ORIGINAL ARTICLE

Optimized Tube Dilution Technique and Sole Carbon Utilization Assay for Anti-leptospiral In Vitro Screening of Plant Extracts

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ABSTRACT

Introduction: Leptospirosis is one of the neglected re-emerging zoonoses that is of public health concern globally. The need to discover novel therapeutic alternatives for leptospirosis through screening for and elucidating the mechanism/s of the anti-leptospiral activity of plant extracts is therefore necessary. This study analyzes the optimized tube dilution technique and the Biolog™ sole carbon utilization phenotype microarray as screening tool for anti-leptospiral activity of plant extracts.

Methods: The suitability of the optimized tube dilution technique was evaluated by determining the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and motility inhibition property of a plant extract and an antimicrobial control (pen G) against 4 dominantly circulating *Leptospira* serovars/serogroup in the Philippines. Likewise, the suitability of the Biolog™ sole carbon utilization assay was evaluated using a plant extract and selected antimicrobials against *L. interrogans* serovar Manilae strain K64 *and L. interrogans* serovar Losbanos strain K37.

Results: The MIC, MBC, and motility inhibition property of a plant extract and the antibiotic controls as well as its effect on the carbon utilization phenome of the *Leptospira* serovars gave consistent results, within and between several runs. With standard deviation = 0 for all serovars. The MIC and MBC of the antimicrobial control (pen G), the positive control, was 10 ug/ml. The growth control (leptospires without treatment), the negative control, showed presence of motile leptospires. The MIC and the MBC of the test plant extract was 250 ug/ml - 500 ug/ml. Results of the carbon utilization phenome or pattern of carbon utilization were consistent within the 3 replicates and between two runs.

Conclusion: The optimized tube dilution technique and the Biolog™ sole carbon utilization assay is a potential in vitro screening tool for determining anti-leptospiral activity of plant extracts.

KEYWORDS: *anti-leptospiral, tube dilution technique, phenotype microarray*

INTRODUCTION

Leptospirosis, an acute febrile disease caused by genus *Leptospira,* is a preventable and treatable disease in animals and humans. It is now considered an emerging global disease due to re-emergence of the disease in non-endemic areas and becoming an urban problem in highly endemic areas.[1,2] In most of the developing countries in the Asia Pacific region, leptospirosis is largely a water-borne disease and in the Philippines, poor sanitation and increase in urban slums along with frequent typhoons, contribute to the risk of infection.[3] Currently, studies are focused on the need for continued monitoring of the prevailing serovars in a given geographical area and improvement of diagnostic capabilities to elucidate the current disease burden of leptospirosis.[4-13] However, there are limited studies on the in vitro and in vivo screening to find alternative treatments (i.e. use of herbal plants) for leptospirosis. The Leptospirosis Task Force issued the Philippine Clinical Practice Guidelines on the Diagnosis, Management and Prevention of Leptospirosis (2010) on antibiotic use and it states that for mild cases of leptospirosis, doxycycline (hydrochloride or hyclate) is the drug of choice. Alternative drugs include amoxicillin and azithromycin dihydrate. Although there is still no problem on the antimicrobial resistance on the current recommended antimicrobials, the "One Health" Operational Concept of the Global Leptospirosis Environmental Action Network (GLEAN), has drawn the global and local efforts on research, development and innovation on genomics, diagnosis, vaccines and on therapeutic alternatives in addressing leptospirosis, as an emerging global disease.

Plants have been used for health and medical purposes for several centuries. Numerous studies have been conducted on the antibacterial properties of plants on various pathogenic and multi-drug resistant organisms.[14-16] However, screening for anti-leptospiral activity of plants is limited. Since leptospires have surface structures that share features of both gram-positive and gram-negative bacteria, evaluating plants with known antibacterial property in terms of their ability to either inhibit the growth or directly kill the said bacteria can be an initial approach in order to find alternative means for treating leptospirosis.

