

*Full Length Research Paper*

## Does seasonal variation influence the phytochemical and antibacterial properties of *Carpobrotus edulis*?

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*Carpobrotus edulis* L. (family Aizoaceae) has been used by locals over the years to treat microbial infections. Extracts of varying polarities were prepared from the leaf debris and filtrate of a spring and an autumn harvest of *C. edulis*. Thin layer chromatography was used to analyze the phytochemicals of the extracts as well as to assay the plant for antioxidant compounds. The spring harvest showed equal distribution of the phytochemicals within the leaf debris and the filtrate, but there was a high prevalence of phytochemicals within the leaf debris extracts of the autumn sample. An antioxidant compound was intensely pronounced in the autumn extracts of intermediate polarity and in the polar extract. The plant was evaluated for antibacterial activity against *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* by using a two-fold serial microdilution method as well as bioautography. The spring extracts were more potent against all test organisms, having MIC values that were lower than the autumn extracts. When taking the total activity of each extract into account, the autumn extracts showed higher efficacy than the extracts from the spring sample. The antibacterial activity observed in the extracts of both seasons somewhat validated the ethnomedicinal use of *C. edulis*.

**Key words:** *Carpobrotus edulis*, antibacterial, antioxidant, minimum inhibitory concentration, bioautography, seasonal.

### INTRODUCTION

The search for new drugs to combat diseases and infections has roused the interest of scientists all over the world in herbal remedies. Due to the emergence of multi-drug-resistant microorganisms, a huge field of research has arisen for novel antimicrobial agents against which microorganisms have not yet acquired resistance (Rojas et al., 2006; Samie et al., 2005). These antimicrobial agents are best obtained from medicinal plants because many of the diseases treated with these plants are of microbial origin (Nascimento et al., 2000). In order to promote the use of medicinal plants as potential sources of antimicrobial compounds, it is pertinent to thoroughly investigate their composition and activity, and thus validate their use (Nair and Chanda, 2006). Some phytochemicals, produced by plants, have antimicrobial

activity allowing these plants to be studied and used for the development of new antimicrobial drugs (Nascimento et al., 2000). The effectiveness of phytochemicals in the treatment of various diseases may lie in their antioxidant effects (Akinmoladun et al., 2007). Bioactive phytochemicals isolated from plants include flavonoids, phenolic acids, tannins, etc, and according to Korotkova et al. (2003) many of these possess antioxidant activity.

According to van der Watt and Pretorius (2001) the family Aizoaceae is one of the most abundant and diverse plant families in Southern Africa. The genus *Carpobrotus* falls within this family and is well identified by its trailing structure and thin, blade-like succulent leaves (Springfield and Weitz, 2006). For decades, traditional healers all over South Africa have been utilizing various species of *Carpobrotus* genus to treat fungal and bacterial infections (van der Watt and Pretorius, 2001).

The antibacterial activity of *Carpobrotus edulis* L. (Aizoaceae) has been studied before (van der Watt and

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Pretorius, 2001). Six active compounds were purified from an ethyl acetate extract and were all identified as flavonoids. These individual compounds showed antibacterial activity against both gram-negative and gram-positive bacteria (van der Watt and Pretorius, 2001).

The aim of the current study was to qualitatively and quantitatively evaluate and compare the antibacterial activity of *C. edulis* leaves harvested in two different seasons (spring and autumn) and to further use a more sensitive method to assess its antibacterial activity. Therefore, this information may help in identifying the season that is more suitable for harvesting the plant for pharmacological evaluation.

## MATERIALS AND METHODS

### Plant collection and Storage

The leaves of the wild fig (*C. edulis*) were collected from University of Limpopo (Turffloop campus) gardens in spring and in autumn. The leaves were homogenized in a Waring Commercial Blender (Model 32BL79, Dynamics Corporation, New Hartford, CT, U.S.A) and filtered through Whatman No. 3 filter paper. The leaf extract and filtrate were both dried under a stream of air at room temperature and stored in a dark cupboard in separate covered glass beakers at room temperature.

