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RESEARCH ARTICLE

In-vitro susceptibility of pathogenic and intermediate *Leptospira* species towards antibiotics and herb extracts

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ABSTRACT

Leptospirosis is a severe and potentially fatal re-emerging zoonotic and waterborne disease caused by pathogenic and intermediate species of *Leptospira*. Given the high global rates of morbidity and mortality associated with this disease, there is an urgent need to explore alternative therapeutic agents to enhance treatment options. This study investigates the anti-leptospiral efficacy of several common antibiotics-penicillin G, doxycycline, ampicillin, amoxicillin, cefotaxime, chloramphenicol, and erythromycin, as well as extracts from local herbs, *Hydnophytum formicarum* Jack and *Boesenbergia stenophylla*, against pathogenic and intermediate *Leptospira* strains. A broth microdilution method determined the minimum inhibitory concentration (MIC) for the antibiotics and herb extracts. Both herbs were extracted using four different solvents: ethyl acetate, methanol, hexane, and chloroform. The extracts were then analysed using gas chromatography-mass spectrometry (GC-MS) to identify their phytochemical compounds. The results demonstrated that cefotaxime and erythromycin exhibited the highest anti-leptospiral activity, with MIC values of 0.2 µg/mL. This was followed by amoxicillin and ampicillin (0.2–0.39 µg/mL), penicillin G (0.39–3.13 µg/mL), chloramphenicol (0.78–3.13 µg/mL), and doxycycline (0.78–12.5 µg/mL*). H. formicarum* Jack and *B. stenophylla* extract extractions displayed the lowest MICs (62.5 µg/mL) for the ethyl acetate, methanol, and hexane extracts. They contained various phytochemical constituents, including some with anti-leptospiral properties. These findings indicate that different strains of *Leptospira* respond with varying levels of inhibition to the antibiotics and herb extracts studied. The extracts from *H. formicarum* Jack and *B. stenophylla* may have potential as anti-leptospiral drugs. However, further in-vivo studies are needed to better understand their efficacy against *Leptospira*.

Keywords: Microbial resistance; zoonotic diseases; antimicrobial stewardship; medical pharmacology; complementary medicine.

INTRODUCTION

The genus *Leptospira* comprises helical, motile, bacterial spirochetes of the Leptospiracae Family that cause debilitating diseases called "leptospirosis" or "rat urine disease", which is an emerging tropical zoonotic disease (Bilung *et al*., 2018; Soo *et al*., 2020). Leptospirosis impacts 1.03 million people and causes 58,900 mortalities worldwide (Costa *et al*., 2015), resulting in 2.90 million disability adjusted life years (DALYs), the majority of which are concentrated in low and middle income tropical countries (Torgerson *et al*., 2015). The risk is higher for communities in rural and urban areas living in tropical and temperate climates. Many regions have recorded leptospirosis outbreaks. However, human infections are concentrated in tropical and subtropical areas, with endemicity in developing countries (Soo *et al*., 2020).

Leptospira species are categorised as human-infectious (pathogenic or intermediate pathogenicity) or non-infectious environmental saprophytes based on biological characteristics and molecular phylogeny. Presently, analysis of lipopolysaccharides (LPS) composition in the bacterial outer membrane has classified the genus *Leptospira* into 69 species, comprising more than 260 serovars grouped into 24 serogroups (de Oliveira *et al*., 2023). Human infection occurs when pathogenic *Leptospira* spp. are transmitted through direct contact with infected animals or indirect contact with the environment (e.g. soil and water) contaminated with the body fluids of the infected animals (e.g. urine) that can enter through abraded skin, open wounds, mucosal membranes (conjunctiva, oral, or genital) (Samrot *et al*., 2021). The incubation period of *Leptospira* is usually 2–30 days after exposure. (Chacko *et al*., 2021), in which the clinical symptoms exhibit significant variability and lack specificity, ranging from subclinical and asymptomatic to severe fatal pulmonary haemorrhage and Weil's syndrome. Most of the cases are asymptomatic or briefly present as a flu-like febrile disease of unknown origin, which complicates clinical diagnosis, especially in countries such as Malaysia, where dengue, malaria, and other infectious diseases are endemic and express overlapping

clinical presentations. (Chacko *et al*., 2021). Rodents are the main *Leptospira* reservoir. However, a wide range of mammals can also be a host (Cordonin *et al*., 2020), while the transmission is intensified by flood, low levels of sanitation, environmental exposure, and uncontrolled rodent population (Neela *et al*., 2020). In Malaysia, leptospirosis is endemic and has been declared a notifiable disease since 2010 (Neela *et al*., 2020).