Traditional methodologies for evaluating antimicrobials, such as the tube and agar-diffusion based assays, to determine the minimum inhibitory (MIC) and minimum bactericidal concentration (MBC) had been utilized in the screening for anti-leptospiral properties of some plants.[17-21] However, plant extracts have distinct properties that need to be considered when being tested using the tube and agar-diffusion based assays such as the interference of the leaf pigments. The Biolog™ sole carbon utilization phenotype microarray is a unique cellular analysis tool that offers a comprehensive approach to identify how a natural product such as plant extracts prevents microbial growth.[22-24] To the knowledge of the authors, there has not been any published paper yet reporting the use of this technology to test for antimicrobial activity of plant extracts against *Leptospira.* Moreover, since suitability of the growth requirements of the microorganism (test system) as well as the inherent properties of a plant ethanolic leaf extract (test item) is crucial to the accuracy and precision of the tube or agar-diffusion based assays and the Biolog™ sole carbon utilization phenotype microarray, therefore, optimizing the standard tube dilution technique and the Biolog™ phenotype microarray intended to screen for anti-leptospiral activity of plant extracts may contribute to the initial efforts for discovering therapeutic alternatives for leptospirosis.

MATERIALS AND METHODS

Equipment and Reagents

Dark field microscope (Olympus BX43) was used to observe the presence or absence of viable leptospires in the in-vitro screening assays for anti-leptospiral property. The Biolog™ GEN III MicroStation System (Biolog™ Inc., Hayward, CA, USA) was used in the sole carbon source utilization phenotype microarray assay. The GEN III Microplate™ and the inoculating fluid (IF-C) used in the sole carbon source utilization assay were all obtained from Biolog™. The USP-grade antimicrobial standards, penicillin (Pen G), doxycycline (Doxy), and polymixin B (PolB), were purchased from Sigma-Aldrich Pte. Ltd. Singapore. The ethanol, phosphate buffered saline (PBS), and *Leptospira* culture medium (i.e., Korthof's medium) were of analytical grade.

Plant material, quality control testing, and extraction procedure

To optimize the tube dilution technique and the Biolog™ Gen III sole carbon source utilization phenotype

microarray for its suitability as in vitro screening tool for anti-leptospiral activity of plant extracts, a matrix-specific test item i.e. plant material known to have an antibacterial property to various pathogenic organisms and with reported MIC to *L. interrogans* serovar Manilae strain K6 was used. The test plant material (coded as "PELE" to protect its patent potential) was collected from the agricultural unit of the National Integrated Research Program on Medicinal Plants (NIRPROMP) in UPLB, Laguna. Particularly, pesticides were not used during cultivation and the farm where the plant material was grown was located far from highways and industrial areas. Standard methods were used in preparing the leaves and processing into powder form. Only the leaves from the 1st through 3rd apical shoots of the plant were collected. Inclusion criteria for the tops include absence of insects and absence of wilting and dark spots. The leaves were then washed with distilled water, air dried and ground into powdered form through a blender and packed properly until further use. In addition to the test plant material, reference antibiotic control/s and a growth control (leptospire without treatment) served as the positive and negative control, respectively during the optimization of the methods.

Subsequently, as required for plants with potential for registration as traditionally-used herbal products in the Philippines, based on FDA Issuance AO 184 Series of 2004, quality control testing of the plant material was done.[25] A standard procedure for plant extraction was adapted. The plant ethanolic leaf extract was prepared by soaking 100 g of the dried powdered leaves in 300 ml absolute ethanol for 24 hours, with occasional stirring. Afterwards, it was filtered using Whatman filter No. 1 and the remaining residue was re-extracted with additional 300 ml of absolute ethanol. The final ethanolic leaf extract was collected and placed in a rotary evaporator at 50^{0}C for 2-3 hours. The residue was then evaporated to dryness in a dish placed in a water bath at 50^{0}C. The leaf extract powder was stored in a sealed amber bottle and kept in the freezer (-20^{0}C) until use.

Leptospira *strains*

*S*tock cultures of *L. interrogans* serovar Manilae strain K64, *L. interrogans* serovar Losbanos strain K37, *L. interrogans* serovar Ratnapura strain K5, and *L. borgpetersennii* serogroup Javanica strain K6, were obtained from the Leptospirosis Prevention and Control Laboratory (LepCon), Department of Medical Microbiology, College of Public Health, University of the Philippines Manila. These strains represented the groups of *Leptospira* that were previously reported to be predominantly circulating in the Philippines.[5,6] All organisms were maintained by continuous culture in Korthof's medium. The inoculum was prepared by adding the stock strain of leptospires into Korthof's medium (1:16) and incubated at 30^{0}C for 4-7 days to a density of approximately 1 x 10^8 cells/ml verified by dark field microscopy and enumerated using the Thoma counting chamber.

