ORIGINAL ARTICLE

Antibacterial Activity of Crude *Momordica charantia*, *Cassia alata*, and *Allium sativum* Methanolic Extracts on *Leptospira interrogans* serovar Manilae

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ABSTRACT

Background and Objective. Leptospirosis is a disease caused by pathogenic *Leptospira* prevalent in tropical countries like the Philippines. Some studies have shown that the role of currently used antibiotics for leptospirosis is unclear since trials have found no significant benefit to patient outcomes compared to placebo. This signals the need for alternative therapies, such as herbal medicines, which may provide effective therapeutic regimens in treating this infection. In this study, we characterized the antibacterial potential of three Philippine herbal medicines against *Leptospira interrogans*.

Methods. Crude methanolic extracts of *Momordica charantia*, *Cassia alata*, and *Allium sativum* were subjected to an optimized broth microdilution assay against *L. interrogans*, utilizing the resazurin-resorufin reaction as a cell proliferation and viability indicator.

Results. The respective minimum inhibitory concentrations of the plants were found to be as follows: 1.25 mg/mL (*M. charantia*), 2.5 mg/mL (*C. alata*), and >5 mg/mL (*A. sativum*).

Conclusions. Among the three herbal medicines, *M. charantia* and *C. alata* proved to have antibacterial activity against *L. interrogans*. Given the promising potential of two of these plant extracts, exploring the use of other solvents to extract natural compounds from these plants, and discovering possible synergistic effects between these plants and conventional antibiotics may be worthwhile.

Keywords: Leptospira interrogans, herbal medicine, M. charantia, C. alata, A. sativum

INTRODUCTION

Leptospirosis is a zoonotic disease primarily transmitted by contact with contaminated urine of reservoir hosts such as rats.[1] While rodents are the primary reservoir for these bacteria, domestic animals are also known to be sources of infection.[2,3] At the same time, humans are accidental hosts infected when pathogenic *Leptospira* spirochetes enter the body through cuts, abrasions, or mucosal breaks that are exposed to tissue or body fluids of an infected animal or contaminated soil or water.[1,2] Due to its mode of transmission, leptospirosis tends to be prevalent in tropical countries like the Philippines, which experience high levels of rainfall annually.[1] Further, individuals from developing countries are at a higher risk of infection, especially in areas with inadequate sanitation and high rodent exposure.[1,3] When infected, humans present with an acute onset fever, usually associated with headache, abdominal pain, nausea and vomiting, jaundice, oliguria or anuria, and muscle pain.[1,2] Severe disease may lead to dysfunction in major organs.[1]

* *Ms. Vista and Mr. De Galicia shared first authorship for this manuscript.*

eISSN 2094-9278 (Online)
Published: December 13, 2024
https://doi.org/10.47895/amp.vi0.8362

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While most cases are mild and can spontaneously resolve, severe leptospirosis is usually treated with antibiotics such as penicillin, ampicillin, ceftriaxone, or cefotaxime.[1] Antibiotics such as doxycycline are also recommended as chemoprophylaxis.[1] However, studies have found insufficient evidence to show the benefit of using these antibiotics in leptospirosis management, especially in severe disease and disease prevention.[4,5] One meta-analysis of clinical trials showed that penicillin is not significantly different from placebo in reducing mortality or length of hospital stay when used to treat leptospirosis infections.[5] The same study looked into clinical trials on chemoprophylaxis and found that while there was a significant benefit for taking doxycycline upon flood water exposure, none of the usual antibiotic regimens against leptospirosis were beneficial in reducing symptomatic cases.[5] Another systematic review of clinical trials concluded that based on data on mortality, duration of illness, duration of fever, and need for dialysis, the benefit of currently used antibiotics in treating leptospirosis is unclear.[4] Thus, there is a need to look for alternative therapeutic regimens that may improve or complement current therapeutic regimens for this disease.