The current treatment guidelines for treating mild to moderate leptospirosis are oral doxycycline 100 mg twice a day for five to seven days, amoxicillin 500–750 mg four times a day, or azithromycin 500 mg one daily for three days (Chacko *et al*., 2021). Generally, the emergence of antimicrobial resistance (AMR) lowers the effectiveness of first-line medications, necessitating second or third-line medications that may be less effective, more toxic, pricier, and associated with adverse effects such as disruption of gut microbiota balance. The rate of new antimicrobial development has slowed markedly in the last two decades, and compounded by the increased emergence of AMR, treatments are hindered with gradually less effective antimicrobial treatments. Previous efforts to eliminate *Leptospira* using penicillin, ciprofloxacin, and doxycycline from tubules of the chronic stage were unsuccessful due to the biofilm formation (Vinod Kumar *et al*., 2016), suggesting *Leptospira* develops resistance during the late stage of infection (Ratet *et al*., 2014). A systematic review concluded that it remains uncertain whether antibiotics are effective in treating leptospirosis due to a lack of robust data from randomised trials (Mukadi *et al*., 2022). Compounded by these scenarios, exploring alternative therapeutic agents that can enhance treatment options remains imperative.

Boesenbergia spp., perennial rhizomatous herbs in the *Zingiberaceae* family, are widely distributed in the Philippines, Indonesia, Malaysia, Brunei, and Thailand, comprising 70 estimated total species. *B. stenophylla* is a species largely indigenous to Sarawak's Borneo Highland region (Saptu *et al*., 2021) and has been used to treat seizures, drunkenness, and food poisoning, along with to relieve coughs and boost libido (Jing *et al*., 2010; Atiekah & Ibrahim, 2018; Sussana Primus *et al*., 2022). *Hydnophytum formicarum* Jack., commonly called the ant plant, is a medicinal species belonging to the *Rubiaceae* family and is a tuberous epiphytic medicinal plant. It is predominantly located in Southeast Asia (Malaysia and Indonesia), Papua New Guinea, and the Pacific Islands. The tuber exhibits cardiovascular, anti-inflammatory, and antiparasitic properties and is utilised for alleviating skin rashes, serving as a neurotic, and treating headaches, hepatitis, rheumatism, and diarrhoea (Prachayasittikul *et al*., 2008). Given the known antimicrobial properties of these plants, we sought to explore their anti-microbial activity against *Leptospira*, which might be the first study using these endemic herb plants. This the study was conducted to i) determine antibiotic susceptibility toward *Leptospira* spp. in Sarawak by using broth microdilution techniques, ii) to investigate the antimicrobial activity of *H. formicarum* Jack and *B. stenophylla* against local *Leptospira* spp. in Sarawak by using broth microdilution techniques, and iii) to determine the chemical compound in *H. formicarum* Jack and *B. stenophylla* by using gas chromatography-mass spectroscopy (GC-MS).

MATERIALS AND METHODS

Preparation of *Leptospira* cultures

We previously isolated *Leptospira* strains with varying pathogenicity from the environment in Sarawak, Malaysia, in 2014–2015, as published in Pui *et al*. (2017). The cultures were later stored and maintained in the Molecular Microbiology Laboratory, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak. A total of six pathogenic and six intermediate *Leptospira* strains from that maintained culture collection were used in this current study (Table 1). Resuscitation was performed by growing the bacteria in *Leptospira* Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Difco BD, USA) containing 0.1 g of 5-fluorouracil and enrichment media at room temperature. All cultures were incubated at 30°C for 30 days. As previously mentioned, the final concentration of the culture was 106 to 108 CFU/mL (Ristow *et al*., 2008).

Preparation of the antibiotics

Antibiotic stock solutions of seven antibiotics, namely penicillin G (Fisher Bioreagents, USA), doxycycline (Acros Organics, USA), ampicillin (Fisher Bioreagents, USA), amoxicillin (Fisher Bioreagents, USA), cefotaxime (Acros Organics, USA), chloramphenicol (Acros Organics, USA), and erythromycin (Fisher Bioreagents, USA) were prepared from the reagent-grade powders using solvents and diluents to produce 1 mg/ml solutions as recommended by the Clinical and Laboratory Standards Institute document M100–S22. The antibiotics were aliquoted into smaller volumes from the stock solutions and stored at -20°C until further use.