### Extraction procedure

Two grams (2 g) of each of the leaf samples was extracted once with 20 ml of one of the following solvents: hexane, dichloromethane (DCM), ethyl acetate, acetone and methanol (Rochelle Chemicals, South Africa). The samples were shaken vigorously at room temperature for 40 min and filtered into pre-weighed beakers. The solvents were evaporated overnight under a stream of air at room temperature.

### Phytochemical Analysis

Phytochemical properties of the plant were analyzed by loading 10 µl of each filtered sample, redissolved in acetone to make 10 mg/ml, onto four aluminium-backed TLC plates (Merck, silica gel 60 F<sub>254</sub>). The plates were then developed separately in CEF (5:4:1, chloroform : ethylacetate : formic acid), EMW (10:1.35:1, ethylacetate : methanol : water), BAW (4:1:5, butanol : acetic acid : water), and BEA (18:2:0.2, benzene : ethanol : ammonia) (Kotze and Eloff, 2002). The chromatograms were visualized under ultraviolet light to detect the UV-active compounds. The plates were sprayed with vanillin-sulphuric acid reagent (0.1 g vanillin, 28 ml methanol, 1 ml sulphuric acid) and heated with a heat gun (PowaForce 2000 W) to develop colour.

### TLC-DPPH antioxidant screening

The plant was assayed for antioxidant activity by loading 10 µl of 10 mg/ml of each filtered sample in acetone onto four aluminium-backed TLC plates. The plates were developed separately in CEF, EMW, BAW and BEA and later sprayed with 0.2% 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) in absolute methanol to visualize the antioxidant compounds that may be present in the extracts (Deby and Margotteaux, 1970).

## Antibacterial Activity Assays

### Test organisms

The antibacterial assays were done using two gram-negative and two gram-positive bacteria that have been maintained on agar slants and sub-cultured in Luria broth prior to use. The gram-negative cultures tested were *Escherichia coli* (ATCC 27853) and *Pseudomonas aeruginosa* (ATCC 25922); and the gram-positive cultures were *Enterococcus faecalis* (ATCC 21212) and *Staphylococcus aureus* (ATCC 29213). These four bacterial strains are recommended for use by the National Committee for Clinical Laboratory Standards (NCCLS, 1992) as these species are the major causes of nosocomial infections in hospitals (Sacho and Schoub, 1993).

### Microdilution Assay

Antibacterial activity was evaluated by estimating the minimum inhibitory concentration (MIC) of each extract on *E. faecalis*, *E. coli*, *P. aeruginosa* and *S. aureus* using the microdilution method developed by Eloff (1998a). Each culture was assayed in triplicate. The extracts were redissolved in acetone to a final concentration of 10 mg/ml. Two-fold serial dilutions of the extracts (2.5 mg/ml to 0.02 mg/ml) were prepared in 96-well microtiter plates. The wells in each plate were inoculated with 100 µl of the relevant culture and incubated at 37°C overnight. Forty microlitres (40 µl) of 0.2 mg/ml iodinitrotetrazolium chloride (INT) (Sigma-Aldrich) was added to each well and the plates were incubated for 30 min at 37°C for *S. aureus* and *P. aeruginosa*, 1.5 h for *E. coli* and 24 h for *E. faecalis*. The formation of a red colour signified microbial growth.