Herbal medicines are one such alternative, with many plants already proven to have good antibacterial activity.[6–8] Further, in indigenous settings where herbal medicines are often used, this treatment may be more cost-effective and acceptable than conventional prescription drugs.[9] In the Philippines, ten herbal medicines are endorsed by the Department of Health as traditional and alternative medicine.[10] They have various known health benefits, from treating infections to managing chronic diseases.[10] Microbiological assays done by our colleagues on these plants showed that the methanolic extracts of *M. charantia, C. alata,* and *A. sativum* exhibit antibacterial activity against laboratory-maintained isolates of *Bacillus subtilis, Escherichia coli, Staphylococcus aureus,* and *Pseudomonas aeruginosa* (unpublished data). In this study, we explore if these methanolic extracts have antibacterial activity against *Leptospira interrogans*. To our knowledge, no other studies have been published evaluating the antibacterial activity of these plants against leptospires. The findings of this study may aid in determining potential alternative treatments against leptospirosis infections that could be especially significant in regions where these plants are widely available and readily accessible.

METHODS

Preparation of Plant Extracts

All raw plant materials used were acquired from different Philippine provinces. Leaves of *M. charantia* were collected from the Leonie Agri Corporation Farm, Sta. Rosa, Nueva Ecija; *C. alata* leaves were collected from Morong, Bataan; *A. sativum* cloves were collected from Ilocos Norte. Authentication was done by the Institute of Biology Jose Vera Santos Herbarium, College of Science, University of the Philippines Diliman. Plant products were then processed following a previously established protocol.[11] Briefly, the collected leaves were dried; then, leaves and cloves were cut, ground, and soaked in 100% methanol for 16-18 hours at room temperature. The resulting filtrate was concentrated with a rotary evaporator at room temperature, and the resulting extract was placed in a dry bath. Extracts were placed in a tightly sealed container and stored at -20°C until use. Our extraction method resulted in percent yields of *M. charantia (Mch), C. alata (Cal)* and *A. sativum (Asa)* with the following values respectively, 18.18%, 13.34% and 13.57%.

Stock solutions of *Mch, Cal,* and *Asa* were prepared by dissolving 5 g of each methanolic extract in 1 mL of dimethyl sulfoxide (DMSO). Nine mL of McCoy's 5A nutrient medium was subsequently added to the three stock solutions to produce 10 mL of a 500 mg/mL solution. This culture media was used as a diluent to mimic the mammalian physiologic milieu where these plant extracts, if effective, are intended to be used. This was further diluted with 10% DMSO to achieve a 10 mg/mL concentration.

Growth and Maintenance of Bacterial Cultures

A laboratory-maintained environmental isolate of *L. interrogans* serovar Manilae strain K64 were acquired from the LepCon Laboratory, Department of Medical Microbiology, College of Public Health, University of the Philippines Manila. Leptospires were maintained in liquid media (Korthof's Medium) inside a 30°C incubator. From optimization experiments, we found that seven-day old cultures of leptospires produce a bacterial density of around 1 x 10^8 leptospires/mL, which allows the resazurin reaction (at 0.5X concentration) to be detectable both spectrophotometrically and by the naked eye of an observer. All experiments were done under biosafety level 2 (BSL-2) in accordance with institutional guidelines and supervision.

Broth Microdilution Assay

To determine the minimum inhibitory concentration (MIC) of our three plant extracts on leptospires *in vitro*, we adapted a broth microdilution assay from published literature.[12,13] The MIC is defined as the lowest concentration of the plant extract that visibly prevents bacterial growth.[14] Five rows of a 96-well plate were used for this assay. Three rows were allotted for the plant extracts (*Mch, Cal, Asa*), and two rows were used for the controls, +*Growth* containing uninhibited leptospires and -*Growth* containing culture media only. The schematic diagram is shown in Figure 1.

