

**Effect of an alkaloidal fraction of *Tabernaemontana elegans* (Stapf.)  
on selected micro-organisms**

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## **Abstract**

*Ethnopharmacological relevance:* Bacterial infections remain a significant threat to human health. Due to the emergence of widespread antibiotic resistance, development of novel antibiotics is required in order to ensure that effective treatment remains available. There are several reports on the ethnomedical use of *Tabernaemontana elegans* pertaining to antibacterial activity.

*Aim of the study:* The aim of this study was to isolate and identify the fraction responsible for the antimicrobial activity in *T. elegans* (Stapf.) root extracts.

*Materials and methods:* The active fraction was characterised by thin layer chromatography (TLC) and gas chromatography – mass spectrometry (GC-MS). Antibacterial activity was determined using the broth micro-dilution assay and antimycobacterial activity using the BACTEC radiometric assay. Cytotoxicity of the crude extract and fractions was assessed against primary cell cultures; lymphocytes and fibroblasts; as well as a hepatocarcinoma (HepG2) and macrophage (THP-1) cell line using the Neutral Red uptake and MTT assays.

*Results:* The crude root extracts were found to contain a high concentration of alkaloids (1.2% w/w). GC-MS analysis identified the indole alkaloids, voacangine and dregamine, as major components. Antibacterial activity was limited to the Gram-positive bacteria and *Mycobacterium* species, with MIC values in the range of 64 – 256 µg/ml. When combined with antibiotics, additive antibacterial effects were observed. Marked cytotoxicity to all cell lines tested was evident in the MTT and Neutral Red uptake assays, with IC<sub>50</sub> values < 9.81 µg/ml.

*Conclusions:* This study confirms the antibacterial activity of *T. elegans* and supports its potential for being investigated further for the development of a novel antibacterial compound.

**Keywords:**

Antibacterial; indole alkaloids; methicillin-resistant *Staphylococcus aureus*; mycobacteria; respiratory; *Tabernaemontana elegans*; Tuberculosis

**List of Abbreviations**

|          |  |
|----------|--|
| AF       | alkaloidal fraction                                  |
| CFU      | colony forming units                                 |
| cps      | counts per second                                    |
| FIC      | fractional inhibitory concentration                  |
| GC-MS    | gas chromatography – mass spectrometry               |
| LLE      | liquid-liquid extraction                             |
| MRSA     | methicillin-resistant <i>Staphylococcus aureus</i>   |
| MBC      | minimum bactericidal concentration                   |
| MIC      | minimum inhibitory concentration                     |
| M/XDR-TB | multi/extensively drug-resistant tuberculosis        |
| NIST     | National Institute of Standards and Technology       |
| PHA      | phytohaematoglutinin                                 |
| PMA      | phorbol 12-myristate 13-acetate                      |
| TB       | tuberculosis   |
| TLC      | thin layer chromatography                            |
| UV       | ultraviolet  |
| VISA     | vancomycin-intermediate <i>Staphylococcus aureus</i> |
| VRSA     | vancomycin-resistant <i>Staphylococcus aureus</i>    |

## 1. Introduction

A crisis of antibiotic resistance is looming globally. Resistance mechanisms have been identified for all classes of antibiotics available (Gold and Moellering, 1996) and in certain bacterial strains, multiple resistance mechanisms have been acquired (Hawkey and Jones, 2009). Infections caused by such strains are difficult and more costly to treat, and are associated with higher incidences of mortality and morbidity, clearly demonstrating the clinical impact of antibiotic resistance (Sefton, 2002).

Tuberculosis (TB) is a bacterial infection where new antibacterial drugs are desperately needed. Infection with *Mycobacterium tuberculosis* occurs every second (World Health Organisation, 2010a), and in 2009, it was estimated that there were 11 million cases of TB globally, resulting in 1.3 million deaths annually. Cases of multidrug resistant tuberculosis (MDR-TB), defined as *M. tuberculosis* infections that are resistant to treatment with isoniazid and rifampicin, are rising, with approximately half a million cases reported in 2008 (World Health Organisation, 2010b). The treatment of MDR-TB requires the use of second-line anti-tuberculosis agents, which are 10 times more expensive due to the longer duration of treatment required (Dorman and Chaisson, 2007), and is associated with greater toxicity (Johnson et al., 2006). The implications of the multi/extensively drug-resistant tuberculosis (M/XDR-TB) for public health are not fully known, but as the treatment of TB intensifies globally, the proportion of *M. tuberculosis* strains that are drug-resistant is bound to rise. This is especially true in high-burden, low-resource settings, where the majority of M/XDR-TB cases are found.