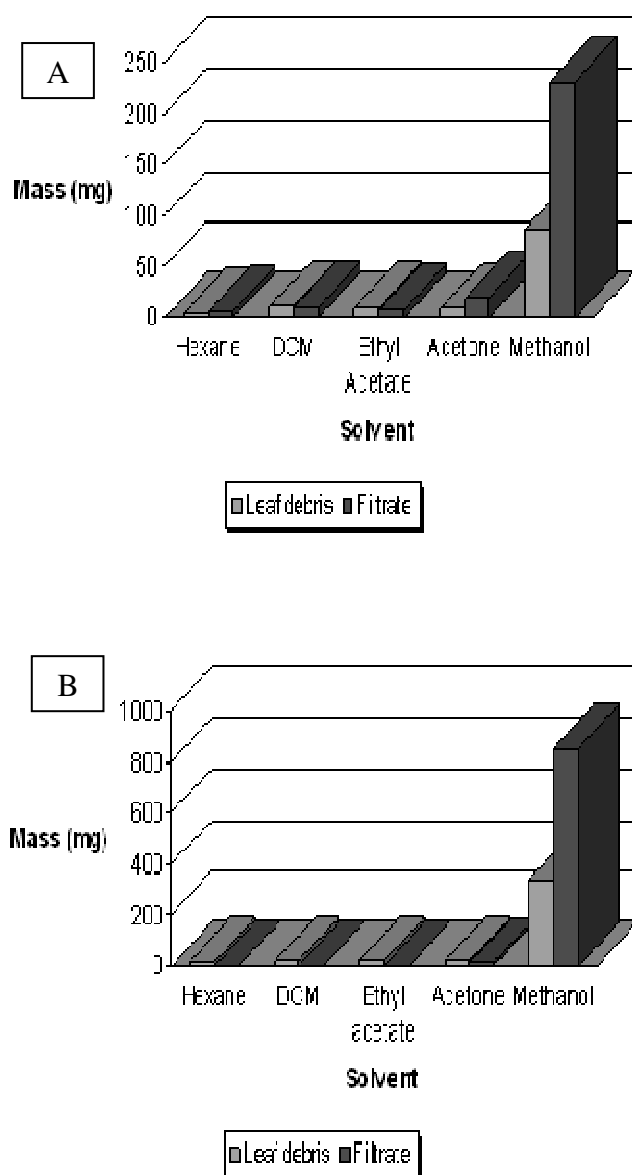
### Bioautography

Bioautographic procedure was done according to Begue and Kline (1972). Briefly, sixteen TLC plates were loaded with 10 mg/ml of each of the extracts dissolved in acetone. The plates were developed in CEF, EMW, BAW and BEA in groups of four and left to dry for a week at room temperature under a stream of air. The test cultures used for the serial microdilution assay were sprayed on one plate (for each mobile phase) and the plates were incubated overnight at 37°C and 100% humidity. The plates were sprayed with 2 mg/ml INT and incubated for 1 h. Clear zones on the plates indicated areas of growth inhibition.

## RESULTS AND DISCUSSION

### Extraction

Due to the vast differences in the nature of the chemical constituents found in a plant, there is no solvent that is able to extract all the compounds on its own. In this study, 5 solvents of varying polarity were used in the extraction to accommodate the range of polarities of the compounds obtained from *C. edulis*. The choice of solvent played a crucial role in the amount of material that was extracted from each of the samples. There was a general increase in the mass of material, in both the spring and autumn samples, as the polarity of the extracting solvent increased (Figure 1). The methanolic extract yielded more material because methanol extracts more compounds of varying polarity (Eloff, 1998b). More



**Figure 1.** Mass of *Carpobrotus edulis* leaf samples collected in spring (A) and autumn (B) extracted with various solvents. (DCM: dichloromethane).

material was found to have been extracted from the autumn sample, suggesting that the compounds that were extracted by the selected solvents are more abundant in the plant during the autumn than spring season.

### Phytochemical Analysis

More vanillin-reacting compounds of *C. edulis* were extracted by the solvents with intermediate polarity, i.e., DCM, ethyl acetate and acetone (Figure 2). The phyto-

