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In vitro antibacterial activity of andrographolide and hordenine against methicillin-resistant *Staphylococcus aureus* and *Staphylococcus aureus*#

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Abstract

Bacterial infections are difficult to treat, especially those infected with resistant bacteria. Due to the haphazard use of antibiotics in humans and animals, methicillin-resistant Staphylococcus aureus (MRSA) has developed resistance to practically all commercially available antibacterial drugs. Exploring natural plant compounds with antibacterial activity could be beneficial in the treatment of infections with MRSA and Staphylococcus aureus. Andrographolide, a labdane diterpenoid derived from Andrographis paniculata, and Hordenine, a phenethylamine alkaloid found in Hordeum vulgare, are reported to have antibacterial activity. In the present study, the antibacterial activity of andrographolide and hordenine was tested against MRSA and S. aureus using the Kirby-Bauer agar disc diffusion method and the modified resazurin microtiter plate assay. The results of the study revealed that both andrographolide and hordenine at various concentrations did not show any zone of inhibition against MRSA and S. aureus. Hordenine showed an MIC of 1000 µg/mL against MRSA and S. aureus, but andrographolide had no effect on both MRSA and S. aureus.

Keywords: *Andrographolide, Hordenine, antibacterial activity, MRSA, S. aureus*

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Bacterial infections are difficult to treat, especially those infected with resistant bacteria. Methicillin-resistant *S. aureus* (MRSA) is the one of the most common multidrug-resistant *S.aureus* (MRSA) in India, with rates varying from 25 per cent in the west to 50 per cent in the south (Joshi *et al*., 2013). Vancomycin-intermediate *S. aureus* (VISA) emerged as a result of *S. aureus*' adaptive mutation to vancomycin, which had long been the last line of defence against MRSA infection (Hiramatsu *et al*., 2014). Due to the haphazard use of antibiotics in humans and animals, MRSA has developed resistance to practically all commercially available antibacterial drugs. Furthermore, the systemic toxicity associated with the administration of high antibiotic doses is a major concern. The lack of development of newer antibiotics in recent years adds to the concern.Exploring natural plant compounds with antibacterial activity could be beneficial in the treatment of infections with MRSA and *S. aureus.* Andrographolide, a labdane diterpenoid, is an anti-inflammatory, antibacterial, antiviral, anti-asthmatic, anti-cancer, anti-malarial, immunosuppressive, hepato-protective, cardio-protective, anti-obesity, anti-diabetes, and anti-leukaemic compound derived from *Andrographis paniculata* (Dai *et al*. 2019). *Hordeum vulgare* seeds have long been used to aid wound healing. This purported application of *H. vulgare* seeds has not been scientifically validated. According to studies, a phenethylamine alkaloid, hordenine, found in *H. vulgare*, has antibacterial, anticancer, and anti-melanogenic properties (Rashid *et al.* 2017). Hence, the present study is envisaged to assess the antibacterial activity of andrographolide and hordenine, which in future could be utilised as promising agents in the treatment of infections.

Materials and methods

Bacterial isolates

The bacterial isolates used in the study included gram-positive bacteria *S. aureus* and methicillin-resistant *S. aureus*. The characterised isolates were obtained from the Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Pookode. Bacterial isolate stock cultures were kept on brain heart infusion agar and stored at 4°C until used as inoculum.

Preparation of stock solutions

Andrographolide, being insoluble in water, dimethyl sulphoxide (DMSO) was used as a solvent. Andrographolide stock solution was prepared using five per cent DMSO. Two-fold serial dilution of andrographolide was made by sequential transfer of 500 µL stock solution to the subsequent microcentrifuge tubes containing 500 µL of sterile distilled water after proper mixing, and finally 500µL was discarded from last tube. Hordenine, being insoluble in water, DMSO was used as a solvent. Hordenine stock solution was prepared using three per cent DMSO. Two-fold serial dilution of hordenine was prepared in a similar fashion as that of serial dilution of andrographolide.