It has been over 60 years since the first strains of methicillin-resistant *Staphylococcus aureus* (MRSA) were identified. Previously, the strains of MRSA were overwhelmingly associated with nosocomial settings (Salgado et al., 2003), however, the rapid evolution of MRSA has garnered it the ability to cause community-acquired infections in previously-healthy young adults and children. In case studies of patients with invasive (i.e. non skin and soft-tissue) community-acquired MRSA infections, mortality rates have been found to be as high as 35% (Hageman et al., 2006). Vancomycin remains the agent of choice in serious MRSA infections (Gemmell et al., 2006). This reliance on vancomycin for the treatment of serious MRSA infections is not a sustainable situation, as vancomycin-intermediate and vancomycin-resistant *Staphylococcus aureus* (VISA and VRSA, respectively) strains have been reported in the literature (Nannini et al., 2010). These infections, without the development of new antibiotics, will represent a growing source of mortality in the years to come.

*Tabernaemontana elegans* (Stapf.) (syn. *Conopharyngia elegans* (Stapf.)), a member of the *Apocynaceae* family, is a small tree found in evergreen river fringes at low altitudes and in coastal scrub forest (Coats Palgrave et al., 2003). It is known in English as the toad tree due to the brown, wart-like skin of its fruit. There are several reports of the ethnomedical use of *T. elegans* pertaining to antibacterial activity: a root decoction is applied as a wash to wounds, and drunk for pulmonary diseases and chest pains by the VhaVenda (Arnold and Gulumian, 1984) and Zulu (Watt and Breyer-Brandwijk, 1962) people of South Africa. Other ethnomedical usages include treatment of heart diseases with the seeds, stem-bark and roots, the root-bark and

fruits for cancer treatment, and a root decoction is said to have aphrodisiac properties (Neuwinger, 1966).

*T. elegans* has previously been identified as having antibacterial activity against *S. aureus* and antimycobacterial activity against *M. smegmatis* (Pallant and Steenkamp, 2008), as well as anti-fungal activity against *Candida albicans* (Steenkamp et al., 2007). An earlier study identified *T. elegans* as one of eight *Tabernaemontana* species possessing antibacterial activity against Gram-positive bacteria (Van Beek et al., 1984). In these studies, the compound(s) responsible for the reported biological activity was not identified.

Previous phytochemical research has shown *T. elegans* to be particularly rich in monoterpenoidindole alkaloids (Gabetta et al., 1975; Bombardelli et al., 1976; Danieli et al., 1980; Van der Heijden et al., 1986). These alkaloids, which are structurally diverse, are commonly found in many members of the genus and are considered chemotaxonomically important (Zhu et al., 1990). To date, over 300 of these alkaloids have been isolated (Cardoso et al., 1998).

The biological activity of *T. elegans*, especially in terms of antibacterial activity, which has thus far been reported for crude extracts, remains less thoroughly investigated. Due to the clear need for new agents which may be used for the treatment of tuberculosis and other bacterial infections, this study was aimed at isolating the active fraction responsible for the antibacterial activity of this plant.

## **2. Materials and methods**

### 2.1 Plant material and extraction procedures

Roots of *Tabernaemontana elegans* (Stapf.) were collected from the Venda region of Limpopo, South Africa. The plant material was authenticated and a voucher specimen (NH 1920) deposited at the Soutpanbergensis Herbarium (Makhado, Limpopo). Powdered root was extracted by maceration in ethanol at a ratio of 100 g plant material to 1 l solvent. The flask was placed in an ultrasonic bath for 1 h, then left to stand overnight at ambient temperature. The extract was centrifuged (1000g, 5 min) and vacuum-filtered through 0.44 µm filters. The plant material was macerated twice under similar conditions and the three extracts combined.

#### 2.1.1 Crude extract

The ethanol maceration was concentrated to dryness with a rotary evaporator at 40°C under reduced pressure. Liquid-liquid extraction (LLE) was performed using distilled water and hexane. The aqueous fraction was lyophilized and stored at 4°C in a desiccator until use.

#### 2.1.2 Alkaloidal fraction

An alkaloidal fraction (AF) was obtained by an acid-base extraction method. An aliquot of the ethanol maceration was partitioned between 2% acetic acid and hexane, with the hexane fraction being discarded. The aqueous layer was adjusted

to pH 10 with ammonia in order to precipitate the alkaloids present. Using LLE, the precipitated alkaloids were collected into chloroform and dried using sodium sulphate, (1.2% dry weight/weight). The AF was dried at 40°C and reconstituted in ethanol to a concentration of 100 mg/ml and stored at -20°C until use.

## 2.2 Chemical characterisation

### 2.2.1 Phytochemical screening of the crude extract

Compounds in the crude extract were separated on silica TLC plates (10 x 10 cm) using a mobile phase of 17:2:1 ethyl acetate:2-propanol:ammonia. Developed TLC plates were assessed for the presence of various phytochemical groups, by utilization of visible and ultraviolet (UV) light, in addition to various spray reagents (Stahl, 1969; Harborne, 1998) which were selected to visualise specific classes of compounds (alkaloids, coumarins, essential oils, flavonoids, phenols and sterols/steroids).

### 2.2.2 Gas chromatography mass spectrometry (GC-MS) analysis of the alkaloidal fraction

GC-MS analysis of the crude extract and alkaloidal fraction was performed using an Agilent 7890 GC-MS equipped with a DB5-MS column (30m x 0.32 µm i.d., film thickness 0.25 mm, J&W Scientific). Helium was used as the carrier gas at a



constant flow, and the injection split ratio was 50:1. The injector temperature was 280°C. Column temperature was programmed to rise from 80°C to 310°C at 10°C/min for 23 min, then maintained at 310°C for 7 min (Total run time 30 min).