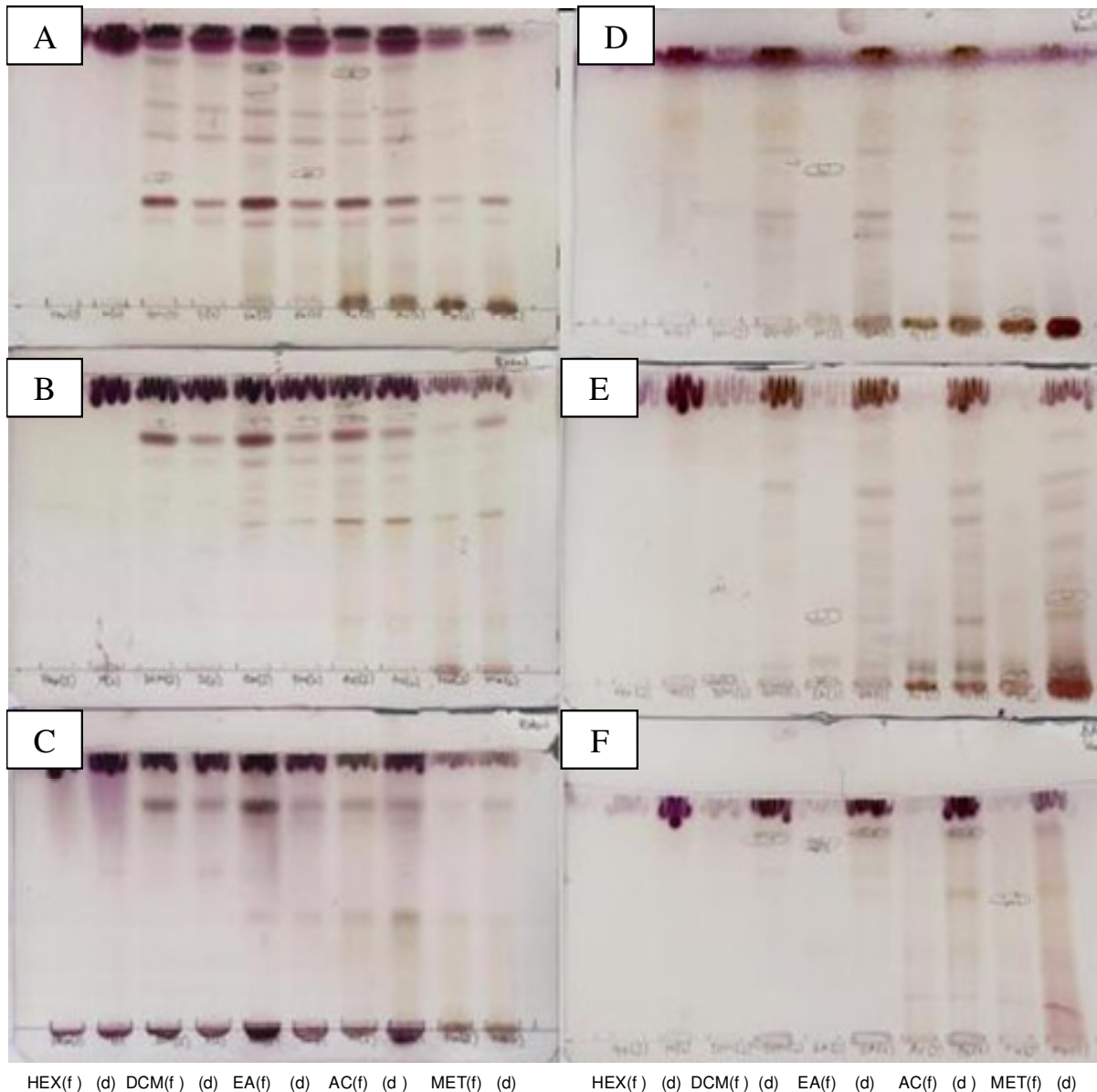
chemical compounds were best separated in the CEF mobile phase, which separates acidic compounds and those of intermediate polarity. Previous studies using this species and other species of the *Carpobrotus* genus reported that many of the compounds present in the leaves of the plant are tannins and flavonoids (Springfield et al., 2003; Springfield and Weitz, 2006), which are both of intermediate polarity. Malic and citric acids were also abundant (Springfield et al., 2003). There was an equal distribution of the phytochemicals within the leaf debris and the filtrate of the spring harvest (Figure 2). However, the prevalence of phytochemicals within the autumn leaf debris samples, regardless of the extracting solvent used, suggests that there is a higher concentration of phytochemicals within the leaf tissue of the plant during autumn and less of them are being circulated around the plant.