Preparation of the culture media

Dehydrated culture media (Muller Hinton agar) used for the study were reconstituted in distilled water as per manufacturer's instructions and sterilised by autoclaving at 121°C and 15 psi pressure for 15 min. Cooled the sterilised molten medium to 40-50°C and poured into sterile, dry petri plates on a leveled surface, to a depth of 4± 0.2 mm and allowed to solidify and then incubated at 37°C for 24 h to check sterility. The sterile agar plates were refrigerated at 4°C, with agar side up to avoid the condensed water drip from the lid onto the agar.

Preparation of inoculum

Direct colony suspension was prepared from 18-24 h old pure culture of the isolates by transferring five isolated colonies with a sterile wire loop into a test tube containing 5 mL phosphate buffered saline and vortexed it. The turbidity of the suspension density was compared visually with 0.5 McFarland turbidity standard (equivalent to $1.5x10^8$ CFU/mL).

Kirby-Bauer agar disc diffusion method procedure

Antibacterial activity of andrographolide and hordenine was determined by agar disc diffusion method according to Clinical and

Laboratory Standards Institute (CLSI, 2020). A sterile cotton swab on a wooden applicator was dipped into the standardized inoculum (0.5 McFarland) and excess fluid was removed by pressing and rotating the swab against the side of the tube above the suspension. The entire agar surface of the Muller Hinton agar (MHA) plates was spread with the swab three times, turning the plate at 60° angle between each spreading to ensure even distribution. Under aseptic conditions six sterile discs were placed on MHA agar plates using sterile forceps with distance of 24 mm (centre to centre). A sterile pipette was used to place 10 µL of various concentrations of andrographolide and hordenine ranging from 1000 to 62.5 µg/mL into each disc. Five and three per cent DMSO was used as the vehicle control for andrographolide and hordenine respectively. The plates were then incubated at 37°C for 24 h. The diameter of the zone of complete inhibition was measured. The experiment was repeated thrice.

Preparation of bacterial inoculum

The stock microbial suspension was prepared from 18-24 h old pure culture of the isolates by transferring five isolated colonies with a sterile wire loop into a test tube containing 5 mL sterile cation adjusted Muller Hinton broth and vortexed. It was then diluted to get a final concentration of $1.5x10^8$ CFU/mL. The turbidity of the suspension density was compared visually with 0.5 McFarland standard (equivalent to $1.5x10^8$ CFU/mL).

Preparation of resazurin solution

Resazurin dye was prepared at concentration 0.015 per cent by dissolving 0.015 g of Resazurin sodium salt powder in 100 mL sterile distilled water, sterilised by filtration, and then stored at 4°C for a maximum of two weeks after preparation.

Modified resazurin microtiter plate assay procedure

In a 96 wells flat bottom microtiter plate, 100 µL of the individual test cultures (at a final concentration of $1.5x10^8$ CFU/mL were co-incubated with decreasing concentrations of andrographolide and hordenine (1000, 500, 250, 125 and 62.5 µg/mL) in 100 µL of cation adjusted Muller Hinton broth. After the incubation at 37 °C for 18 to 24 h, 20 µL of the resazurin dye (0.015 per cent) was added to all the wells to determine the dye reduction (from purple to pink) and thereby the bacterial inhibition. The lowest concentrations of andrographolide and hordenine without visible growth were designated as MIC (Mohammed *et al*., 2020). The experiment was repeated thrice.

Results and discussion

Kirby-Bauer Agar disc diffusion method

The results of the present study are depicted in Table 1 and 2. Agar disc diffusion assay was conducted to analyse the *in vitro* antibacterial activity of andrographolide and hordenine against *S. aureus* and MRSA (Fig.1, 2). In the present study, both andrographolide and hordenine at concentrations of 1000, 500, 250, 125 and 62.5 µg/mL did not show any zone of inhibition against MRSA and *S. aureus.* The results of the present study are in accordance with the observations made by Xu *et al.* (2006), who reported that andrographolide