The acquisition mode selected for mass spectrometry was scan mode, with a scan range of 35 - 550 Da and a threshold of 100 counts per second (cps). Solvent delay was 4 min. Quadrupole temperature was 150°C, with the transfer line temperature set to 280°C. GC and mass spectrometry parameters, data collection and analysis were performed by Agilent Chemstation. Identity of the compounds was determined using the National Institute of Standards and Technology (NIST) library.

## 2.3 Anti-microbial assays

### 2.3.1 Micro-organisms

The crude extract and alkaloidal fraction were tested for antibacterial activity against the Gram-positive bacteria *Bacillus subtilis* (ATCC 6633), *Enterococcus faecalis* (ATCC 29212) and *Staphylococcus aureus* (ATCC 12600), the mycobacteria *M. tuberculosis* H<sub>37</sub>R<sub>V</sub> (ATCC 25177) and *M. smegmatis* (ATCC 14468), and the Gram-negative bacteria *Escherichia coli* (ATCC 35218), *Klebsiella pneumoniae* (ATCC 13883) and *Pseudomonas aeruginosa* (ATCC 9027). Samples were also tested against a clinical isolate of methicillin-resistant *S. aureus* (NHLS 363), obtained from the National Health Laboratory Service, Pretoria, and a clinical isolate of *M. tuberculosis* displaying resistance to isoniazid (MRC 3366), obtained from the Tuberculosis Unit, Medical Research Council, Pretoria.

Microbial cultures were maintained on Lowenstein Jensen medium (*M. tuberculosis*), Mannitol Salt Agar (*S. aureus*), Mueller-Hinton agar supplemented with 5% sheep blood (*E. faecalis*, *P. aeruginosa*) and Nutrient agar (*B. subtilis*, *E. coli*, *K. pneumoniae*, *M. smegmatis*).

### 2.3.2 Bacterial inocula

Bacterial inocula for all micro-organisms apart from *M. tuberculosis* were prepared by transferring colonies from 24 h, freshly prepared subcultures (72 h subcultures in the case of *M. smegmatis*) to an aliquot of Mueller Hinton broth. Inocula were diluted with additional broth to a 0.5 McFarland turbidity standard, and then further diluted with liquid media to obtain a concentration of  $5 \times 10^5$  colony forming units (CFU/mL).

Inocula of *M. tuberculosis* were prepared by transferring colonies from a culture to test tube containing sterile glass beads and diluting fluid (0.1% Tween 20). A homogeneous suspension was obtained by vortex mixing. After the large particles had settled, the supernatant was transferred to a test tube and adjusted to a 1 McFarland turbidity standard. An inoculum (100 $\mu$ l) was added to a BACTEC vial, incubated at 37°C, with Growth Index (GI) readings taken daily. Once the GI reached 400 -500, contamination of the inoculum was assessed by overnight growth on blood agar. If free of contamination, the inocula were used on the BACTEC assay described below.

### 3.3.3 Broth micro-dilution assay

Determination of the minimum inhibitory concentration (MIC) for the all the bacterial species, apart from the strains of *M. tuberculosis*, was performed using the broth micro-dilution assay (National Committee for Clinical Laboratory Standards, 2008).

Stock solutions of the test samples and antibiotic standards were prepared in distilled water. The concentrations of the stock solutions were 4.096 mg/ml for the crude extract, 2.048 mg/ml for the alkaloidal and basic fraction, 32 µg/ml for ampicillin and 2 µg/ml for ciprofloxacin. In order to increase the solubility of the alkaloidal fraction in distilled water, the stock solution was acidified to  $\approx$  pH 6.5 with acetic acid. All stock solutions were kept at -20°C until use.

Ampicillin and ciprofloxacin were serially diluted in the media and served as the positive control for the Gram-positive bacteria and Gram-negative bacteria and *M. smegmatis*, respectively. Wells containing 50 µl of media without plant extracts or antibiotics were used as growth controls. Wells containing 100 µl of medium served as the sterility control. Inocula (50 µl) was added to the all wells except the sterility controls to give a final volume of 100 µl before incubating at 37°C in an ambient atmosphere for 24 h (72 h in the case of *M. smegmatis*). The MIC was defined as the lowest concentration of the sample that was able to visibly inhibit the growth of the bacteria.

From all wells that demonstrated no apparent bacterial growth, the entire well contents (100 µl) were streaked in a straight line on a Mueller Hinton agar plate. After allowing the broth to dry, which prevented antibiotic carry-over, the plate was streaked to form a lawn of bacterial growth and incubated for 24 h at 37°C (72 h in the case of *M. smegmatis*). A 100-fold dilution of the starting inoculum was used to determine the starting colony count (Anhalt et al., 1980). The minimum bactericidal concentration (MBC) was defined as the concentration of a compound or extract that resulted in >99.9% killing of bacterium relative to the concentration of the bacterium present in the inoculum (National Committee for Clinical Laboratory Standards, 2008). Each assay was performed in triplicate and on at least three different occasions.