On a 96-well plate, 100 uL of sterile Korthof's medium were added onto each of the seven wells of four rows labeled *Mch, Cal, Asa,* and *"+Growth"* (Appendix). A total of 200 uL of medium were added onto each of the seven wells of the row labeled *"-Growth"*. Subsequently, 100 uL of the corresponding plant extract stock solution in 10% DMSO were introduced into the leftmost wells of rows *Mch, Cal, and Asa*. Two-fold serial dilutions were then performed on these

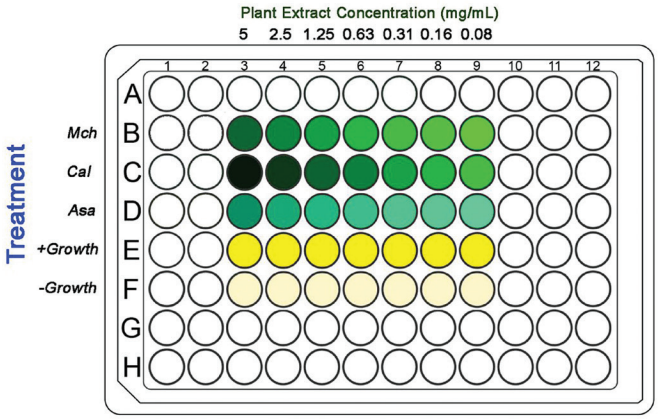

Figure 1. Schematic diagram of broth microdilution assay plate layout.

plant extracts, starting from a concentration of 5 mg/mL until 0.08 mg/mL. Then, 100 uL of Korthof's medium containing 1.3 x 10^8 leptospires/mL were introduced onto each well of rows *Mch, Cal, Asa,* and *+Growth.* The row "*+Growth*" contained no plant extract and allowed for the unopposed growth of our bacterial species, readily showing the colorimetric reaction as there was no growth inhibition. The 96-well plate was then covered and incubated at 30°C for seven days.

After seven days, the plates were retrieved, and 20 uL of 0.5X resazurin solution (Catalog No. B6098, ApexBio Technology, Houston, Texas, USA) were added onto each well of the five rows (*Mch, Cal, Asa, +Growth, -Growth)*, introducing a dark blue hue to these wells. In contrast to previous broth microdilution assays, with the indicator being an observer-determined colorimetric reaction endpoint, we decided to spectrophotometrically determine the change in the concentration of the reduction product, resorufin. The plate was loaded onto a spectrophotometric plate reader, with absorbance measurements of the wells taken at 575 nm, to take a baseline measurement. Plates were immediately re-incubated thereafter.

After one day, the plates were retrieved, and absorbance was again measured at 575 nm. During the 24-hour incubation, viable proliferating bacterial cells would have metabolized resazurin, increasing the concentrations of the reduction product, resorufin. All experiments were done in triplicate.

Statistical Analysis

The change in the absorbance readings between Days 7 and 8 were calculated as follows:

$$\Delta\text{Absorbance} = (\text{Absorbance}_{\text{Day8}} - \text{Absorbance}_{\text{blank}}) - (\text{Absorbance}_{\text{Day7}} - \text{Absorbance}_{\text{blank}})$$

This was done for three independent experiments, and error bars signify the variation in their ΔAbsorbance. Dunnett's one-way ANOVA was performed to compare the mean absorbance values of each extract concentration to the mean absorbance values of the *-Growth* control. The MIC is reported as the lowest concentration at which there was no significant growth compared to the *-Growth* wells ($p \geq 0.01$).

RESULTS

MIC of *M. charantia*, *C. alata*, and *A. sativum*

Results show that when exposed to aqueous extracts of *M. charantia* at concentrations as low as 1.25 mg/mL, leptospires exhibit no statistically significant growth (Figure 2A). For *C. alata*, a concentration of 2.5 mg/mL could still inhibit the growth of leptospires (Figure 2B). However, statistically significant leptospiral proliferation could be observed beyond this. Among the three plants, *A. sativum* was the only plant that did not exhibit inhibition of leptospiral growth at concentrations as high as 5 mg/mL. All concentrations of this plant extract showed statistically significant growth of leptospires (Figure 2C).