Table 1. *In vitro* antibacterial activity of antibiotic discs against MRSA using Kirby- Bauer agar disc diffusion method, mm

Antibiotics	Mean zone of inhibition (diameter)*	
	MRSA	
Oxacillin 5 µg	0	R
Cefoxitin 30 µg	17 ± 0.57	R
Ceftriaxone:sulbactam 30:15 µg	27.66 ± 0.33	S
Vancomycin 30 µg	0	R
Gentamicin 50 µg	19.66 ± 0.33	S
Azithromycin 30 µg	14.66 ± 0.88	I

Resistant, S- Susceptible, I- Intermediate, *- Value expressed as Mean ± SE

Table 2. *In vitro* antibacterial activity of antibiotic discs against *S. aureus* using Kirby- Bauer agar disc diffusion method, mm

Antibiotics	Mean zone of inhibition (diameter)*	
	S. aureus	
Oxacillin 5 µg	26.66 ± 0.88	S
Cefoxitin 30 µg	23.66 ± 0.33	S
Ceftriaxone:sulbactam 30:15 µg	22 ± 0	S
Vancomycin 30 µg	21.33 ± 0.33	S
Gentamicin 50 µg	17 ± 0	S
Azithromycin 30 µg	20.66 ± 0.33	S
Linezolid 30 µg	18.33 ± 0.66	S
Doxycycline 30 µg	23.66 ± 0.66	S

S-Susceptible, *- Value expressed as Mean ± SE

Table 3. Minimum inhibitory concentration of andrographolide and hordenine estimated using modified resazurin microtiter plate assay, µg/mL

Test substances	Bacterial isolates	
	MRSA	*S. aureus*
Andrographolide	-	-
Hordenine	1000	1000
Ceftriaxone-sulbactam	30	30

at concentrations of 5 mg, 0.5 mg, 50 µg did not show any antibacterial activity against *S. aureus.* Leelarasamee *et al.* (1990) also reported that the crude powder suspension of *Andrographis paniculata* in water, at 25 g/L didn't show any antibacterial activity against *S. aureus in vitro*. Ganapathy and Karpagam (2016) studied the antibacterial activity of *A. paniculata* against MRSA using the agar disc diffusion method and reported that chloroform and ethanol extracts showed the antibacterial activity but hexane and water extracts did not show antibacterial activity against MRSA.

There are limited published data on the antimicrobial properties of hordenine or extracts of *H. vulgare* against *S. aureus* and no data on their antimicrobial activities against MRSA. Jaounil and Selim (2017) stated that the methanol extract of *H. vulgare* showed the antimicrobial activity against *S. aureus*. The reported work contradicts our findings. Ong (2020) stated that antibiotic migration into the agar has been one of the important factors in the agar disc diffusion method. The possible reason for the absence of hordenine's antimicrobial activity in this study could be attributed to a lack of migration of hordenine into the Mueller Hinton agar. Hence, a modified resazurin microtiter plate assay has been opted, which is a test in which the microorganism has a direct interaction with plant compounds.

Modified resazurin microtiter plate assay

Modified resazurin microtiter plate assay determines the minimum inhibitory concentration (MIC) of the test substance. The MIC of an antimicrobial is the lowest concentration that will prevent the visible growth of a microorganism after an overnight incubation period. In the present study, andrographolide did not show any activity against MRSA and *S. aureus* whereas hordenine exhibited MIC of 1000 µg/mL (Table 3, Fig.3). The results of the present study are in accordance with the observations made by Chen *et al.* (2011) who reported that andrographolide did not inhibit the growth of *S. aureus* or MRSA. Limited data are available on the antimicrobial activities of hordenine or extracts of *H. vulgare* against *S. aureus* using modified resazurin microtiter plate assay. Jaounil and Selim (2017) stated that the methanol extract of *H. vulgare* showed the antimicrobial activity against *S. aureus* with MIC of 125 and 500 µg/mL. There is no data on the antibacterial activity of hordenine or extracts of *H. vulgare* against MRSA using modified resazurin microtiter plate assay. Our data revealed that andrographolide had no effect on