Antimicrobial and plant extract stock preparation

Penicillin G, doxycycline and polyymixin B were used as antimicrobial control/ positive controls, depending on the assay. Pen G was used as the reference antimicrobial control in the tube dilution technique. Pen G, doxycycline and polymixin B were used as the reference antimicrobial controls in Biolog™ Gen III sole carbon source utilization phenotype microarray. Stock antimicrobial solutions (2,500 ug/ml) were prepared using the USP-grade powders of penicillin G, doxycycline, and polymixin B diluted in sterile distilled water and stored in one-time-use aliquots at −80^{0}C. Similarly, stock plant leaf extract solution (125,000 ug/ml) was freshly prepared daily and vortex-mixed to ensure homogeneity. The concentrations of the stock solutions were prepared so that at least a 1:10 ratio of the antimicrobial control to Korthof's medium can be achieved to ensure that enough growth media is available for the leptospires to grow.

MIC and MBC by Tube Dilution Technique

In this study, the minimum inhibitory concentration (MIC) is defined as the lowest concentration of plant extract or antimicrobial agent that showed inhibition of growth as indicated by the absence of motile leptospires after incubation for 7 days at 30°C. The minimum bactericidal concentration (MBC) is defined as the lowest concentration of plant extract or antimicrobial agent that showed absence of motile leptospires, when an inoculum from the tubes without motile leptospires from an MIC set up was transferred into fresh Korthof's medium without test agent and further incubated for 7 days at 30°C. The presence or

absence of motile leptospires was checked using the dark field microscopy.

The optimized tube dilution technique in this study was a modification of the broth microdilution technique.[17,26] The optimized tube dilution technique used the Korthof's growth medium in tubes (macrodilution) and dark field microscopy to check for the presence or absence of motile leptospires. While the broth microdilution used the Ellinghausen-McCullough-Johnson-Harris (EMJH) growth medium in microwells and spectrophotometry with alamar dye to determine the viability of leptospires. Based on the MICs of the test plant material at 250 ug/ml (Yabes et al., *unpublished*) and pen G (10 ug/mL) against *L. interrogans* serovar Manilae strain K64,[27,28] stock solutions were prepared and added to Korthof's medium (1:10) to achieve the desired highest concentration. Subsequent concentrations of the plant extract and pen G were prepared in tubes by serial dilution. Antimicrobial-containing tubes included final concentrations of pen G (i.e., 0.5, 5, and 10 ug/ml). Plant extract-containing tubes had final concentrations ranging from 250 - 10,000 ug/ml.

To determine the MIC, one part of the *Leptospir*a inoculum (1 x 10^8 cells/ml) was added to 9 parts of Korthof's medium containing treatments (plant extract and antimicrobial control) at desired concentrations. The growth control or negative control (leptospires in Korthof's without treatment) was prepared similarly and included in every run. The tubes were incubated for 7 days at 30°C and observed for motility of the leptospires using dark field microscopy daily from days 4-7. The lowest concentration that showed absence of motile leptospires on the 7th day was reported as the MIC.

To determine the MBC, inoculum was taken from the tubes in the MIC set up with the lowest concentration of plant extract or antimicrobial agent that showed absence of motile leptospires verified by dark field microscopy. The inoculum was transferred to freshly prepared Korthof's medium (1:10) and subsequently incubated for another 7 days. The lowest concentration that showed absence of motile leptospires on the 7th day was reported as the MBC.

Test for Motility Inhibition Property

In this study, positive for motility inhibition is defined as the lowest concentration of plant extract or antimicrobial agent that showed the reduction in the percentage of motile leptospires to approximately 50% on the 24th hour as compared to the growth control. To determine the motility inhibition property of the extract, a method described previously was adapted.[17,29] Briefly, 10 ul sample from each tube in the MIC set up was placed on the slide and observed under dark field microscope at 0, 3, 5 and 24 hr incubation. Results were scored as follows; a score of "100%" indicated that the leptospires were motile and comparable with the growth control (leptospires without treatment). A score of "0%" indicated absence of motile leptospires. While a score of "50%" indicated that the number of motile leptospires was reduced to approximately 50% as compared with the growth control. Thus, the reduction in the percentage of motile leptospires to approximately 50% on the 24th hour was reported as motility inhibition. Images of leptospires under the dark field microscope were electronically captured and the "percentage and motility" of leptospires were evaluated by at least 2 researchers.