Preparation of the local herb crude extracts

Tubers of *H. formicarum* Jack were purchased from Lachau local market, Sarawak, while *B. stenophylla* was collected from Bario, Sarawak. Tubers of *H. formicarum* Jack, and leaves and rhizomes of *B. stenophylla* were finely ground before 20 g of the powders were used in the maceration extraction. The extraction process used four different solvents: chloroform, ethyl acetate, hexane, and methanol, to yield the corresponding chloroform, ethyl acetate and methanol extracts.

Table 1. A list of pathogenic and intermediate *Leptospira* strains was used in this study

Leptospira pathogenicity	Strain ID	*Leptospira* strains	Source of isolation in Sarawak, Malaysia
Pathogenic strains	CFP16	*Leptospira noguchii* strain LT796	Rait River, Miri Camp
	CFP18	*Leptospira noguchii* strain LT796	Kubah National Park
	CFP21	*Leptospira noguchii* strain LT796	Samunsam Wildlife Sanctuary
	CFP3	*Leptospira borgpetersenii* serovar mini	Bako National Park
	CFP34	*Leptospira borgpetersenii* serovar mini	Rait River, Miri Camp
	CFP35	*Leptospira borgpetersenii* serovar mini	Kubah National Park
Intermediate strains	CFG4	*Leptospira wolffii* serovar Khorat strain Khorat-H2	Bako National Park
	CFG12	*Leptospira wolffii* serovar Khorat strain Khorat-H2	Rait River, Miri Camp
	CFG14	*Leptospira wolffii* serovar Khorat strain Khorat-H2	Kubah National Park
	CFG3	*Leptospira inadai* serovar Aguaruna strain MW4	Tanjung Datu National Park
	CFG15	*Leptospira inadai* serovar Aguaruna strain MW4	Kubah National Park
	CFG18	*Leptospira inadai* serovar Aguaruna strain MW4	Barieng Village, Bau

Minimum inhibitory concentration of antibiotics and the local herbs against *Leptospira*

The minimum inhibitory concentration (MIC) was tested using the broth microdilution testing (Murray & Hospenthal, 2004b; Chakraborty *et al*., 2010). Each plate included positive control (*Leptospira* only), negative controls (EMJH broth only), and serial two-fold dilutions of each of the seven antibiotics diluted in EMJH broth. The final concentration of each antibiotic in the antibiotic-containing well was 0.20–50 µg/ml (unit/ml for penicillin). The broth microdilution testing on the herbs was performed similarly, except the antibiotics were substituted with the herb crude extracts. As initially described, *Leptospira* culture grown in liquid EMJH medium was used in this testing. A volume of 100 µL of *Leptospira* inoculum (with a concentration of 2×10^6 CFU/mL) was added into the antibiotic-containing and positive control wells, producing the final volume of each well was 200 µL. The plates were incubated at 30°C for three days. Then, a volume of 20 µL of 10× AlamarBlue was added to each well to determine the presence of bacterial growth, in which AlamarBlue is a cell viability indicator dye that turns from dark blue to bright pink when bacterial growth exists. On the fifth day of incubation, the colour of each well was observed. The MICs were determined based on the lowest concentration of the antibiotics or local herbs that caused no colour changes in the medium.

GC-MS analysis

Phytochemical constituents of *H. formicarum* Jack and *B. stenophylla* crude extract were determined via GC-MS profiling using Shimadzu 2010 Plus GC-MS instrument (Amzad Hoss & Rahman, 2010) with a little modification. The carrier gas was helium, and the capillary column was BPX-5 (30.0 mm × 0.25 mm). The diluted sample was then injected into a splitless injector. The column was heated to 60°C for 2 min. The temperature was then increased to 300°C at 10°C/min for 5 min. The temperature of the injector and detector was 250°C and 300°C, respectively. Finally, the substance was identified by matching its mass spectrum to the NIST library.

RESULTS

Antibiotic susceptibility testing towards pathogenic and intermediate *Leptospira* spp.

