A proposal towards a rational classification of the antimicrobial activity of acetone tree leaf extracts in a search for new antimicrobials

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Dedicated to Professor Arnold Vlietinck on the occasion of his 80th birthday

Abstract

Many scientists investigate the potential of finding new antibiotics from plants leading to more than a thousand publications per year. Many different minimum inhibitory concentrations (MIC) of extracts have been proposed to decide if an extract has interesting activity that could lead to the discovery of a new antibiotic. To date no rational explanation has been given for the selection criteria different authors have used. The cumulative percentage of plant extracts with different activities from a large experiment determining the activity of 714 acetone tree leaf extracts of 537 different South African tree species against four nosocomial pathogenic bacteria and two yeasts was calculated using a widely accepted serial dilution microplate method with *p*-iodonitrotetrazolium violet as indicator of growth. All the extracts were active at a concentration of 2.5 mg/mL. The formula, % of active extracts = 439xMIC in mg/mL[1,5385] describes the results for MICs below 0.16 mg/mL with a correlation coefficient of 0.9998. A rational approach could be to determine the MICs of the most active 1%, 3%, 9%, 25%, 50% and > 50% of a large number of plant extracts investigated against these six important microbial pathogens. Starting with an extract concentration of 10 mg/ml, I propose the following classification based on MICs: outstanding activity < 0.02 mg/mL, excellent activity 0.021 - 0.04 mg/mL, very good activity 041 - 0.08 mg/mL, good activity 0.081 - 0.16 mg/mL, average activity 0.161 - 0.32 mg/mL and weak activity > 0.32 mg/mL. Higher MICs may still be effective in ethnopharmacological studies.

Keywords: antimicrobial; minimum inhibitory concentration; tree leaves; classification of activity

Introduction

The discovery of antibiotics is one of the major advances in human medicine. Before the application of antibiotics, infections were one of the major causes of death. Even a simple wound could become infected and fatal. After the successful application of antibiotics, the situation has changed drastically with many microbes developing resistance against antibiotics [1-3]. The development of resistance has a large effect on human health and also on the production of food using antibiotic feed additives. The development of resistance may be mainly caused by improper use of antibiotics leading to microbial mutations. With slow-growing organisms such as tuberculosis bacteria, requiring medication over a long period, patient non-compliance also leads to the development of resistance against even last-line antibiotics.

There has been a decrease in the development of new antibiotics by the pharmaceutical industry possibly by the dearth of new scaffold molecules. Most new antibiotics are based on modifications of an existing scaffold molecule. The last widely-used antifungal scaffold molecule, amphotericin B was discovered in 1956 [4]. It has also been stated that pharmaceutical companies are more interested in developing medicines for chronic use, because it is financially more rewarding to deliver a product that will be used for a very long time than a product that will heal the patient within a week or two [5].

It is surprising that in the order of 25% of prescription medicine in the United States, especially those used to treat cancer or malaria, are based on plant-derived products [6] yet no plant-based antibiotic has been commercialized to date. Several authors have indicated the potential of developing antibiotics from plants because plants contain such a tremendous number of different compounds [7-9]. Of the 868 new chemical entities approved between 1981 and 2002, 52% were natural or created around natural product structures [2]. This lack of discovering new antimicrobial compounds from plants is not because scientists have not been investigating plants for antimicrobial activity. There have been more than 1700 publications per year in this field between 2005 and 2015 [10]. This includes a number of very good review papers [11-15]. One of the aspects that these papers addressed was, what activity of a plant extract should be considered as good activity for follow-up work.

Many of the papers written on investigating plants for antimicrobial activity have the stated or implied aim of discovering a new antibiotic. One approach to select the MIC of an interesting plant extract is by considering the concentration required in the blood of a patient to control the pathogen. If a metabolite has a concentration of 0.01% of an extract e.g. for taxol in *Taxus* extracts [16], there is only one active compound in the extract, and a concentration of 1 μg/mL is required to inhibit the growth of the microbe, the plant extract should have an MIC of 0.1 mg/mL or lower. This calculation is however based on no loss due to absorption, distribution, metabolism and excretion of the compound in the host. These assumptions, especially that a plant extract has only one active compound or than a new potential antibiotic has an MIC of 1 μg/mL are not necessarily valid. Another reason why these assumptions may not be valid is because it has frequently been shown that synergism between different compounds in plant extracts play an important role in the antimicrobial activity. This appears to be in contrast to the situation with many fungi and bacteria that produce only one or a few antimicrobial compounds leading to the discovery of practically all antibiotics.