Biolog^{TM} phenotype microarray (PM) sole carbon utilization technology

The Biolog^{TM} Gen III microplate was used to analyze the ability of the leptospires to metabolize 71 major classes of biochemicals belonging to sugars, hexose phosphates, amino acids, hexose acids, esters, carboxylic acids, and fatty acids. Significant respiration in the wells was observed when the carbon source is utilized. The increased respiration caused the reduction of the tetrazolium redox dye, forming an irreversible purple color, which was then used to colorimetrically indicate utilization of the carbon sources or resistance to inhibitory chemicals. These carbon sources are necessary for the growth of mostly gram negative and gram-positive bacteria.[22-24,30-33]

The inoculum i.e., PBS-washed *Leptospira* cells in IF-C, was prepared using *Leptospira* grown in 5 ml Korthof's at 30^{o}C for 4-7 days with bacterial density of approximately 1 x 10^8 cells/ml. The bacterial solution was centrifuged for 10 minutes at 1500 x g. The supernatant was discarded carefully to remove the Korthof's medium. The *Leptospira* cells were then washed 2 times with 5 ml of PBS and centrifuged for 10 minutes at 1500 x g. After discarding the supernatant, the *Leptospira* cells were re-suspended in 5 ml of IF-C. Simultaneously, to prepare the IF-C spiked with

treatments containing sub-MIC (0.25x and 0.5x the MIC) of the plant extract or antimicrobial controls (penG, doxy and pol B), stock solutions were added to the IF-C (10 ml) to achieve the desired concentration of the treatments. Subsequently, 1.5 ml of the *Leptospira* cells in IFC was added to the IF-C spiked with treatments. The inoculum (100ul) was aseptically dispensed to each well of the Biolog™ Gen III microplates and incubated at 30°C. The microplates were read from day 0 to 7 using the Biolog™ Microstation 2 set at 590 and 750 nm. Based on the Biolog Gen III kit protocol, results may be interpreted in three ways, visual inspection of color formation in the wells, as well as the graphic result and the optical densities (OD) produced by the Biolog™ Microstation. OD readings at 590 nm were encoded in Microsoft Excel to generate the corrected ODs and subsequent data analysis. Corrected ODs were calculated by subtracting the OD of the negative control well from the OD of each test well in the Biolog™ Gen III microplate. The use of corrected OD ($\geq$0.100) as basis for setting the threshold for "positive for carbon source utilization" was adapted from previous studies.[30-33] Readings of the negative control wells (n=60), positive control wells (n=60) and the tetrazolium wells (n=120) in this study were also considered in setting up the threshold. Those wells with corrected OD of $\geq$0.100 were considered well utilized and interpreted as positive for carbon source utilization while those wells with corrected ODs <0.100, although above the corrected OD of the negative control, were considered negative or borderline and interpreted as negative for carbon source utilization.

Quality Control and Internal Validity

Quality control samples (i.e., growth control/negative control, positive/antimicrobial control, and plant extract control) were used in the pilot run and during the actual experiments. Freshly prepared plant extract and suspension of actively growing leptospires were used in each run. The suitability of the tube dilution technique for its intended use as anti-leptospiral screening tool of plant extracts was evaluated using the plant extract and the antimicrobial controls (pen G) against the 4 *Leptospira* serovars/serogroups. The determination of MIC, MBC and motility inhibition using the tube dilution technique were performed, in different runs over several days (3 runs in triplicates using strain K64 and 1 run in triplicates using the other 3 *Leptospira* serovars/serogroups). Similarly, the Biolog™ Gen III sole carbon utilization phenotype microarray was also performed in 2 runs in triplicates using strain K64 and strain K37. The suitability of the Biolog™ Gen III for its intended use as anti-leptospiral screening tool of plant extracts was evaluated by the consistency of the results of the carbon source utilization phenome and the inhibition of the carbon source utilization of the three replicates using the 2 *Leptospira* serovars, as well the results of the negative control wells, positive control wells and the tetrazolium wells. Only three replicates per serovar was used since the manufacturer's claim for reproducibility in the package insert of the Biolog™ Gen III test kit is excellent due to controlled conditions required in the whole procedure. Similarly, at least three replicates for each serovar was also used in the tube dilution technique since pilot study of the optimized tube dilution method also showed consistent MIC when used in plant extracts.