The antibiotic susceptibility level of the *Leptospira* strains towards antibiotics was expressed in MIC value. Pathogenic and intermediate *Leptospira* strains exhibited MIC values ranging from 0.2 to 6.25 µg/mL towards seven tested antibiotics (Table 2). Doxycycline exhibited the highest MIC value (6.25 µg/mL), whilst amoxicillin, ampicillin, cefotaxime and erythromycin yielded the lowest (0.20 µg/mL). Cefotaxime and erythromycin inhibited well against all tested environmental isolates with MIC 0.2 µg/mL. The isolates CFP3, CFP34, CFP35, CFG3, CHG15, and CFP18 were susceptible to amoxicillin, ampicillin, cefotaxime and erythromycin with the lowest MICs value of 0.2 µg/mL. In contrast, doxycycline had MIC values ranging from 0.78 to 12.5 µg/mL, where CFP18 and CFP21 showed higher MIC values of 6.25 µg/mL and 12.5 µg/mL, respectively. Overall, all tested isolates *Leptospira* spp. more susceptible toward cefotaxime and erythromycin with MIC 0.2 µg/mL, compared to amoxicillin and ampicillin with MIC 0.39 µg/mL.

Antimicrobial activity of crude extract of *H. formicarum* Jack

Antimicrobial activity of crude extract of *H. formicarum* Jack against pathogenic *Leptospira* spp. showed different MIC values across the type of chemical extractant used (Figure 1) (Table 3). Ethyl acetate crude extract showed the lowest MIC (125–250 µg/mL), followed by methanol crude extract (125–250 µg/mL), hexane extract (250–500 µg/mL) and chloroform extract (500 µg/mL). For intermediate *Leptospira* strains, ethyl acetate crude extract showed the lowest MIC (62.5–250 µg/mL), followed by methanol crude extract (125–250 µg/mL), hexane extract (125–250 µg/mL), and chloroform extract (250–500 µg/mL).

Twelve different crude extracts (extracts derived from *H. formicarum* jacks, *B. stenophylla* leaves, and *B. stenophylla* rhizomes) produced using four different solvents (ethyl acetate, methanol, hexane, and chloroform) were tested against pathogenic and intermediate *Leptospira* isolates. The lowest value of the minimum inhibitory concentration (MIC) was 62.5 µg/mL, as highlighted in boldface, indicating the highest anti-leptospiral activity.

Antimicrobial activity of crude extract of *B. stenophylla*.

Antimicrobial activity of *B. stenophylla* (leaves) crude extract against pathogenic *Leptospira* spp. showed different MIC values across the type of chemical extractant used (Figure 1) (Table 3). Ethyl acetate crude extract showed the lowest MIC (62.5–125 µg/mL), followed by methanol crude extract (62.5–250 µg/mL), hexane extract (62.5–250 µg/mL) and chloroform extract (125–500 µg/mL). For intermediate *Leptospira* strains, ethyl acetate crude extract showed the lowest MIC 62.5–250 µg/mL), followed by methanol crude extract (62.5–250 µg/mL), hexane extract (62.5–250 µg/mL) and chloroform extract (125–500 µg/mL).

Antimicrobial activity of *B. stenophylla* (rhizomes) crude extract against pathogenic *Leptospira* spp. showed different MIC values across the types of chemical extractants used (Table 3).

Table 2. Antibiotic susceptibility testing of pathogenic and intermediate *Leptospira* spp.

Leptospira pathogenicity	Strain ID	Minimum inhibitory concentration (µg/mL)*						
		Penicillin G	Amoxicillin	Ampicillin	Doxycycline	Cefotaxime	Chloramphenicol	Erythromycin
Pathogenic strains	CFP3	0.39	**0.20**	**0.20**	0.78	**0.20**	1.56	**0.20**
	CFP16	0.78	0.39	0.39	1.56	**0.20**	1.56	**0.20**
	CFP18	3.13	0.39	0.39	6.25	**0.20**	3.13	**0.20**
	CFP21	3.13	0.39	0.39	12.5	**0.20**	3.13	**0.20**
	CFP34	0.39	**0.20**	**0.20**	0.78	**0.20**	1.56	**0.20**
	CFP35	0.39	**0.20**	**0.20**	0.78	**0.20**	1.56	**0.20**
Intermediate strains	CFG3	0.39	**0.20**	**0.20**	0.78	**0.20**	0.78	**0.20**
	CFG4	0.78	0.39	0.39	1.56	**0.20**	1.56	**0.20**
	CFG12	0.78	0.39	0.39	1.56	**0.20**	1.56	**0.20**
	CFG14	3.13	0.39	0.39	3.13	**0.20**	3.13	**0.20**
	CFG15	0.39	**0.20**	**0.20**	0.78	**0.20**	1.56	**0.20**
	CFG18	0.39	**0.20**	**0.20**	0.78	**0.20**	0.78	**0.20**

*Indicates the minimum inhibitory concentration (MIC) was expressed in µg/mL except the penicillin G values expressed in units/mL. Seven antibiotics were tested against pathogenic and intermediate *Leptospira* isolates. The lowest MIC was highlighted in boldface.