DISCUSSION

M. charantia, C. alata, and *A. sativum* are three plant species widely available and readily accessible throughout the Philippines and are used locally for their medicinal value[10], with reference to indigenous knowledge passed down from generations. Our institution aims to empirically test the biofunctionalities of these plant species *in vitro*. In the present study, we aim to determine the antibacterial activity of these three medicinal plants against *L. interrogans*. Antimicrobial susceptibility testing against leptospires grown on solid media would have methodologically been easier and has been conducted in published literature.[15] Nevertheless, the specialized CO_2 incubator and specially prepared solid media (LVW media) precluded us from performing inhibition testing on solid media. Albeit with relatively slower growth and the requirement of a bacterial proliferation and viability indicator (resazurin), a broth microdilution assay adapted from published literature was used to determine the MIC of our plant treatments against leptospires *in vitro.*[12,13]

MIC is a commonly used measure of antimicrobial activity. While a standard interpretation criteria is lacking, conventional antibiotics are typically effective at MICs ranging from 0.01 to 10 µg/mL. At the same time, plant compounds are generally considered to have antimicrobial activity at MICs between 100 and 1000 µg/mL.[16] The MIC of *M. charantia,* with a value of 1.25 mg/mL, was lower than the other treatments, *C. alata* and *A. sativum,* which had MICs of 2.5 mg/mL and >5 mg/mL, respectively. MICs of conventionally used drugs in clinical medicine to treat leptospirosis, such as doxycycline and penicillin G, have MICs of approximately 0.1-0.2 µg/mL and 0.4 µg/mL, respectively.[17] Meanwhile, the crude methanolic extract of *Boesenbergia rotunda,* an herbal medicine used in India to