#### 3.3.4 BACTEC radiometric assay

The BACTEC radiometric broth dilution assay was used to determine the MIC of the samples for the two strains of *M. tuberculosis* (Siddiqi et al., 1981). Starting concentrations were 1.052 mg/ml, 512 µg/ml and 0.1 µg/ml for the crude extract, alkaloidal fraction and isoniazid, respectively. Three vials were used as controls, two containing only medium and the vehicle, representing solvent controls, while the third contained only medium and was the growth control. From the BACTEC vial containing the inoculum of *M. tuberculosis*, 100 µl was transferred to the vials containing the samples and the two solvent control vials. In the growth control vial, a 1:100 dilution of the inoculum was added, representing  $1 \times 10^3 - 1 \times 10^2$  CFU/ml, or 1% of the initial mycobacterial population.

Inoculated vials were incubated at 37°C, with each vial assayed at 24 h intervals until the growth control vial reached a GI of greater than 30. The following day, final readings were taken and the  $\Delta$ GI determined for all samples. The MIC was defined as the lowest concentration of a sample for which the  $\Delta$ GI was less than the  $\Delta$ GI of the growth control. If the GI of the sample was greater than 100, *M. tuberculosis* was deemed to be resistant to the sample, even if the  $\Delta$ GI was less than that of the growth control.

#### 2.4 Synergistic antimicrobial activity assays

Assessment of the synergistic activity between the alkaloidal fraction and the antibiotics utilized as controls for the broth micro-dilution assay was performed using a chequerboard synergy testing protocol (Bonapace et al., 2000). This was performed for all susceptible bacterial strains, however, the methodology was adapted for used in the BACTEC assay for *M. tuberculosis*.

A dilution series of the alkaloidal fraction and of the antibiotics was made up in Mueller Hinton broth, with the concentration range representing 1/8 x MIC – 1 x MIC for the sample against the specific bacteria, as previously determined in the broth micro-dilution assay.

In a four-by-four grid on a 96 well microtitre plate, 25 µl of the dilution series of the alkaloidal fraction was added along the x-axis of the grid, while 25 µl of the antibiotic dilution series was added to the y-axis of the grid. Wells containing 50 µl of sample-free medium served as growth controls and wells containing 100 µl of medium served as sterility controls. Each well containing 50 µl of the alkaloidal fraction/antibiotic mixture was inoculated with 50 µl of the bacterial inocula. The microtitre plates were incubated at 37°C in an ambient atmosphere for 24 h (72 h in the case of *M. smegmatis*).

For *M. tuberculosis*, BACTEC vials were arranged in a four-by-four grid, and 50 µl of the alkaloidal fraction was added along the x-axis of the grid, while 50 µl of the antibiotic dilution series was added to the y-axis of the grid. The rest of the BACTEC assay was performed as described above.

The mean fractional inhibitory concentration (FIC) was used to interpret the results of the chequerboard synergy assay. The FIC was defined as:

$$\text{FIC} = \frac{\text{MIC of drug A in combination}}{\text{MIC of drug A alone}} + \frac{\text{MIC of drug B in combination}}{\text{MIC of drug B alone}}$$

The FIC was calculated for each sample indicating no growth along the growth/inhibition interface of the grid. Synergism between the samples was defined as a mean FIC of  $\leq 0.5$ , additivity as a value of  $> 0.5 - 4$  and antagonism as a value

of > 4 (Bonapace et al., 2000). Each four-by-four grid was repeated in triplicate on at least three different occasions.

## 2.5 Cytotoxicity assays

Stock solutions of the test reagents were prepared by re-constitution in distilled water. The concentrations of the stock solutions were 410 µg/ml for the crude extract and the basic aqueous fraction, 205 µg/ml for the alkaloidal fraction, and 1% (w/v) for saponin. In order to increase the solubility of the alkaloidal fraction in distilled water, the stock solution was acidified to pH 6.5 with acetic acid. All stock solutions were kept at -20°C until use.

### 2.5.2 Cell lines

Cytotoxicity of the crude ethanol extract and alkaloidal fraction was determined against normal human dermal fibroblasts (Southern Medical, SA), human lymphocytes, HepG2 hepatocytes (ATCC HB 8065) and THP1 macrophages (ATCC TIB 2.2).