Table 3. Antimicrobial activity of crude extracts derived from *H. formicarum* jacks, *B. stenophylla* leaves, and *B. stenophylla* rhizomes against pathogenic and intermediate *Leptospira* spp.

Leptospira pathogenicity	Strain ID	Minimum inhibitory concentration (µg/mL)											
		Crude extract of *H. formicarum* jacks				Crude extract of *B. stenophylla* leaves				Crude extract of *B. stenophylla* rhizomes			
		Ethyl acetate extract	Methanol extract	Hexane extract	Chloroform extract	Ethyl acetate extract	Methanol extract	Hexane extract	Chloroform extract	Ethyl acetate extract	Methanol extract	Hexane extract	Chloroform extract
Pathogenic strains	CFP16	125	125	250	500	**62.5**	125	125	250	125	250	250	500
	CFP18	250	250	500	500	125	250	250	500	250	500	500	>500
	CFP21	250	250	500	500	125	250	250	500	250	500	500	>500
	CFP3	125	250	250	500	**62.5**	125	125	250	250	250	250	500
	CFP34	125	250	250	500	**62.5**	**62.5**	**62.5**	125	125	125	125	250
	CFP35	125	250	250	500	**62.5**	125	125	250	250	250	250	500
Intermediate strains	CFG4	125	125	125	250	125	125	125	250	125	250	250	250
	CFG12	125	250	125	250	125	125	125	250	125	250	250	250
	CFG14	250	250	125	500	250	250	250	500	250	250	250	500
	CFG3	**62.5**	250	250	250	**62.5**	**62.5**	**62.5**	125	**62.5**	**62.5**	**62.5**	125
	CFG15	**62.5**	125	125	250	**62.5**	**62.5**	**62.5**	125	**62.5**	**62.5**	**62.5**	125
	CFG18	**62.5**	125	125	250	**62.5**	**62.5**	**62.5**	125	**62.5**	**62.5**	**62.5**	125

Ethyl acetate crude extract showed the lowest MIC (125–250 µg/mL), followed by methanol crude extract (125–500 µg/mL), hexane extract (125–500 µg/mL) and chloroform extract (250–500 µg/mL). For intermediate *Leptospira* strains, ethyl acetate crude extract showed the lowest MIC (62.5–250 µg/mL), followed by methanol crude extract (62.5–250 µg/mL), hexane extract (62.5–250 µg/mL) and chloroform extract (125–500 µg/mL).

GC-MS analysis

Phytochemical analysis of *H. formicarium Jack* and *B. stenophylla* extracted using four different solvents (methanol, ethyl acetate, chloroform, and hexane) using GC-MS revealed a variety of phytochemical components (Supplementary Table S1-S8).

A total of 17 compounds were identified in the crude ethyl acetate extract of *H. formicarum,* comprising n-hexadecanoic acid (22.11%), followed by octadecanoic acid (11.86%) and 1,3,5-triphenyl-cyclohexane (6.02%) and other 14 chemical compounds ranging from 0.72 to 5.43%. The crude methanol extract of *H. formicarum* showed that (E)-4-methoxy-6-styryl- 2H-pyran-2-one is the major compound (23.80%), followed by 3-phenyl-2-propenoate (12.03%), isospathulenol (8.33%) and other seven chemical compounds with compositions ranging from 1.06% to 3.50%. Variety of compounds (16) identified in hexane crude extract for *H. formicarum,* whilst tetracontane represented the major composition (39.36%), followed by Bis(2-ethylhexyl) phthalate (11.94%), and Decanedioic acid, bis(2-ethylhexyl) ester (11.26%). The other 13 compounds ranged from 0.62 to 9.32%. Chemical compositions for chloroform extract are mostly composed of hexadecanoic acid methyl ester (15.48%), followed by tetracontane (12.08%) and dotriacontane (6.82%). Other 10 compounds constituted 1.33-5.92% of the crude extract.

