Development of a reproducible method to determine minimum inhibitory concentration (MIC) of plant extract against a slow-growing mycoplasmas organism

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Abstract

Mycoplasma species are fastidious bacteria that require a specialized medium for their growth, isolation and identification. There are no standardized tests to evaluate the in vitro susceptibility of mycoplasmas to medicinal plant extracts. A widely used in-broth, microtitre plate, minimum inhibitory concentration (MIC) assay was adapted and evaluated using acetone extracts of Anoigeissus leiocarpus on the isolates of Mycoplasma mycoides subsp. mycoides small colony variants (MmmSC). Several problems were encountered including the contamination of the medium by Bacillus species found in plants and the fact that the slow-growing mycoplasmas proved to be poor reducers of the indicator tetrazolium salt or resorcinol. We then examined a pH indicator-dependant technique to detect the acid production caused by the growth of the organism after glucose utilization from the broth medium. The method gives a clear cut-off point that was easy to read and interpret and was also reproducible.

The MIC value for acetone extract of A. leiocarpus was 0.16 mg/ml. The development of this method now makes it possible to evaluate extracts of several plant species for antimycoplasmal activity.

Keywords: *Mycoplasma mycoides* subsp. *mycoides*; *Anoigeissus leiocarpus*; MIC; pH; Glucose and broth

Introduction

Mycoplasmas are obligate parasites of animals and humans that occasionally cause respiratory, urogenital and ocular disease. *Mycoplasma mycoides* subsp. *mycoides* small colony (SC) variant is the cause of contagious bovine pleuropneumonia (CBPP) in cattle. This disease is common in West and East Africa as well as parts of Central and southern Africa. Since cattle are considered by people in

these countries as a source of wealth and afford social status, huge social and economic losses are incurred. Furthermore, CBPP severely limits the exportation of cattle and their products. It is usually controlled by the slaughtering of positive and in-contact cattle or by vaccination (Nicholas and Bashiruddin, 1995). A stamping-out policy is not possible in countries where CBPP is widespread and the live vaccine is often difficult to obtain and must be protected a cold-storage chain. Thus, many farmers have resorted to the treatment of their animals with antimicrobials.

It is known that many plants especially those used by traditional healers produce pharmaceutically active compounds that have antimicrobial, antihelminthic, antifungal, antiviral, anti-inflammatory and antioxidant activity (McGaw et al., 2000). Medicinal plants are usually easier to obtain than pharmaceutical products. Furthermore, the emergence of antibiotic resistance in both Gram-positive and Gram-negative bacteria has heightened interest in medicinal plants as a source of unique antibacterial compounds (Cowan, 1999). This led to a plethora of publications on the *in vitro* efficacy of plant extracts from various parts of the globe.

Unfortunately, different test methods and conditions were used making it difficult to compare results. The agar diffusion and dilution tests, and the tube and microplate dilution tests have been employed for the determination of minimum inhibitory concentration (MIC) of plant extracts on bacteria and fungi but with attendant problems and limitations (Eloff, 1998). A quick and sensitive microplate method utilizing tetrazolium red or thiazole blue as indicators of bacterial metabolism, developed by Eloff (1998) is a robust and widely used technique which works well for fast-growing bacteria such as *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli* (Eloff, 1998). It has also worked very well with human and plant fungal pathogens (Masoko et al., 2007; Eloff et al., 2006). It has also worked well with a small adaptation to microaerophilic acidogenic oral bacteria (Eloff et al., 2005). However, its suitability for fastidious, slow-growing bacteria such as mycoplasmas is less known.

Thus the aim of this project was to evaluate and adapt the method of Eloff (1998) so that the antimicrobial susceptibility of mycoplasmas could be reliably tested. Acetone extracts of the leaves of *Anoigeissus leiocarpus* and the T1/44 vaccine strain of *Mycoplasma mycoides* subsp. *mycoides* SC was used as a model.

Materials and methods

The leaves of *A. leiocarpus* (DC) Guill. & Perr. used traditionally to treat CBPP was collected around Zaria in Kaduna State of Nigeria. The identity was confirmed at the Herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria where voucher specimen number 167 was deposited. Leaves were dried, ground in a mill and extracted by vigorously shaking with acetone at a ratio of 1:15 w/v, with a yield of approximately 15%.