Human lymphocytes were isolated from whole blood (Anderson et al., 1993) obtained after ethical approval (University of Pretoria) from healthy volunteers. The media and cell concentrations used in the assays were DMEM-F12 and  $5 \times 10^4$  cells/ml, RPMI-1640 and  $2 \times 10^6$  cells/ml, EMEM and  $5 \times 10^5$  cells/ml, THP-medium

and  $1 \times 10^5$  cells/ml for the normal human dermal fibroblasts, human lymphocytes, HepG2 and THP-1 cells, respectively. All media was supplemented with 10% foetal calf serum. To each well of a microtitre plate, 100  $\mu$ l of the cell suspension was added. For the fibroblasts and hepatocytes, an additional 80  $\mu$ l of FCS-supplemented medium was added to the microtitre plates which were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> to allow for cellular adhesion. Lymphocytes (100  $\mu$ l) were added to each well of a microtitre, together with 60  $\mu$ l of medium. Following 1 h incubation at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>, 20  $\mu$ l of a 5  $\mu$ g/ml solution of phytohaemagglutinin (PHA) in medium was added to selected microtitre plates to produce populations of stimulated lymphocytes, while other microtitre plates received a further 20  $\mu$ l of medium and represented resting lymphocytes. For the THP-1 cells, 100  $\mu$ l of the cell suspension and 80  $\mu$ l THP medium was plated into the wells of a 96-well microtitre plate. Plates were incubated for 1 h at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Differentiation of the THP-1 monocytes into macrophages was induced by the addition of 20  $\mu$ l of a 0.1  $\mu$ g/ml solution of phorbol 12-myristate 13-acetate (PMA) in THP medium, followed by a further 48 h incubation period.

### 2.5.3 Cytotoxicity assays

Two-fold serial dilutions of the crude extract (1.6 – 204.8  $\mu$ g/ml), alkaloidal fraction (0.1 – 12.8  $\mu$ g/ml) and aqueous fraction (3.2 – 410  $\mu$ g/ml) were prepared. Saponin (0.0008 – 0.1% w/v), served as the positive control. Each well containing cells received either 20  $\mu$ l of the sample or for the growth controls, 20  $\mu$ l of medium. The cells were incubated for 72 h at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> before



the cytotoxicity assays were conducted. The MTT assay was performed as described by Mosmann (1983), and the Neutral Red uptake assay as described by Borenfreund and Puerner (1986). All assays were conducted in triplicate, on three different days.

The concentration of sample that prevented the survival of 50% of the cell population ( $IC_{50}$ ) was determined by non-linear regression from optical density measurements. Sample optical density values were converted to a percentage of the average growth of control wells. Non-linear regression, using a sigmoidal dose-response curve model with a bottom constraint of 0, was performed using GraphPad Prism 4.0 software.

### **3. Results and discussion**

Phytochemical screening of the crude extract indicated the presence of: alkaloids, amines, essential oils, phenols and steroids/sterols. Phytochemical screening of other *Tabernaemontana* species support these findings. The presence of triterpenes/triterpenoids and steroids (Cardoso et al., 1998), flavonoids, phenylpropanoids and phenolic acids (Chattipakorn et al., 2007) and phytosterols (Mederios et al., 2001) has been reported.

GC-MS analysis of the crude extract and alkaloidal fraction indicated the presence of two major compounds that together accounted for more than 75% of all the detected

compounds. These compounds, identified with a greater than 90% certainty by comparison to the NIST database, were the indole alkaloids, dregamine [20 $\alpha$ , 19,20-dihydro-3-oxoVobasan-17-oic acid methyl ester] and voacangine (12-methoxyibogamine-18-carboxylic acid methyl ester) (Fig. 1). Dregamine has been reported to be present in other species of *Tabernaemontana*, and has previously been isolated from *T. elegans* (Gabetta et al., 1975; Van der Heijden et al., 1986). The presence of voacangine in various *Tabernaemontana* species is also known (Kingston, 1978).

The MICs and MBCs obtained in the broth micro-dilution assay for the crude ethanolic extract and alkaloidal fraction of *T. elegans*, as well as the antibiotic controls, are reported in Table 1. The antimicrobial activity of the crude ethanolic extract was limited to Gram-positive bacteria and the mycobacteria. *B. subtilis* demonstrated the highest susceptibility to the antimicrobial-effects of the crude extract.

These results are supported by reports of antibacterial properties for the ethanolic extract of *T. elegans*, against *B. subtilis* and *S. aureus* (Van Beek et al., 1984). This study indicated antimycobacterial activity against *M. smegmatis* and *M. tuberculosis*, confirming a previous report of antimycobacterial activity against *M. smegmatis* (Pallant and Steenkamp, 2008).

The alkaloidal fraction demonstrated antibacterial activity against the same organisms as that of the crude extract, but at a significantly lower concentration (Table 1). These results support the hypothesis that the alkaloids enriched in the alkaloid subfraction, are primarily responsible for the antimicrobial activity of *T. elegans*. The presence of drug resistance in the bacteria from clinical isolates tested in this study did not have an effect on the susceptibility of the bacteria to the crude extract or the alkaloidal fraction. This effect has been reported for another species of *Tabernaemontana* (Ruttoh et al., 2009).

The alkaloidal fraction of *T. catharinensis* has known activity against 3 species of mycobacteria, as well as Gram-positive and Gram-negative bacteria (Pereira et al., 2005). Isolated alkaloids from various *Tabernaemontana* species: conoduramine, conodurine, and voacamine, possess strong antibacterial activity against Gram-positive bacteria (Van Beek et al., 1984; 1985). The alkaloids ibogamine and voacangine have proven antimycobacterial activity (Rastogi et al., 1998). None of these compounds, however, have been previously identified in *T. elegans*, suggesting that the pharmacophore responsible for the antibacterial activity may be common to a number of alkaloids in this genus.