Van Vuuren [13] reviewed many antimicrobial papers published in South Africa. In the cases where she listed the MICs of extracts that were published, the values varied from 0.01 to 2.5 mg/mL with an average of 0.43 mg/mL (Table 1). In a later review paper [15] more values obtained by several additional authors were listed. Although there has been growing consensus that a value of 100 μg/mL

(Table 1), [17-21] should be used, it appears that the values presented were based on judgment of the authors and there was no clear rationale for the value selected.

In a paper entitled "A classification system for antimicrobial activity based on MIC values: Fake or reality? Roersch [22] questioned why there could be such an enormous variation in what authors considered a good antimicrobial activity for plant extracts. In this paper I will attempt to provide a rational basis for the classification of the activity of plant extracts after determining the antimicrobial activity of 714 acetone extracts of tree leaves from 537 tree species against six important bacterial and yeast pathogens. The motivation for this large study [23] was to determine if there is a correlation between taxonomy and microbial activity to find which taxa had the highest antimicrobial activity and should be targeted to discover new antimicrobial compounds. Some correlations at the order level were indeed found. There was a fivefold increase in probability of finding extracts with interesting activity against Gram-negative bacteria from tree orders with high mean activity than from tree orders with low mean activity [23].

The aim of the paper presented now is to determine the average MIC values of a large number of acetone tree leaf extracts against six important microbial pathogens using a well-established and widely used method and to use the results to classify antimicrobial activity of acetone leaf extracts.

Results

At the highest concentration tested, 2.5 mg/mL, all the extracts inhibited the bacterial or fungal growth and c. 90% of the extracts inhibited growth of the pathogens at a concentration of 1.2 mg/mL. (Fig. 1). Each point on the graph represented the average of more than 12800 results (714 measurements in triplicate against six pathogens). The results obtained had a good fit (R^2 of 0.9592) with the formula calculated by Windows Excel: Average % activity l = 23.126 x lnMIC in mg/mL.

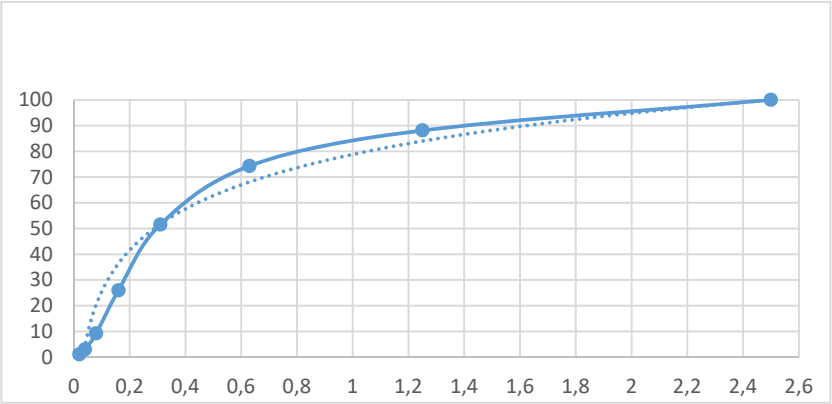

Figure 1 The average cumulative percentage activity (y axis) of 714 acetone leaf extracts from 537 tree species at MICs of 0 to 2.5 mg/mL (x-axis). The markers represent experimental values obtained. The formula obtained by using Excel describing the correlation is % of extracts = 439xMIC in $mg/mL^{1,5385}$ with a correlation coefficient of R^2 = 0.9592 with the experimental data. The broken line represents values calculated from the formula.

The results were redrawn for MIC values of 0.16 mg/mL and lower (Figure 2). There was an excellent agreement of the results (R^2 = 0.9998) with the formula: average % of extracts = 439xMIC in $mg/mL^{1.5385}$

The percentage of extracts with activity at different MICs can be deduced from Figure 2. More accurate values can be calculated from the formula. Approximately one out of eight (12.7%) plant extracts examined had an MIC of 0.1 mg/mL or lower. This is the MIC selected by many authors to identify interesting extracts (Table 1).

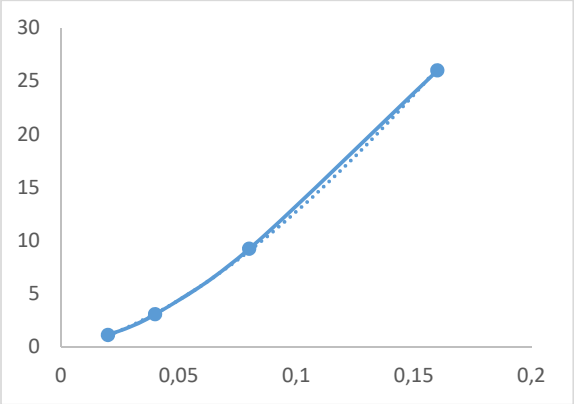

Figure 2 The average cumulative percentage activity (y axis) of 714 acetone leaf extracts from 537 tree species at MICs of 0.16 mg/mL and lower (x-axis). The markers represent experimental values obtained. The formula obtained by using Excel describing the correlation is % of extracts = 439xMIC in mg/mL^{1,5385} with a correlation coefficient of R^2 = 0.9998 with the experimental data. The broken line represents values calculated from the formula.

