Journal of Ethnopharmacology 51: 29-38.

Bruneton, J. (1995). In: ''Pharmacognosy, Phytochemistiy, Medicinal plants.'' New York, USA: Lavoisier Publishing Inc. p.265-334.

Conco, W.Z. (1991). Zulu traditional medicine: its role in modern society. Community Health 5: 8-13.

Cragg, G.M., Boyd, M.R., Cardellina, J.H., Grever, M.R., Schepartz, S.A. and Snader, K.M. (1993). ''The role of plants in the drug discovery program of the US manipulation.'' In: ''International crop science 1'' Madison, USA: Crop Science Society of America. p.178-196.

Eloff, J.N. (1998). Which extractant should be used for the screening and isolation of antimicrobial components from plants, Journal of Ethnopharmacology 60: 1-8.

Ewing, W.H. and Fife, M.A. (1972). Flavanoid compounds, International Journal of Systematic Bacteriology 22: 4-11.

Farnsworth, N.R. (1994). ''Ethnopharmacology and drug development.'' In: Prance, G.T. (Ed.). ''Ethnobotany and the Search for New Drugs Ciba Foundation Symposium''. Chischester: Wiley 185. p.42-59.

Freudenberg, K. and Weinges, K. (1962). ''The chemistry of flavonoid compounds.'' Oxford, UK: Pergamon Press p.1-11.

Hagerman, A.E., Zhao, Y. and Johnson, S. (1997). ''Antinutrients and Phytochemicals in Foods'' Washington DC: American Chemical Society. p.1-30.

Hagerman, A.E. (1998). ''Tannin Handbook'' Oxford, USA: Miami University, p.1-35.

Harborne, J.B. (1991). ''Plant defenses against Mammalian Herbivory'' Boca Raton, Oxford, USA: CRC Press p.503-515.

Hostettman, K., Marston, A.J., Wolfender, L. and Maillard, M. (1995). Screening for flavanoids and related compounds in medicinal plants by LC-UV-MS and subsequent isolation of bioactive compounds'' Akademiai, Kiaho: Budapest p.35-52.

Ihlenfeldt, H.D. (1994). Diversification in an arid world: the mesembryanthemaceae, Annual review of ecology and systematics 25: 521-546.

Jansman, A.J.M. (1993). ''Tannins in Faba beans (*Vicia Faba* L.) — antinutritional properties in monogastric animals.'' In: '' PhD Thesis'' Wageningen: Netherlands: Department of Animal Nutrition Wageningen Agricultural University. p.12-19.

Kinghorn, K. and Balandrin, G. (1993). ''Human medicinal agents from plants,'' In: ''ACS Symposium series 534'' Washington, DC: American Chemical Society. p.639-700.

Mammen, M. 1996. Bridging the gap between traditional and 'modern' medicine. Veld&Flora 2 (2).

Markham, K.R. (1982). ''Techniques of flavonoid identification.''. New York: Academic press p.1-35.

Nigg, H.N. and Seigler, D. (1992). ''Phytochemical Resources for Medicine and Agriculture''. New York: Plenum Press. p.76-363.

Ouellette, R.J. (1992). ''Introduction to general, Organic, and Biological Chemistry''. Third edn, New York: Macmillan publishing Company. p.25.

Rios, J.L., Recio, M.C. and Villar, A. (1988). Screening methods for natural products with antimicrobial activity: a review of the literature, Journal of Ethnopharmacology 32:127-149.

Roberts, M. (1995). ''Indigenous Healing Plants.'' Cape Town: Tafelberg Publishers Inc. p.190-192.

Rojas, A., Hernandez, L., Rogeho, P.M. and Mata, R. (1992). Screening for antimicrobial activity of crude drug extracts and pure natural products from Mexican medicinal plants, Journal of Ethnopharmacology 35: 275-283.

Rood, B. (1994). ''From the veldpharmacy.'' Cape Town: Tafelberg Publishers Inc. p. 72.

Shimamura, T. (1999). Antimicrobial activities of tea and its clinical applications, World)-CHA (tea) Festival 2001, pp.1–2.

Singleton, P. (1997). ''Bacteria in Biology, Biotechnology and Medicine''. fourth edn. New York: John Wiley and Sons.p.1-20.

Smith, G.F., Chesselet, P., Van Jaarsveld, E.J., Hartmann, H., Hammer, S., Van Wyk, B., Burgoyne, P., Klak, C. and Kurzweil, H. (1998). ''Mesembs of the world'' Pretoria: Briza Publications. p.252-255.

Van Wyk, B. (1997). ''Medicinaiplants of South Africa.'' Pretoria: Briza Publications. p.70-71.

Vlahov, G. (1992). Flavanoids in three olive (*Olea europea*) fruit varieties during maturation, Journal of the Science of Food and Agriculture 58:157-159.

Wagner, H. and Bladt, S. (1996). ''Plant Drug Analysis. A Thin Layer Chromatography Atlas''.. New York: Springer p.195-245.

Watt, J.M. and Breyer-Brandwijk, M.G. (1962). ''The Medicinal and Poisonous Plants of Southern and Eastern Africa'' London: Livingstone.p.35.

Wisura, W. and Glen, H.F. (1993). The South African species of *Carpobrotus* (Mesembryanthema, Aizoaceae) Contribution, Boletim Herbage 15: 76-107.