### Antioxidant Screening

One antioxidant compound was extracted by solvents that were of intermediate polarity, i.e., ethyl acetate and acetone, as well as by methanol, which is a polar solvent. The compound's prevalence in the methanol extract suggests that this compound must be of polar nature because it was best extracted by a polar solvent. The compound was more pronounced in the samples that were harvested in autumn, leading to the understanding that it is abundant in autumn than in spring. The antioxidant compound in *C. edulis* was best visualized after separation with BAW (Figure 3). Separation with CEF and EMW demonstrated antioxidative activity only at the origin of migration of the fractions, which suggested that the compound was not well soluble in these two solvent systems. The constituents, and ratios thereof, of BAW make the mobile phase more polar than CEF and EMW (results not shown) and the better migration of the antioxidant compound in BAW was due to this polarity.

### Antibacterial Activity Assays

The antibacterial activity of *C. edulis* has previously been evaluated by van der Watt and Pretorius (2001) using the agar plate diffusion assay. This method only gives a qualitative indication of the antibacterial activity of compounds. It does not give the optimum concentration of the compound(s) that inhibits bacterial growth. The microdilution assay employed in this study measures the lowest concentration of the compound(s) that inhibits microbial growth, i.e., minimum inhibitory concentration (MIC). This is a quantitative assay and it is said to be rapid and much more sensitive than many other previously developed antibacterial assays (Eloff, 1998a). The spring filtrate fractions were more active against all test organisms, demonstrating MIC values that were lower than all leaf debris fractions (Table 1). The phyto-



**Figure 2.** Chromatograms of spring-collected (A - C) and autumn-collected (D - F) *C. edulis* extract developed in CEF (A and D), EMW (B and E) and BAW (C and F). The chromatograms were visualized by spraying with vanillin-sulphuric acid reagent [HEX: hexane, DCM: dichloromethane, EA: ethyl acetate, AC: acetone, MET: methanol, (f): filtrate, (d): leaf debris]. More vanillin-reacting compounds were observed in the spring leaf debris samples.

chemicals that elicit strong antibacterial properties in *C. edulis* leaves could be abundant in the aqueous part of the leaves and form only a minor portion of the tissue of the leaves during spring. The hexane fraction did not show strong bacterial inhibition. Table 1 also indicates that dichloromethane was the most active extract for both seasons with average MIC values for the four pathogens at 0.31 mg/ml (Spring filtrate), 0.45 mg/ml (Spring leaf debris) and 0.65 mg/ml (autumn leaf debris). Phytochemicals with antibacterial properties in *C. edulis* were expected to be extracted by DCM, ethyl acetate, acetone

and methanol and not by hexane, as it is a non-polar solvent. Hexane extracts compounds that are non-polar, e.g. lipids, which are not active against bacteria (Eloff, 1998b). The spring extracts were more potent than the autumn extracts as they had lower average MIC values (Tables 1a and 1b).

When taking the total activity of each extract into account (Tables 2a and 2b), the autumn extracts had higher efficacy than the spring sample, having higher average total activities. A high total activity means that less plant material is required to elicit antibacterial activity

**Table 1a.** Minimum inhibitory concentrations of *C. edulis* spring extracts against test bacterial cultures. The MIC was recorded as the highest concentration at which there was bacterial growth inhibition.