Crude extract of *B. stenophylla* using ethyl acetate produced remarkable high amount of (E)-4-Methoxy-6-styryl-2H-pyran-2-one (46.27%), 2,4-Di-tert-butylphenol (8.95%), 2-Propenoic acid, 3-phenyl-methyl ester (7.39%), and other compounds ranging from 0.72 to 2.7%. In methanol crude extract *B. stenophylla,* the major compound was (E)-4-Methoxy-6-styryl-2H-pyran- 2-one (17.88%), followed by kaurene (14.36%), and 3-Phenyl-2-Propenoic acid methyl ester (10.16%). Other compounds ranging 0.69 to 7.49%. Extraction using hexane produced a significantly high amount of Kaur-16-ene (62.90%), and other compounds with amounts ranging from 0.86 to 3.78%. Whereas seven compounds were identified from chloroform extraction with bis (2-ethylhexyl) phthalate constituted the highest (5.23%), phthalic acid, bis(2-ethylhexyl) ester (6CI,8CI) (4.9%) and others (0.95-2.18%).

DISCUSSION

This study determined the minimum inhibitory of antibiotics and local herbs against six local strains of serovars Sejroe, Icterohaemorrhagiae, Grippotyphosa and Pomona, belonging to species *Leptospira noguchii, L. borgpetersenii, L. wolffii,* and *L. inadai* (Table 1). We utilised the microbroth dilution method to determine MICs since the agar diffusion method is suboptimal for evaluating the antimicrobial activity of plant extracts. This is due to insensitivity, poor diffusion of non-polar molecules into the agar matrix, and lack of reproducibility. (Eloff, 2019). Currently, no breakpoint for *Leptospira* is established in the standards and guidelines from the Clinical and Laboratory Standards Institute (CLSI). Therefore, anti-*leptospiral* activities were interpreted based on the demonstrated inhibitions in the assays.

Antibiotic susceptibility testing of this study suggests cefotaxime and erythromycin exhibited the strongest anti-leptospiral activity among the seven studied antibiotics, as shown with the lowest MIC (0.2 µg/mL) against all tested isolates of pathogenic and intermediate *Leptospira*, which agrees with the previous findings (Murray & Hospenthal, 2004a), indicating that these two antibiotics have the highest efficacy against all Leptospira strains studied. Nevertheless, the MICs of these two antibiotics have been consistently lower than those of the current leptospirosis drugs of choice, penicillin G and doxycycline. Doxycycline exhibited a significantly high MIC (0.78–12.5 µg/mL), and penicillin G exhibited a relatively high MIC (0.39–3.13 µg/mL).

The MICs for penicillin G, ampicillin, and amoxicillin ranged from 0.39 to 3.13 µg/mL. Unlike doxycycline, penicillin G demonstrated better inhibition against *Leptospira* spp. and is commonly prescribed for treating serious leptospirosis due to the low level of toxicity and the ability to deliver the drug intravenously at high dosages at the initial stage of infection (Watt *et al*., 1988). In contrast, ampicillin and amoxicillin exhibited lower MICs than penicillin G. This finding is in tandem with previous studies where ampicillin and amoxicillin had lower MICs than penicillin G or doxycycline for almost all tested strains (Murray & Hospenthal, 2004a). Previous studies have reported using ampicillin as a possible alternative for *in vivo* and *in vitro* treatment of leptospirosis. However, the ampicillin reaction against leptospirosis is limited. It cannot permeate all organ tissues (such as the heart and kidneys), rendering it ineffective in clearing leptospires in the protected sites (Truccolo *et al*., 2002).

To our knowledge, this is the first study on the anti-leptospiral properties of *H. formicarum* Jack and *B. stenophylla*. The findings showed that both *H. formicarum* Jack and *B. stenophylla* had potent anti-leptospiral activity; however, they exhibited variations according to the type of solvent used. Besides plant species, extraction procedure and solvent are major criteria for producing plant extract with high anti-microbial properties. The lowest MIC of the plant extracts (*H. formicarum* Jack and *B. stenophylla*) in this study (62.5 µg/mL) like another study utilising *Boesenbergia rotunda* (Chander *et al*., 2016), however, lower than another similar study utilising *Phyllanthus amarus* extract which obtained 100 µg/mL (Ismail *et al*., 2021).