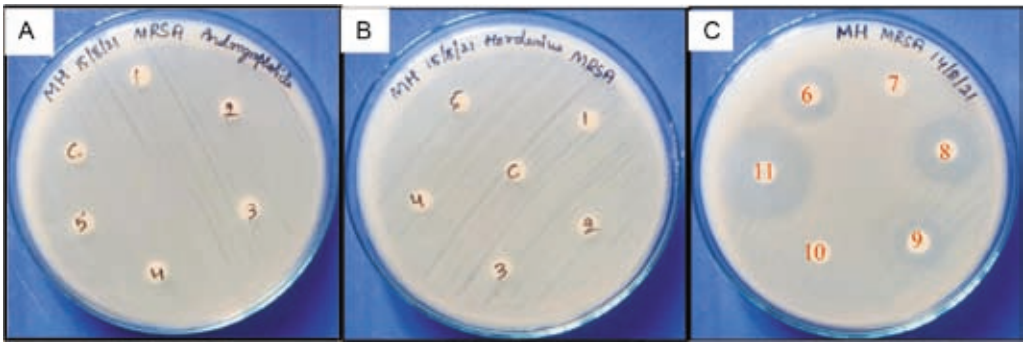

Fig. 1. *In vitro* antibacterial activity of (A) andrographolide and (B) hordenine (C) antibiotics against MRSA: C- control, 1-1000 µg/mL, 2- 500 µg/mL, 3- 250 µg/mL, 4- 125 µg/mL, 5- 62.5 µg/mL,6- Cefoxitin, 7- Oxacillin, 8- Gentamicin, 9- Azithromycin, 10- Vancomycin and 11- Ceftriaxone-sulbactam

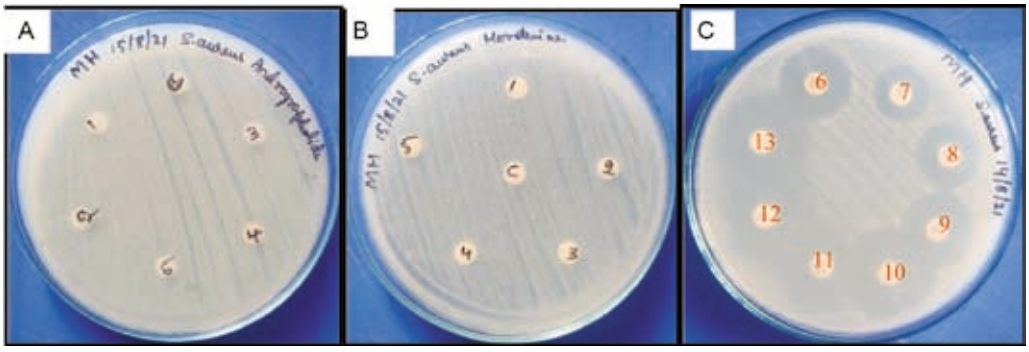

Fig. 2. *In vitro* antibacterial activity of (A) andrographolide and (B) hordenine (C) antibiotics against *S. aureus*: C- control, 1-1000 µg/mL, 2- 500 µg/mL, 3- 250 µg/mL, 4- 125 µg/mL, 5- 62.5 µg/mL, 6- Doxycycline, 7- Vancomycin, 8- Azithromycin, 9- Gentamicin, 10- Linezolid, 11-Ceftriaxone-sulbactam, 12- Oxacillin, 13- Cefoxitin

MRSA or *S. aureus*, whereas hordenine had a MIC of 1000 µg/mL.

Conclusion

The present study revealed that hordenine at 1000 µg/mL was effective against MRSA and *S. aureus*. Andrographolide did not show any activity against MRSA and or *S. aureus*.