Since *Mycoplasma* species do not grow in Mueller–Hinton broth, pleuropneumonia-like organism basal medium (PPLO) (Difco Laboratories, Detroit, USA) with added foetal calf serum and yeast extract was prepared according to the method of Hayflick (Hayflick, 1965; Thiaucourt and Di-Maria, 1992). This medium has been found to be effective for *in vitro* antimicrobial susceptibility testing (Hannan, 2000). Filter-sterilized, amoxycillin-clavulanic acid was added at a concentration of 5×10^5 IU/l growth medium. One percent glucose and phenol red as an indicator was added to an aliquot of the enriched PPLO.

Each well of a sterile 96-well flat-bottomed, microtitre plate was inoculated with 100 µl of media, one plate with enriched PPLO medium with glucose and phenol red and the other without. Using a starting concentration of 10 mg/ml, 12 serial two-fold dilutions of the plant extract were made in 100 µl of enriched PPLO medium in a sterile, microtitre plate. Thereafter, 100 µl of 1:10 dilution of 48 h actively growing *Mycoplasma mycoides* subsp. *mycoides* (T1/44 vaccine strain) broth culture containing approximately 6×10^{12} cfu/ml was added to each well. The antibiotic tylosin at concentration of 1.28 mg/ml was also serially diluted and used as drug positive control while half strength acetone was used as negative control. One well on each plate also had a positive growth control containing no plant extract or tylosin. The microtitre plates were covered with thin aluminium foil, to prevent light-associated damage of the plant extract, and incubated at 37 °C for 24–36 h before the results were read. After 24 h of incubation, 40 µl of freshly prepared 0.2 mg/ml INT (*p*-iodonitrotetrazolium) was added to each well of the plate that had the medium that did not contain glucose and phenol red. It was incubated for a further 4–5 h before being read. In this case, a change of the medium to pinkish-red indicated that the bacteria were metabolically active. The MIC was taken as the last well where no change of colour (i.e. no bacterial growth) was observed for both methods. The MIC results were then compared. This test was repeated in triplicate over a period of 3 successive weeks to test for reproducibility.

Result and discussion

The MIC value of the acetone extract of *A. leiocarpus* for both methods was 0.16±0 mg/ml. It was observed that the yellow from red colour produced by the first method (pH dependant) was more pronounced and the cut-off point was easier to read and interpret on the plate than the pinkish colour of the second (modified Eloff's) method which was very faint and not consistent in duplicate wells making it difficult to determine the cut-off point.

In preliminary studies it was found that the rich media and slow growth (2–3 days) of mycoplasmas tended to allow the endospores of *Bacillus* species present in the plant material to germinate and overgrow. Amoxycillin with clavulanic acid is not active against *Mycoplasma* species and was found not to interact with the antimicrobial activity of the plant nor of the control antibiotic (tylosin).

Earlier phytochemical studies on some species of *Anogeissus* have yielded various classes of compounds such as sugars and their derivatives (Aspinall and Christenen, 1961; Aspinall and Chudhari, 1975), acids (Anderson et al., 1987), glycosides (Nduji and Okwute, 1988), flavones and flavonoidal glycosides (Rimando et al., 1994), lignans (Lin et al., 1991) and tannins (Bate-Smith, 1954). Adigun et al. (2000) evaluated the antimicrobial activities of the extract of *A. leiocarpus* on *S. aureus*, *E. coli*, *P. aeruginosa* and *Candida albicans* using an agar dilution method. They were able to isolate and identified the active antimicrobial constituents as 3,4,3′-tri-0-methylflavellagic acid and its glucoside. It is possible that the antimicrobial (antimycoplasmal) activity observed in the present study with the plant extract could be attributed to the presence of this compound.

In conclusion, though the two methods gave the same MIC against *Mycoplasma mycoides* subsp. *mycoides*, the pH-dependant method is more accurate, consistent, reproducible and MIC was easier to determine when compared to using INT. Thus the pH-dependant method was found to be more reproducible when used to identify the MICs of plant extracts to glucose-fermenting mycoplasmas. Other sugars can be used as substrates for those mycoplasmas that do not ferment glucose.

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