All MBC values of the crude extract and alkaloidal fraction were within two serial dilutions of the respective MICs obtained for the bacterium, indicating that the antibacterial alkaloids of *T. elegans* are bacteriocidal.

The combination of the alkaloidal fraction and either ampicillin for Gram-positive bacteria, ciprofloxacin for *M. smegmatis* or isoniazid for *M. tuberculosis*, reduced the required concentration of both agents to inhibit the growth of all bacteria assayed indicating additive activity (FIC 0.76-1.26).

The combination of 16 µg/ml (1/2 x MIC) of the alkaloidal fraction and 0.06 µg/ml ampicillin produced an inhibitory effect on growth of the methicillin-resistant strain of *S. aureus*. The controls for the experiment, which consisted of either the alkaloidal fraction or ampicillin at the above concentrations alone, did not produce the same effect. This effect was not seen, however, in isoniazid-resistant *M. tuberculosis*, where a mixture of 16 µg/ml of the alkaloidal fraction and 0.1 µg/ml of isoniazid failed to inhibit the growth of the mycobacteria. The results obtained in this study indicate that the alkaloids present in *T. elegans* possess additive activity when combined with ampicillin or isoniazid, and that these alkaloids may be able to reverse resistance acquired against ampicillin. Furthermore, this finding implicates that the mechanism(s) of action responsible for the antibacterial activity of the alkaloidal fraction differs from those employed by antibiotics used in the assay. No reports could be found in the literature of other studies assessing the synergistic antibacterial effects of extracts or alkaloids of the *Tabernaemontana* genus in combination with antibiotics.

The effects of the crude ethanolic extract and the alkaloidal fraction of the roots of *T. elegans* on cell viability for the cell lines and primary cultures are presented in Table 2. The IC<sub>50</sub> values for the crude ethanolic extract in both assays ranged from 1.93

$\mu\text{g/ml}$  –19.27  $\mu\text{g/ml}$  and showed dose-dependent response curves. Smaller  $\text{IC}_{50}$  values were obtained for the alkaloidal fraction, ranging from 1.11  $\mu\text{g/ml}$  –9.81  $\mu\text{g/ml}$ . The lower  $\text{IC}_{50}$  values obtained for the alkaloidal fraction, combined with the non-toxicity of the basic fraction, demonstrate that it is primarily the indole alkaloids of *T. elegans* that are responsible for the cytotoxicity observed in the crude extract.

Previous research on crude extracts of *Tabernaemontana* species has shown conflicting evidence of cytotoxicity. An aqueous extract of *T. elegans* demonstrated weak toxicity to human lymphocytes, with  $\text{IC}_{50}$  concentrations ranging between 90 - 160  $\mu\text{g/ml}$  (Pallant and Steenkamp, 2008). The earliest investigation of the cytotoxic effects of *Tabernaemontana* species showed that an aqueous methanolic extract of *T. arborea* possessed an  $\text{IC}_{50}$  of 8  $\mu\text{g/ml}$  against P-388 cells (murine lymphocytic leukaemia) (Kingston et al., 1978). Methanolic and dichloromethane root extracts of *T. divaricata* were found to be cytotoxic in COR L23 (human non-small cell lung cancer) and MCF7 (human breast adenocarcinoma) cell lines, however, the aqueous extract did not produce an effect even at the highest concentration tested ( $> 25 \mu\text{g/ml}$ ) (Lee and Houghton, 2005). Another study on *T. divaricata* confirmed these results (Thind et al., 2008). On the contrary, a methanol extract of *T. divaricata* had no effect on resting human mesangial cells (Kuo et al., 1999). An ethyl acetate extract of the stem bark of *T. laeta*, and leaf extracts of *T. amygdalifolia* exhibited no cytotoxicity ( $\text{IC}_{50} > 20 \mu\text{g/ml}$ ) in a KB human oral epidermoid carcinoma cell line (You et al, 1994; Ankli et al., 2002). A methanol extract of the leaves of *T. obliqua* was reported to have cytotoxic effects against promonocytic U937 cells, however, the  $\text{IC}_{50}$  value (231.6  $\mu\text{g/ml} \pm 2.7 \mu\text{g/ml}$ ) provided is substantially higher than what is deemed to be physiologically significant (Weniger et al., 2001).

Several possible explanations exist for the lack of cytotoxicity activity reported in some of the crude extracts. It may be related to factors influencing the concentration of the alkaloids in the plant, such as pre-cursor availability (Dagnino et al., 1995; Lucumi et al., 2001), microbial attack (Fulton et al., 1994), or other environmental stressors, and factors influencing the concentration of alkaloids in the crude extract, such as plant part as well as the method of extraction and solvent system selected for extraction (Pereira et al., 2004).