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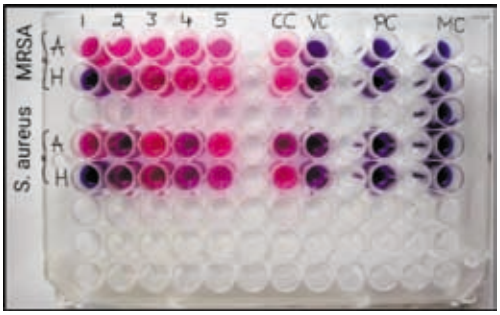

Fig. 3. Minimum inhibitory concentration of andrographolide and hordenine estimated using modified resazurin microtiter plate assay: A- andrographolide, H- hordenine, from left to right: 1-1000 µg/mL, 2- 500 µg/mL, 3- 250 µg/mL, 4- 125 µg/mL, 5- 62.5 µg/mL, CC- Culture control, VC- Vehicle control, PC- Positive control, MC- Media control

Conflict of interest

The authors disclose that they do not have any conflict of interest.

References

Chen, Z.G., Zhu, Q., Zong, M.H., Gu, Z.X. and Han, Y.B. 2011. Enzymatic synthesis and antibacterial activity of andrographolide derivatives. *Process Biochem*. **46**: 1649-1653.

CLSI [Clinical and laboratory standards institute]. 2020. *Performance standards for antimicrobial susceptibility testing*: *CLSI supplement M 100*. (30th Ed). Wayne, P A. 332p.

Dai, Y., Chen, S.R., Chai, L., Zhao, J., Wang, Y. and Wang, Y. 2019. Overview of pharmacological activities of *Andrographis paniculata* and its major compound andrographolide. *Crit. Rev. food Sci. Nutr.* **59**: S17-S29.

Ganapathy, S. and Karpagam, S. 2016. *In vitro* evaluation of antibacterial potential of *Andrographis paniculata* against resistant bacterial pathogens methicillin resistant *Staphylococcus aureus* (MRSA) and multiple drug resistant *Escherichia coli* (MDR E. coli). *Int. J. Bioassays*. **5**: 4879-4881.

Hiramatsu, K., Katayama, Y., Matsuo, M., Sasaki, T., Morimoto, Y., Sekiguchi, A. and Baba, T. 2014. Multi-drug-resistant *Staphylococcus aureus* and future chemotherapy. *J. Infect. Chemother.* **20**: 593-601.

Jaounil, S.A. and Selim, S. 2017. Antimicrobial and antimutagenicity potentials of *Hordeum vulgare* L. extract. *Biosci. Res*. **14**: 139-142.

Joshi, S., Ray, P., Manchanda, V., Bajaj, J., Chitnis, D.S., Gautam, V., Goswami, P., Gupta, V., Harish, B.N., Kagal, A. and Kapil, A. 2013. Methicillin resistant *Staphylococcus aureus* (MRSA) in India: prevalence & susceptibility pattern. *The Indian J. Med. Res*. 137: 363-369.

Leelarasamee, A., Trakulsomboon, S. and Sittisomwong, N. 1990. Undetectable anti-bacterial activity of *Andrographis paniculata* (Burma) wall. Ex ness. J. *Med. Assoc. Thai.* **73**: 299-304.

Mohammed, T.K., Aqel, N. and Al-Dujaili, E.A. 2020. Antimicrobial activity of liquid residues of *Cymbopogon citratus* oil extracts. *J. Phys: Conf. Ser*. **1660**: 12006-12023.

Ong, I. 2020. Zone of inhibition test: why bigger isn't always better. *Microban.* [on line]. Available: https://www.microban.com/blog/zone-inhibition-test-bigger-not-always-better. [16 April 2020].

Rashid, K., Kumar, C.S. and Haleel, P.M. 2017. Healthcare benefits of *Hordeum vulgare* L (Barley): A phyto-pharmacological review. *Res. J. Pharmacol. Pharmacodynamics*. **9**: 207-210.

Xu, Y., Marshall, R.L. and Mukkur, T.K. 2006. An investigation on the antimicrobial activity of *Andrographis paniculata* extracts and andrographolide *in vitro*. *Asian J. Pl. Sci*. **5**: 527-530.

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