Table 1 MICs proposed by different authors for extracts and isolated compounds

Author	Year	MICs extracts proposed in µg/mL			Isolated
		strong	moderate	weak	compounds
Fabry *et al.* [17]	1998			8000	
Alligianis *et al.*[18]	2001	500	600-1500	>1600	
Holetz *et al.* [19]	2002	100	100-500	500-1000	
Eloff [20]	2004		100		
Rios and Recio [21]	2005		100		
Cos *et al.*[12]	2006		100 (IC_{50})		10
Van Vuuren [13]	2008		200		200
Kuete [14]	2010	<100	100-625	>625	

Discussion

Some of the six pathogens investigated are certainly more resistant to plant extracts than others. Vlietinck and colleagues [24] found major differences in the activity of plant extracts against different microorganisms using the agar diffusion assay. It was surprising that despite the difference in sensitivity of the different pathogens, there was an excellent correlation between the percentage of plant extracts with different average activities. This may be due to the serial microplate dilution assay used or because all the results for the different pathogens were combined and sorted in classes.

The aim of this paper was to indicate the range of MICs found on a large number of plant extracts using a well-established method on different important pathogenic microorganisms and then classifying activity based on the percentage of plants with different activities. I propose that five stages of classification varying from weak to outstanding activity should be used (Table 2). It may be more

rational to select exactly 1%, 5% 10%, 25% and 50% as the discriminating values, but starting with an extract of 10 mg/mL after serial dilution the microbes would be subjected to 2.5, 1.25. 0.63, 0.32, 0.16, 0.08, 0.04 and 0.02 mg/mL of the extract [19]. These concentrations are therefore proposed to classify the antimicrobial activity of plant extracts (Table 2).

Table 2 Classification of activity of plant extracts based on MIC range and approximate percentage of plants with that activity

Classification	MIC range in mg/mL	Approximate percentage of plants
Outstanding	0.02 and lower	Within the top 1%
Excellent	0.021 - 0.04	Within the top 3%
Very good	0.041 - 0.08	Within the top 9%
Good	0.08 - 0.16	Within the top 25%
Average activity	0.161 - 0.32	Within top 50%
Weak activity	> 0.32	Outside the top 50%

This classification should provide a reality check to authors that hope to isolate a plant-based compound that can become a new antibiotic. It appears that pharmaceutical companies prefer developing products from microbe-derived compounds because fermentation of the microbe with established techniques could resolve supply and quality control issues [11]. In contrast to microorganisms, the strategy of plants to inhibit infections in many cases appear to be by using several compounds acting synergistically. Another factor decreasing the interest of pharmaceutical companies is the potential variation in biological activity and content of active compound in plants.

There are additional factors that come into play in deciding how important a low MIC is. If a plant extract is to be used as a topical application, products with higher MICs may still be very efficient. The toxicity and selectivity of a compound or an extract may be more important than a low MIC. If the extract or an active compound contains a general metabolic toxin that affects a basic metabolic pathway, there would be high activity against different pathogens but also high cytotoxicity against human or animal cells. It therefore makes much sense to compare the activity against different organisms and to determine cellular toxicity of interesting extracts or compounds [10]. The selectivity index can be calculated by dividing the cytotoxicity (LD_{50}) by the MIC in the same units.

These criteria do not necessarily mean that extracts of plants with MICs of higher than say 0.1 mg/mL used as herbal medicines or in traditional medicine to treat infections are not effective in combating pathogens. These extracts may contain compounds that act in synergism in the human or animal body and not on the microbe. These extracts may also contain compounds that have an influence on the immune system of the host adding an additional level of defence.

The procedure used to determine the MIC will have an effect on the outcome. We have found in many cases that acetone extracts the largest variety of compounds from leaves, has the lowest toxicity to microorganisms and is easy to remove from an extract [26]. If authors use different extractants this could lead to different activities. In the most of our studies comparing different extractants we have found that acetone extracts have the highest activity and also extracts the most antimicrobial compounds based on bioautography [27, 28].