Ethical Statement

This study was performed in adherence to Good Laboratory Practice (GLP) standards. All experiments using *Leptospira* cultures were performed in a university-based BSL-II facility. Strict compliance to the regulations of the UP Manila Institutional Biosafety and Biosecurity Committee (IBBC), as well as technical review and research registration procedures with UPM REB (Research Ethics Review Board) and UP Manila RGAO (Registration Grants Administration Office) were observed. The data on the optimization of the anti-leptospiral in vitro screening methods for plant extracts described in this paper was the preliminary data of the two subsequent researches funded by the UP National Institutes of Health and the Philippine Institute of Traditional and Alternative Medicine (PITAHC), Department of Health that used these optimized methods in screening for anti-leptospiral activity of plant extracts.

RESULTS

The optimized tube dilution technique described in this study gave consistent results of MIC, MBC, and motility inhibition property of the antimicrobial control and the plant extract, within runs and between runs over several days, with standard deviation = 0 for all serovars (Table 1). The MIC and MBC of the antimicrobial control

(pen G), which also served as the positive control, was 10 ug/ml against *L. interrogans* serovar Manilae strain K64*, L. interrogans* serovar Losbanos strain K37*, L. interrogans* serovar Ratnapura strain K5, and *L. borgpetersennii* serogroup Javanica strain K6. The growth control (leptospires without treatment) which served as the negative control showed presence of motile leptospires after incubation for 7 days at 30°C. The MIC and the MBC of the test plant extract was 250 ug/ml against *L. interrogans* serovar Manilae strain K64*, L. interrogans* serovar Losbanos strain K37, and *L. interrogans* serovar Ratnapura strain K5*,* while the MIC against *L. borgpetersennii* serovar Javanica strain K6 was 500 ug/ml (Yabes et al., *unpublished*). In this study, the MIC is equivalent to its MBC, thus the antibacterial activity against the four serovars/serogroup is bactericidal.

Table 1. MIC, MBC and motility inhibition property of the plant extract (PELE) and antimicrobial control against four (4) dominantly circulating Leptospira serovars/serogroup in the Philippines.

***Leptospira* serovars/serogroup**	**Plant extract (PELE)**		**Pen G**	
	MIC and MBC (ug/ml)	**Motility inhibition property (ug/ml)**	**MIC and MBC (ug/ml)**	**Motility inhibition property (ug/ml)**
L. interrogans serovar Manilae strain K64 (n=9)	250	5,000	10	>10
L. interrogans serovar Losbanos strain K37 (n=3)	250	1,250	10	>10
L. interrogans serovar Ratnapura strain K5 (n=3)	250	>10,000	10	>10
L. borgpetersennii serogroup Javanica strain K6 (n=3)	500	>10,000	10	>10

Results of the carbon utilization phenome or pattern of carbon utilization of *L. interrogans* serovar Manilae strain K64 and *L. interrogans* serovar Losbanos strain K37 using the optimized Biolog™ Gen III sole carbon utilization phenotype microarray were consistent within the 3 replicates and between two runs. Likewise, when the two *Leptospira* serovars were treated with sub-MICs of the plant extract and antimicrobial controls (pen G, doxycycline and polymixin B), consistent pattern of inhibitions, between and within runs, were also obtained. In addition, the internal validity of the microplates showed that all the negative control wells (n=60) gave an average corrected OD of 0.040, which was also consistent with visual inspection, that no purple color was observed in all the negative control wells. All the positive control wells showed corrected ODs $\geq$0.100 (n=60). Similarly, all the wells containing tetrazolium blue and tetrazolium violet, which are indicators of cell respiration, showed corrected ODs $\geq$0.100 (n=120). Comprehensive results and discussion of the carbon utilization phenome of *L. interrogans* serovar Manilae strain K64 and *L. interrogans* serovar Losbanos strain K37 and the effect on the inhibition pattern of the plant extract and the 3 antimicrobial controls on the 2 *Leptospira* serovars using the optimized Biolog™ Gen III sole carbon utilization phenotype microarray will be discussed in a separate paper.