From the literature, more than 40 isolated *Tabernaemontana* alkaloids have been assayed for cytotoxicity (Pallant, 2010). The majority of these alkaloids have IC<sub>50</sub> values below 20 µg/ml, indicating significant cytotoxic effects regardless of the type of cell or the cytotoxicity assay employed. Regarding the two alkaloids identified in this study, cytotoxicity has been reported for voacangine (Kingston, 1978; Chaturvedula et al., 2003). Furthermore, two alkaloids previously isolated from *T. elegans*, conoduramine and isovoacangine, have also been associated with cytotoxic activity. The cytotoxicity of the alkaloidal fraction of *T. elegans* may potentially be ascribed to the presence of these alkaloids.

#### **4. Conclusions**

The crude extract and the alkaloidal subfraction possessed significant antibacterial activity (MIC ≤ 256 µg/ml) against the Gram-positive bacteria and mycobacteria, but did not influence the viability of the Gram-negative bacteria. This study leads support

to the ethnomedical usage of *T. elegans* for the treatment of respiratory tract infections, and may indicate a potential future source of antibacterial molecules.

## **5. Acknowledgements**

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## **Legends to Figure and Tables**

### **Figure**

Figure 1. GS-MS total ion chromatogram of the alkaloidal fraction.

### **Tables**

Table 1. MIC and MBC values obtained in the broth micro-dilution assay.

Table 2. IC<sub>50</sub> values obtained in the MTT and Neutral Red uptake cytotoxicity assays.

Figure 1 GS-MS total ion chromatogram of the alkaloidal fraction

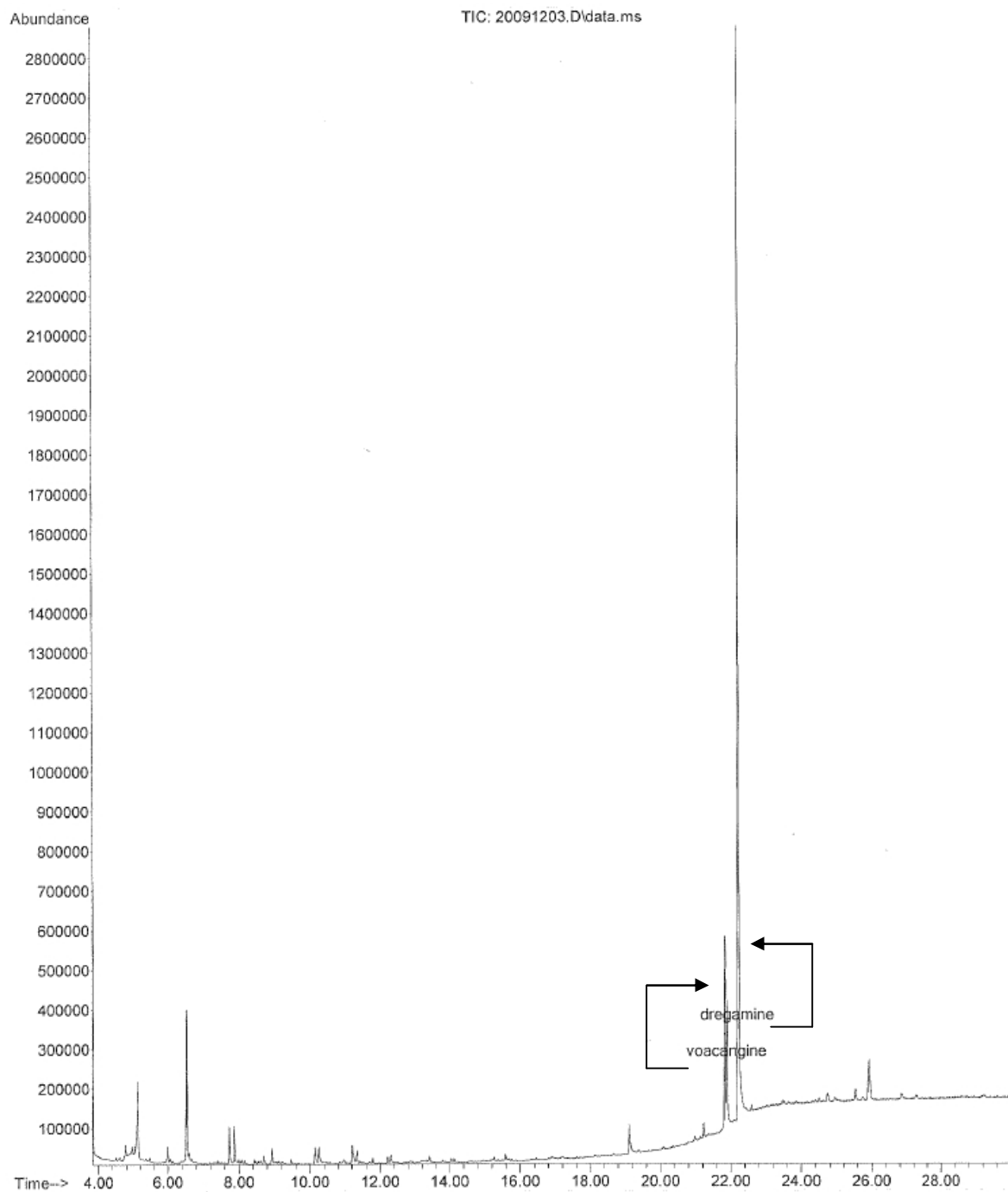


Table 1 MIC and MBC values obtained in the broth micro-dilution assay.