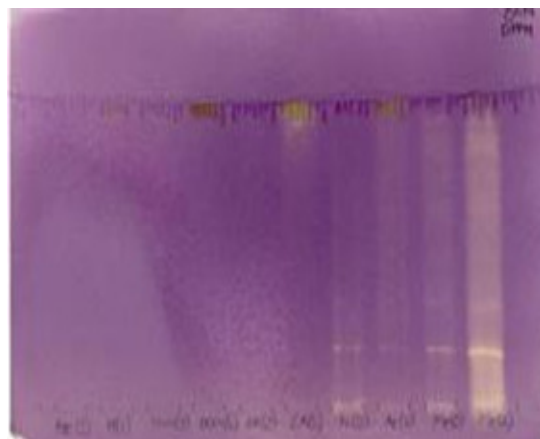
| Microorganism        | MIC value (mg/ml) |      |      |      |      |                      |      |      |      |      | Average |
|----------------------|-------------------|------|------|------|------|----------------------|------|------|------|------|---------|
|                      | Filtrate (Spring) |      |      |      |      | Leaf debris (Spring) |      |      |      |      |         |
|                      | Hex               | DCM  | EA   | Ac   | Met  | Hex                  | DCM  | EA   | Ac   | Met  |         |
| <i>E. coli</i>       | 0.52              | 0.31 | 0.31 | 0.31 | 0.31 | 0.84                 | 0.31 | 0.52 | 0.42 | 0.84 | 0.47    |
| <i>E. faecalis</i>   | 0.52              | 0.26 | 0.31 | 0.42 | 0.63 | 2.08                 | 0.42 | 0.84 | 0.63 | 0.63 | 0.67    |
| <i>P. aeruginosa</i> | 0.52              | 0.52 | 0.63 | 0.63 | 0.63 | 2.5                  | 0.84 | 0.63 | 0.63 | 2.5  | 1.00    |
| <i>S. aureus</i>     | 0.31              | 0.16 | 0.21 | 0.32 | 0.16 | 0.63                 | 0.21 | 0.26 | 0.21 | 2.5  | 0.50    |
| Average              | 0.47              | 0.31 | 0.37 | 0.42 | 0.43 | 1.51                 | 0.45 | 0.56 | 0.47 | 1.62 |         |

Hex: hexane, DCM: dichloromethane, EA: ethyl acetate, Ac: Acetone, Met: methanol

**Table 1b.** Minimum inhibitory concentrations of *C. edulis* autumn leaf debris extracts against test bacterial cultures. The MIC was recorded as the highest concentration at which there was bacterial growth inhibition.

| Microorganism        | MIC value (mg/ml) |      |      |      |      | Average |
|----------------------|-------------------|------|------|------|------|---------|
|                      | Hex               | DCM  | EA   | Ac   | Met  |         |
| <i>E. coli</i>       | 1.67              | 0.52 | 2.5  | 0.31 | 0.63 | 1.13    |
| <i>E. faecalis</i>   | 2.5               | 0.16 | 0.13 | 0.26 | 2.08 | 1.03    |
| <i>P. aeruginosa</i> | 2.08              | 1.67 | 1.67 | 2.5  | 2.5  | 2.08    |
| <i>S. aureus</i>     | 1.25              | 0.26 | 0.21 | 0.16 | 2.5  | 0.88    |
| Average              | 1.88              | 0.65 | 1.13 | 0.81 | 1.93 |         |

Hex: hexane, DCM: dichloromethane, EA: ethyl acetate, Ac: Acetone, Met: methanol.



HEX(f) (d) DCM(f) (d) EA(f) (d) AC(f) (d) MET(f) (d)

**Figure 3.** Chromatogram of autumn-collected leaf extracts from *C. edulis* developed in BAW. The chromatogram was visualized by spraying with 0.2% 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) in absolute methanol. Yellow zones indicate compounds with antioxidant activity. [HEX: hexane, DCM: dichloromethane, EA: ethyl acetate, AC: acetone, MET: methanol, (f): filtrate, (d): leaf debris].

because it is a reflection of the volume to which 1 g of the active compound(s) can be diluted and still inhibit bacterial growth (Eloff, 2004). Therefore, the autumn ex-

tracts elicit the desired activity even when diluted to a high volume. Considering the total activity, the methanolic extract was the most active fraction. However, this extract does not hold any medical value as methanol extracts primary metabolites, which are not active, along with the phytochemicals.

There were no individual compounds present in the spring filtrate extracts that had inhibitory effects on any of the test organisms (Figures 4 and 5). This lack of activity suggests that the antibacterial compounds act synergistically and could therefore not elicit any inhibition as individual bands on the bioautogram. The leaf debris extracts of both seasons exhibited an almost equal distribution of antibacterial compounds for each individual extract, except for methanol. The methanolic extracts did not show any bands that inhibited the growth of the test organisms, although these extracts had the highest total activity values (Tables 2a and 2b). The high total activity was a result of the large quantity of material extracted by the solvent. Thus, the methanol extracts had high efficacy (total activity) despite the low potency (average MIC values).