DISCUSSION

The optimized tube dilution technique described in this study is suitable in vitro screening tool for anti-leptospiral activity of plant extracts. Based on the results of this study, both the optimized tube dilution technique and the Biolog™ sole carbon utilization assay phenotype microarray have the potential of being used as an in vitro screening tool for anti-leptospiral activity of plant extracts. It showed consistent results of MIC, MBC, and motility inhibition property of the antimicrobial control

and plant extract when tested against 4 *Leptospira* serovars/serogroup that are dominantly circulating in the Philippines. Moreover, the obtained MIC of the antimicrobial control (pen G) was within the reported MIC of aminobenzyl penicillin (ampicillin) ranging from 6.25 - 12.5 ug/ml against *L. interrogans* serovar icterohaemorrhagiae using the microdilution technique.[26] Similarly, the obtained MIC of the plant extract was within the range of the MICs of other plant extracts evaluated for anti-leptospiral activity.[17-21] The result was also comparable with the study of another plant with anti-leptospiral property such as the *Andrographis paniculata* Nees (Acanthaceae), which showed an MIC of 200-600 ug/ml against *L. interrogans* serovar australis and *L. interrogans* serovar icterohaemorrhogica.[21] In addition, it has the advantage of circumventing the interference of plant leaf pigments in spectrophotometric assays by using the dark field microscopy as an alternative way of determining the viability of leptospires through the MIC, MBC, and motility inhibition assays.

Although the Biolog™ Gen III sole carbon utilization phenotype microarray was introduced in the 90's, to the knowledge of the authors, there has not been any published paper yet reporting the use of this technology for *Leptospira*. The closest so far was its use to a related anaerobic spirochete, *Brachyspira pilosicoli* 95/100, B2904 and WesB.[34] Its use for Genus *Leptospira* could have been limited because the Biolog™ standard protocol for inoculum preparation was for microorganisms grown on solid media, in which the bacterial density estimation uses a turbidimeter. This however was not applicable for leptospires, since the means of estimating the leptospire's density is via direct enumeration using a counting chamber under a dark-field microscope. Thus, the Biolog™ standard protocol of inoculum preparation appropriate for organisms grown in solid media was completely modified. To satisfy the required actively growing cell inoculum, the use of the *Leptospira* grown in Korthof's medium for 4-7 days was used. To mimic the 40% transmittance used in *B. pilosicoli* study,[34] PBS-washed *Leptospira* was added to the inoculating fluid to obtain an estimated density of 1.3 x 10^7 cells/ml. Interestingly, this was the same bacterial density that was found to be also optimum in the tube dilution technique. Similarly, PBS-washed *Leptospira* suspended in the inoculating fluid was used in order to have a media-free inoculum. This medium-free condition was done to avoid confounders brought about by the *Leptospira* culture media and to allow the leptospires to solely depend and utilize the carbon source in the wells for bacterial cell respiration. Lastly, monitoring the carbon source utilization up to 4th day was also noted to be optimum for leptospires using the Biolog™ sole carbon utilization technology. It considered the slowing of the metabolism of cells and the decreased availability of carbon sources needed to support later growth phases. In addition, the inclusion of growth control (i.e., leptospires without treatment) ensured that the *Leptospira* inoculum used in the runs were actively growing and that the readings were not due to contamination with other bacteria. Likewise, inclusion of plant extract control (i.e., plant extract in Korthof's medium without any treatment) was noted to be a good means of ruling out bacterial contamination and abiotic dye reduction in the IF-C coming from the plant extract since these may potentially interfere in the assay leading to false positive results. The modifications done on the methods, to suit with the growth requirements of *Leptospira* and the inherent properties of plant extract, showed that it can be a standard screening tool for compounds with anti-leptospiral property. Moreover, results from this pioneering study on the use of the Biolog™ Gen III in *Leptospira* suggest that this technology may be used for these bacteria and may possibly serve as basis for other microorganisms grown on liquid media.

CONCLUSION

Traditional methodologies for evaluating antimicrobials are being used in the screening for anti-leptospiral properties. However, crucial to the accuracy and precision of any procedure is its suitability with the growth requirements of the leptospires as well as the inherent properties of a plant extract. This study showed that both the optimized tube dilution technique and the Biolog™ Gen III sole carbon utilization phenotype microarray technology have acceptable reproducibility, within and between runs. The modified tube dilution technique described in this study is a simple, reliable technique, comparable to the commonly used broth microdilution technique. Moreover, although this is the first study reporting the use of Biolog™ sole carbon source utilization phenotype microarray technology for *Leptospira*, this in vitro assay was found to be a

promising tool in evaluating plants with anti-leptospiral property. Lastly, due to the promising results obtained from these methods, these were subsequently used in similar researches that aim to screen for anti-leptospiral properties of a plant extract known to have anti-bacterial property with the aim of discovering novel therapeutic alternatives for leptospirosis.

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