| Micro-organism                                 | Crude extract<br>(µg/ml) |            | Alkaloidal fraction<br>(µg/ml) |            | Antibiotic control<br>(µg/ml) <sup>a</sup> |            |
|--|--------------------------|------------|--------------------------------|------------|--|------------|
|  | <u>MIC</u>               | <u>MBC</u> | <u>MIC</u>                     | <u>MBC</u> | <u>MIC</u>                                 | <u>MBC</u> |
| <b>Gram-positive</b>                           |                          |            |                                |            |  |            |
| <i>B. subtilis</i> (ATCC 6633)                 | 64                       | 128        | 32                             | 64         | 0.06                                       | 0.13       |
| <i>E. faecalis</i> (ATCC 29212)                | 128                      | 512        | 64                             | 256        | 0.50                                       | 1.00       |
| <i>S. aureus</i> (ATCC 12600)                  | 128                      | 128        | 32                             | 64         | 0.06                                       | 0.06       |
| <i>S. aureus</i> (NHLS 363) <sup>b</sup>       | 128                      | 128        | 32                             | 64         | >2   | NA         |
| <b>Gram-negative</b>                           |                          |            |                                |            |  |            |
| <i>E. coli</i> (ATCC 35218)                    | >1024                    | NA         | 512                            | 512        | 0.13                                       | 0.13       |
| <i>K. pneumonia</i> (ATCC 13883)               | >1024                    | NA         | >512                           | NA         | 0.13                                       | 0.13       |
| <i>P. aeruginosa</i> (ATCC 9027)               | >1024                    | NA         | >512                           | NA         | 0.50                                       | 0.50       |
| <b>Mycobacteria</b>                            |                          |            |                                |            |  |            |
| <i>M. smegmatis</i> (ATCC 14468)               | 256                      | 1024       | 32                             | 128        | 0.50                                       | 0.50       |
| <i>M. tuberculosis</i> (ATCC 25177)            | 128                      | ND         | 32                             | ND         | 0.10                                       | ND         |
| <i>M. tuberculosis</i> (MRC 3366) <sup>c</sup> | 128                      | ND         | 32                             | ND         | N/D  | ND         |

<sup>a</sup>Antibiotic for Gram-positive bacteria – ampicillin; Antibiotic for Gram-negative bacteria- ciprofloxacin; Antibiotic for *M. smegmatis* – ciprofloxacin; Antibiotic for *M. tuberculosis* – isoniazid.

<sup>b</sup>Clinical isolate of *S. aureus*– resistant to ampicillin, erythromycin, clindamycin and oxacillin

<sup>c</sup>Clinical isolate of *M. tuberculosis* – resistant to isoniazid

NA: No activity

ND: Not determined

Table 2 IC<sub>50</sub> values obtained in the MTT and Neutral Red uptake cytotoxicity assays.

| Cell line                       | IC <sub>50</sub>              |                                     |                |                               |                                     |                |
|---------------------------------|-------------------------------|-------------------------------------|----------------|-------------------------------|-------------------------------------|----------------|
|                                 | MTT assay                     |                                     |                | Neutral red uptake assay      |                                     |                |
|                                 | Crude extract<br>(µg/ml ± SD) | Alkaloidal fraction<br>(µg/ml ± SD) | Saponin<br>(%) | Crude extract<br>(µg/ml ± SD) | Alkaloidal fraction<br>(µg/ml ± SD) | Saponin<br>(%) |
| <b>Primary cell cultures</b>    |                               |                                     |                |                               |                                     |                |
| Resting lymphocytes             | 4.52 ± 3.20                   | 1.75 ± 1.35                         | 0.006          | 19.27 ± 8.43                  | 4.35 ± 0.58                         | 0.004          |
| PHA-stimulated lymphocytes      | 11.77 ± 4.20                  | 1.67 ± 1.96                         | 0.006          | 9.05 ± 5.10                   | 2.30 ± 1.96                         | 0.005          |
| Normal human dermal fibroblasts | 10.91 ± 2.54                  | 5.91 ± 0.24                         | 0.018          | 13.38 ± 2.59                  | 9.81 ± 1.19                         | 0.015          |
| <b>Cell lines</b>               |                               |                                     |                |                               |                                     |                |
| HepG2 hepatocytes               | 5.81 ± 4.85                   | 1.11 ± 0.49                         | 0.005          | 1.93 ± 0.29                   | 1.27 ± 0.26                         | 0.003          |
| THP-1 macrophages               | 16.77 ± 9.56                  | 9.73 ± 4.10                         | 0.01           | 8.10 ± 2.92                   | 8.23 ± 0.88                         | 0.012          |