*S. aureus* was the most sensitive bacterium to the extracts. It is a gram-positive microorganism and thus has a cell wall composed mostly of peptidoglycan with no protective outer membrane. This allowed easy penetration of the toxic phytochemicals into the cells. Gram-negative organisms (e.g., *E. coli*) have less peptidoglycan

**Table 2a.** Total activity of *C. edulis* Spring extracts against test bacterial cultures.

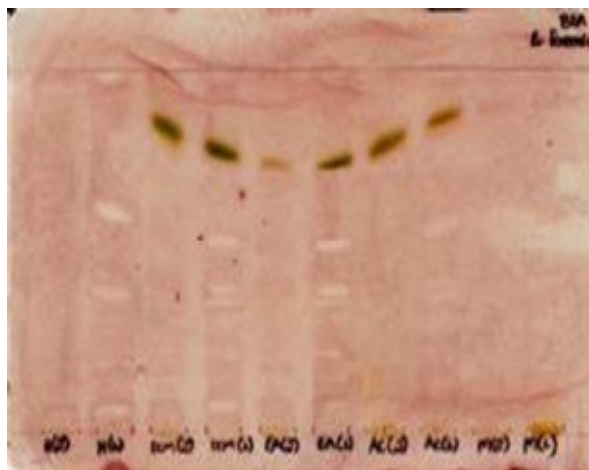
| Microorganism        | Total activity (ml) |     |    |    |     |                      |     |    |    |     | Average |
|----------------------|---------------------|-----|----|----|-----|----------------------|-----|----|----|-----|---------|
|                      | Filtrate (Spring)   |     |    |    |     | Leaf debris (Spring) |     |    |    |     |         |
|                      | Hex                 | DCM | EA | Ac | Met | Hex                  | DCM | EA | Ac | Met |         |
| <i>E. coli</i>       | 6                   | 16  | 13 | 31 | 373 | 2                    | 18  | 10 | 11 | 52  | 53      |
| <i>E. faecalis</i>   | 6                   | 19  | 13 | 23 | 183 | 1                    | 13  | 6  | 7  | 70  | 28      |
| <i>P. aeruginosa</i> | 6                   | 10  | 6  | 15 | 183 | 1                    | 7   | 8  | 7  | 18  | 26      |
| <i>S. aureus</i>     | 10                  | 31  | 19 | 30 | 722 | 3                    | 26  | 19 | 21 | 18  | 90      |
| Average              | 7                   | 19  | 13 | 25 | 365 | 2                    | 16  | 11 | 12 | 28  |         |

Hex: hexane, DCM: dichloromethane, EA: ethyl acetate, Ac: Acetone, Met: methanol.

**Table 2b.** Total activity of *C. edulis* autumn leaf debris extracts against test bacterial cultures.

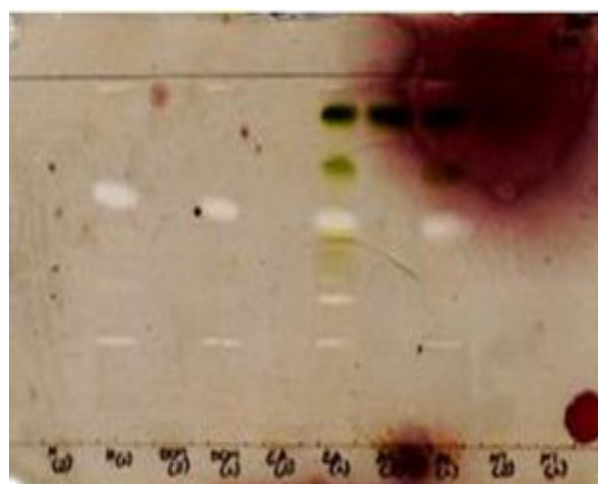
| Microorganism        | Total activity (mg/ml) |     |    |    |     | Average |
|----------------------|------------------------|-----|----|----|-----|---------|
|                      | Hex                    | DCM | EA | Ac | Met |         |
| <i>E. coli</i>       | 5                      | 20  | 4  | 32 | 271 | 66      |
| <i>E. faecalis</i>   | 3                      | 66  | 81 | 39 | 82  | 54      |
| <i>P. aeruginosa</i> | 4                      | 6   | 6  | 4  | 68  | 18      |
| <i>S. aureus</i>     | 6                      | 40  | 50 | 63 | 68  | 45      |
| Average              | 5                      | 33  | 35 | 35 | 122 |         |