MIC values for the plant extracts were higher compared to antibiotics, and this pattern was also observed in another study. Intermediate *Leptospira* was inhibited more than pathogenic *Leptospira* by crude extract *H. formicarum* Jack and *B. stenophylla*, suggesting variations in the structure of the bacterial cell envelope alter permeabilisation and membrane disruption after antibacterial drug exposure (Silhavy *et al*., 2010). Solvents with toxic properties may also affect the microbiological evaluation of substances with poor water solubility. For example, ethyl acetate has high toxicity (Punnam Chander *et al*., 2015). Methanol is typically the preferred solvent because, despite being polar, its amphiphilic properties allow it to dissolve nonpolar compounds.

The major phytochemical compounds in *H. formicarum* Jack were tetracontane (39.36% in hexane extract), n-hexadecanoic acid (22.11% in ethyl acetate extract), hexadecenoic acid methyl ester (15.48% in chloroform extract), and (E)-4-methoxy-6-styryl-2H-pyran-2-one (23.80% in methanol extract). Whilst the major phytochemical constituents in *B. stenophylla* were 16-kaurene (62.9% in hexane extract) and (E)-4-methoxy-6- styryl-2H-pyran-2-one (46.27% in ethyl acetate extract; 17.88% in methanol extract (Table 3). Of the compounds listed, (E)-4-methoxy-6-styryl-2H-pyran-2-one and 16-Kaurene are classified as flavonoid and terpenoid, respectively and known as the major phytochemicals exhibiting antimicrobial properties (Huang *et al*., 2022).

Other compounds, including tannins, alkaloids, lignins, saponins, anthraquinones, and steroids, display antimicrobial properties primarily by distorting the formation or structure of bacteria cells, leading to increased fluidity. This provokes uncontrolled efflux of metabolites, ions, and membrane proteins, ultimately causing cell perforation and death. Flavonoids, saponins, tannins, and anthraquinones cause increased cell permeability and distortion to the microbial cell wall, leading to cytoplasmic leakage. Terpenoids, alkaloids, tannins, and anthraquinones hinder biofilm formation, compromising the resistance mechanism to antibacterial agents. Terpenoids, alkaloids, saponins, and tannins impede bacterial propagation by interfering with bacterial physiology and metabolism, inhibiting protein, nucleic acid, and adenosine triphosphate synthesis (Ismail *et al*., 2021). A previous study demonstrated the antimicrobial activity of *H. formicarum* Jack against many gram-positive and gram-negative bacteria (e.g. *E. coli*, *Salmonella* spp., *Listeria monocytogenes*, and *Bacillus cereus*) (Prachayasittikul *et al*., 2008). However, our study represents the first to demonstrate the antimicrobial activity of *H. formicarum* and *B. stenophylla,* specifically on pathogenic and intermediate *Leptospira* species. This study supports the anti-leptospiral activity of these phytochemical compounds in the study by Ismail *et al*. (2021) performed on different plant species, *Phyllanthus amarus*.

Anti-leptospiral activity of hexane extracts was only observed in *B. stenophylla* but not in *H. formicarum* Jack. Kaur-16-ene is a diterpene compound that possesses a wide range of antimicrobial activities (Oliveira *et al*., 2020; Saha *et al*., 2022), and a significant concentration of Kaur-16-ene contained in the hexane extract of *B. stenophylla* (containing 62.90%) could be the reason for the observed anti-leptospiral properties. Nevertheless, the inhibition of leptospires demonstrated may not be the only action limited to the major compounds. Other compounds present in traces may also synergically inhibit bacterial growth that is not limited to *Leptospira*. Besides the secondary metabolites in the plant extracts, the concentration also causes the antimicrobial effect and its potential interaction with other components. For example, the reaction of tannins with bacterial wall proteins and binding to proline-rich protein disrupts protein synthesis (Hemeg *et al*., 2020).

CONCLUSIONS

Pathogenic and intermediate strains of *Leptospira* were susceptible to the tested antibiotics and two indigenous herbs that demonstrated anti-leptospiral activity. Although *Leptospira* is not currently known to develop antibiotic resistance, it is important to recognise that bacterial resistance often emerges in response to antibiotic use. Therefore, there is a need to explore potential therapeutic alternatives that could enhance treatment options in the future. Additionally, further research involving in-vivo experiments using animal models is necessary to assess the effectiveness and safety of these treatments before they can be applied in clinical settings.

Conflict of interests

No conflict of interest throughout this study.

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