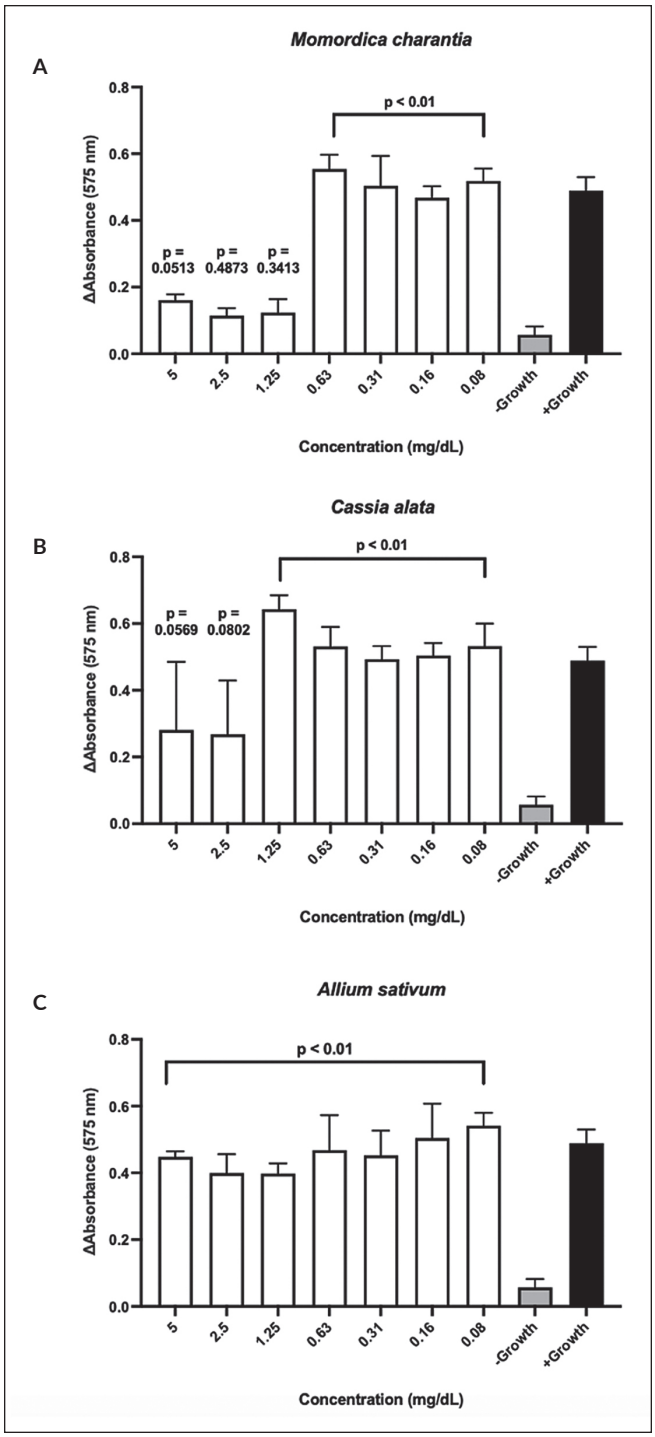

Figure 2. Change in absorbance after resazurin application for **(A)** *M. charantia*, **(B)** *C. alata*, and **(C)** *A. sativum*.

treat fever, headache, and body aches, was found to have an MIC range of 62.5 to 125 μg/mL when tested against 24 leptospiral strains.[18] In addition, another study showed that crude extracts of *Garcinia mangostana* inhibit leptospires at concentrations of 200 to ≥800 μg/mL. Upon looking into purified xanthones of *G. mangostana*, they found that a lower MIC at 100 ug/mL was effective against pathogenic leptospira.[13] Our study's antimicrobial activity is weaker than plant crude extracts, purified plant metabolites, and conventional drugs in published literature. Nonetheless, we observed a clear MIC at the concentrations we tested and thus conclude that antibacterial compounds with activity against *L. interrogans* are present in the methanolic crude extracts of *M. charantia* and *C. alata*. Methanol is the commonly preferred solvent for plant phytochemical extraction; although it is polar, it may also dissolve nonpolar compounds due to its amphiphilic nature. It is possible that the antileptospiral compounds of *M. charantia* and *C. alata* are underestimated in the methanolic fraction, and thus, exploring solvent extraction with water, n-hexane, or other solvents may yield different results. Subsequently, further studies can examine particular fractions or purified *M. charantia* and *C. alata compounds*. Using other solvents to extract plant compounds, such as water or n-hexane, may also be done to test the full spectrum of the plants' metabolites against *L. interrogans*. It can be hypothesized that a lower MIC may be observed with fewer interfering compounds, as seen in the case of purified xanthones in *G. mangostana.*[13] We also acknowledge that the principle of our indicator in the broth microdilution assay is a surrogate marker for bacterial cell reproduction and not bacterial cell viability; thus, we can only report MICs. Further studies may look into assays yielding minimum bactericidal concentrations (MBCs). Moreover, synergism between plant extracts and conventional antibiotics against leptospirosis, as observed in some preclinical studies[13,19] is an avenue worth exploring.

CONCLUSION

The results of our broth microdilution study show the *in vitro* antileptospiral activity of the methanolic extracts of *M. charantia* and *C. alata.* These plant products may not fully replace these conventional antibiotics recommended for treating leptospirosis. Still, it is possible that they may complement current therapeutic regimens to produce better patient outcomes. More preclinical and clinical studies are needed to elucidate the role of herbal medicines in treating cases of human leptospirosis.

Acknowledgments

The authors would like to thank Dr. Sharon Villanueva, Dr. Nina G. Gloriani, the Department of Medical Microbiology, College of Public Health, UP Manila, and the Department of Biochemistry and Molecular Biology, College of Medicine, UP Manila.

Statement of Authorship

Both authors certified fulfillment of ICMJE authorship criteria.

Author Disclosure

Both authors declared no conflicts of interest.

Funding Source

The authors received no financial support for this article's research, authorship, and/or publication.