Other aspects of the methods used to determine the activity will also have an influence on the activity. Van Vuuren and Holl [15] stated that the time after adding *p*-iodonitrotetrazolium violet to the microbial culture before reading the value may influence the results. We have never experienced this with the

large inoculum of 50% that we used in all our studies based on the widely-cited original method [10, 25]. This large inoculum would place the microorganisms directly into the logarithmic growth phase without a lag phase from a small inoculum that could influence the results. For bacteria the inhibition was determined after two hours and for fungi after 24 hours of incubation [23]. It is also clear that environmental conditions and also the season of growth can have an effect on the antimicrobial activity although water stress and temperature stress did not have a major effect on the antimicrobial activity of some plants (29, 30).

With the methods we used, all plant extracts had an MIC of 2.5 mg/mL or lower. A statement that a plant extract has antimicrobial activity without specifying the MIC therefore has no value [10]. Depending on the extractant and method used to determine MIC all plant extracts have antimicrobial activity if the dose is high enough. The problems using agar diffusion studies to determine the antimicrobial activity of plant extracts has been argued by several authors [10, 13].

To evaluate the activity of isolated compounds, Kuete [14] selected 10 μg/mL as MIC for interesting activity. In an excellent review on the anti-Staphylococcal activity of 116 plant derived natural products Gibbons [11] indicated that many compounds had activities below 1 μg/mL. He concluded that MICs of 25-50 μg/mL for compounds should be considered as moderately active. It is disconcerting that with so many compounds with outstanding antimicrobial activity no clinically used antibiotic has been developed from plant products yet [11]. In many cases the acetone leaf extracts had higher activity against the pathogens than the positive controls used. Without testing the extract or isolated compounds in a preclinical or clinical trial the potential value has not been confirmed.

If the distribution in activities of acetone tree leaf extracts are similar to results found in extracts of different parts of other plants and there is an agreement that the top 1%, 3% and 9% are good criteria, then MICs lower than 0.02, 0.04 and 0.08 mg/mL would represent outstanding, excellent and very good activities. If authors and journal editors were happy with plants e.g. in the top 20% they could use the formula to calculate the relevant MIC. Using these values could answer the question how scientific the science in Ethnopharmacology is [31], partly because physiologically non-relevant concentrations should not be classified as active. Scientists may differ on the percentage to be selected to determine which plants should be examined further or which data should be published. The formula should make it possible to calculate the relevant MIC with confidence especially if the same extractant and MIC technique is used. If another extractant or another method to determine the MIC is used, different values would be obtained and the most active 1% could be lower or higher than the MIC of 0.02 mg/mL identified here.

Material and Methods

Tree leaves were collected from 714 labelled trees in several botanical gardens, mainly the Lowveld (Nelspruit), Pretoria, Kirstenbosch (Cape Town) and Harold Porter (Betty's Bay) National Botanical Gardens as well as the Manie van der Schyff Botanical Garden of the University of Pretoria and the Durban Botanical Garden [18]. This represents the different climatic conditions experienced in South Africa. The, 714 samples consisted of 537 tree species representing 350 genera, 101 families and 38 orders. Voucher specimens are stored in the HGW Schweickert Herbarium of the University of Pretoria [18] listed under PMDN numbers 1-537.

Leaves were dried at room temperature in the shade and ground to a fine powder. The powder was extracted with acetone [20] and made up to a concentration of 10 mg/mL. By using a serial microplate

dilution technique with *p*-iodonitrotetrazolium violet as growth indicator [19] the minimum inhibitory concentration (MIC) was determined against the following pathogens: *Enterococcus faecalis*, (ATTC 29212), *Staphylococcus aureus* (ATTC 29213), *Escherichia coli* (ATTC 25922) and *Pseudomonas aeruginosa* (ATTC 27853). The pathogens used are the reference strains recommended by the National Committee for Clinical Laboratory standards Villanova, Pennsylvania, USA to compare the activity of antibiotics. Two yeasts *Candida albicans* and *Cryptococcus neoformans* were also examined. They are associated with opportunistic infections of immune-compromised animals and were obtained from Dr Jackie Picard (Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria). *C. albicans* was isolated from a Goldian finch and *C. neoformans* from a cheetah suffering from systemic mycosis. The positive controls were gentamicin for bacteria and amphotericin B for fungi [18].

The average MIC of the extracts of different trees were determined against each pathogen. and the results were ordered from high to low activity in the classes obtained by serial dilution of a 10 mg/ml extract. The percentage of tree leaf extracts active at different average MICs were calculated. The results for MICs below 0.16 mg/mL were also plotted separately.

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Conflict of interest statement

The author declares that he has no conflict of interest in publishing this paper.

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