Hex: hexane, DCM: dichloromethane, EA: ethyl acetate, Ac: Acetone, Met: methanol.



HEX(f) (d) DCM(f) (d) EA(f) (d) AC(f) (d) MET(f) (d)

**Figure 4.** Bioautogram of spring-collected leaf extracts from *C. edulis*. The TLC plate was developed with BEA and sprayed with *E. faecalis*. After overnight incubation at 37°C the plate was sprayed with INT. Clear zones on the plate indicate bacterial growth inhibition. [HEX: hexane, DCM: dichloromethane, EA: ethyl acetate, AC: acetone, MET: methanol, (f): filtrate, (d): leaf debris].



HEX(f) (d) DCM(f) (d) EA(f) (d) AC(f) (d) MET(f) (d)

**Figure 5.** Bioautogram of Spring-collected leaf extracts from *C. edulis*. The TLC plate was developed with BEA and sprayed with *E. coli*. After overnight incubation at 37°C the plate was sprayed with INT. Clear zones on the plate indicate bacterial growth inhibition. [HEX: hexane, DCM: dichloromethane, EA: ethyl acetate, AC: acetone, MET: methanol, (f): filtrate, (d): leaf debris].

in their cell walls but they contain an outer membrane composed of lipopolysaccharides, lipoproteins and other complex molecules that make their cell walls less penetrable than gram-positive organisms (Dmitriev et al.,

2005). *P. aeruginosa* was the most resistant microorganism to all the extracts from both seasons. It is also a gram-negative bacterium and according to Lagamayo





HEX(f) (d) DCM(f) (d) EA(f) (d) AC(f) (d) MET(f) (d)

**Figure 6.** Bioautogram of leaf debris extracts from *C. edulis*. The TLC plate was developed with BEA and sprayed with *P. aeruginosa*. After overnight incubation at 37°C the plate was sprayed with INT. Clear zones on the plate indicate bacterial growth inhibition. [HEX: hexane, DCM: dichloromethane, EA: ethyl acetate, AC: acetone, MET: methanol, (s): spring, (a): autumn].

(2008), some strains of *P. aeruginosa* are resistant to many of the antimicrobial drugs that they are subjected to. Despite this resistance, *P. aeruginosa* was susceptible to one antibacterial compound that was present in the leaf tissue extracts (Figure 6). Although the compound displayed moderate activity against *P. aeruginosa*, it does warrant further exploration as a potential candidate for designing a novel antibacterial drug against this resistant bacterium.

## Conclusion

*C. edulis* is used by traditional healers throughout South Africa for the treatment of a variety of ailments. This study has confirmed its use by a method that is more sensitive and rapid than other antibacterial activity assays that have been used previously. The activity of the plant was high enough to consider its use for clinical applications and to isolate and characterize more antibacterial compounds from the extracts. The spring harvest showed better antibacterial activity than the autumn harvest and further work would best be done using the leaves harvested in spring. When using the spring leaf sap, it must be used as crude extract as it has low MIC values; none of the phytochemicals elicited antibacterial activity individually, thus suggesting synergistic action. Pure active compounds can be isolated from the spring leaf tissue as this sample also had low MIC values and there were compounds that displayed the ability to inhibit bacterial growth, individually. Indeed, the work has also validated the assertion that the actual con-

tent of the bioactive molecules from plants may vary with both the locality and the season in which they are collected. Further studies aimed at isolating and characterizing the bioactive ingredients of the active fractions are currently underway.

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