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APPENDIX

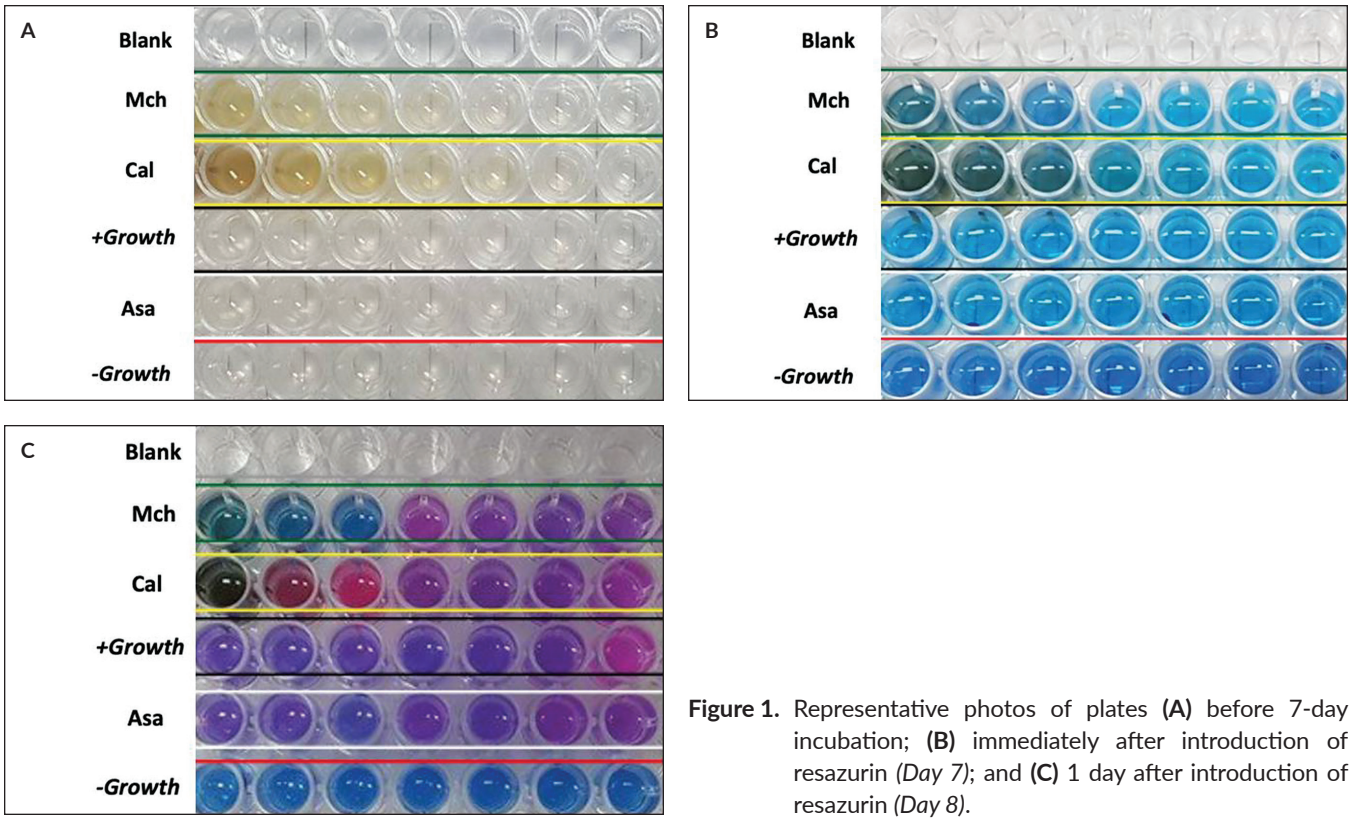

Figure 1. Representative photos of plates **(A)** before 7-day incubation; **(B)** immediately after introduction of resazurin *(Day 7)*; and **(C)** 1 day after introduction of resazurin *(Day 8)*.