

ANTIBACTERIAL ACTIVITY OF ETHANOLIC PLANT EXTRACTS ON MULTIDRUG RESISTANT ACINETOBACTER BAUMANNII CLINICAL ISOLATES

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Abstract

Acinetobacter baumannii is one of the most important human pathogen frequently recovered from different clinical samples and responsible for nosocomial infections. *A. baumannii* contains a repertoire of antibiotic resistance mechanisms including drug modification enzymes, alteration of target sites, efflux pumps and others. There are increasing reports of infections caused by MDR *A. baumannii*, which limits the availability of suitable antibiotics. The present study evaluates the Anti-*Acinetobacter* activity of different plant extracts. The ethanolic extract of 10 different plant materials was screened by agar-well diffusion method and the minimal inhibitory concentration was also determined using broth-macrodilution technique against 15 clinical MDR *A. baumannii* isolates. Our results show that the ethanolic extract of *Piper betle* L. (Betel leaf) has a very strong anti-bacterial activity against MDR *Acinetobacter baumannii* strains, producing a mean zone of inhibition of 31.33 mm±1.799 and MIC₉₀ of 16 µg/ml. The ethanolic extract of garlic also exhibited considerable effect against few isolates, producing zone of inhibition ranges from 9-13 mm and the MIC₉₀ was 32 µg/ml. However, the ethanolic extract of the remaining plant materials did not show any antibacterial activity against the tested strains. These results suggest that ethanolic extract of *Piper betle* L. leaf can be a suitable alternative or adjunct for the treatment of MDR *A. baumannii* infections.

Key words: *A. baumannii*, MDR, Betel leaves, Garlic, Antibacterial activity.

Introduction

Acinetobacter baumannii is widely distributed in nature like soil, sewage, water, vegetables, fish, meat, animals and arthropods (Atrouniet *et al.*, 2016 ; Rado *et al.*, 2019; Trajkovska *et al.*, 2009; Yang *et al.*, 2010). It is an emerging pathogen, mainly associated with nosocomial infections and usually considered as less virulent. *A. baumannii* infections are frequently associated with organs having a high fluid content such as CSF, peritoneal fluid, lungs and urinary tract and infections in immunosuppressed patients are particularly significant (Ehlers *et al.*, 2012). *A. baumannii* has a high mortality rate, about 75% in nosocomial pneumonia cases and 20-60% in bacteremic infections (McConnell *et al.*, 2011).

Among the multidrug resistant (MDR) Gram-negative pathogens, *Acinetobacter baumannii* has acquired significant attention this bug generally resistant to three or more classes of drugs (Atrouni *et al.*, 2016; Adams *et al.*, 2009), and is ranked as third highly resistant organism (Longo *et al.*, 2014). In recent years, studies have been conducted regarding the prevalence of this organism in Pakistan and an increased incidence of *Acinetobacter* infections, more specifically with MDR strains, has been reported. Begum and co-workers reported 91 MDR *A. baumannii* isolates from different wound samples, with 100% resistance to beta-lactam antibiotics.

The emergence of acquired multidrug resistance in *A. baumannii* to conventional antibiotics poses a serious therapeutic problem in the treatment of the infections. This alarming situation has directed the efforts towards the identification of novel antibacterial compounds from natural products including plants. Since ancient times, plants have been used for the treatment of various ailments due to their medicinal properties (Adwan *et al.*,

2006). In recent years, a number of studies have indicated the efficacy of various plant extracts against antibiotic-resistant pathogenic organism (Djeussi *et al.*, 2013; Khameneh *et al.*, 2019)

The present study focuses on determining the antibacterial activity of different plant extracts on clinical MDR *A. baumannii* isolates.

Material and Methods

Bacterial strains: Fifteen clinical isolates of multi-drug resistant *A. baumannii* reported in a previous study (Ain *et al.*, 2019) were tested in this study. *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Bacillus subtilis* strains were included as controls.

Plant material: For this study ten plant materials were used including betel (*Piper betle*) leaves, lemon (*Citrus limon*) seeds, lemon (*Citrus limon*) peels, beal (*Aegle marmelos*) leaf, watermelon (*Citrullus lanatus*) peel, star anise (*Illicium verum*), garlic (*Allium sativum*), bitter gourd (*Momordica charantia*) pulp, bitter gourd (*Momordica charantia*) seeds and vinca rosea (*Catharanthus roseus*) leaves.

Preparation of ethanolic plant extract: Plant extracts were prepared following the protocol of Oskay *et al.*, (2009). Briefly, the plant material was washed with distilled water, oven dried at 60°C and pulverized. The plant material was macerated in 95% ethanol (1:2w/v) for seven days in a dark place. The ethanol fraction was filtered (Whatman No.1 filter paper) and evaporated to dryness in a water bath at 50°C. The extract was weighed and dissolved in 95% ethanol at a concentration of 200 mg/mL and stored at 4°C.

Screening for antimicrobial activity: Antibacterial activity of plant extract was screened by agar well diffusion method according to Balouiri *et al.*, (2016). Bacterial suspension was adjusted to McFarland standard No. 0.5 (1×10^8 CFU/mL) and a bacterial lawn was prepared on Mueller-Hinton agar (MHA) plates using sterile cotton swab. Wells were punched out with the help of sterile metal borer of 8 mm diameter. Twenty microliter (20 μ l) of each plant extract was added in the respective well and 95% ethanol was used as control. The plates were incubated at 37°C for 24 hours and zone of inhibition was measured in “mm”. All the experiments were performed in triplicates and results were recorded by calculating the average.

Minimum inhibitory concentration (MIC) of plant extracts: Following the guidelines of Anon., (2012), MIC values of plant extracts were determined by macro-broth dilution method and compared with that of colistin. We prepared concentrations of the extract ranging from 64 to 0.25 μ g/ml by serial dilutions in Mueller Hinton broth medium. Colistin concentration ranges from 16-0.625 μ g/ml used for positive control.

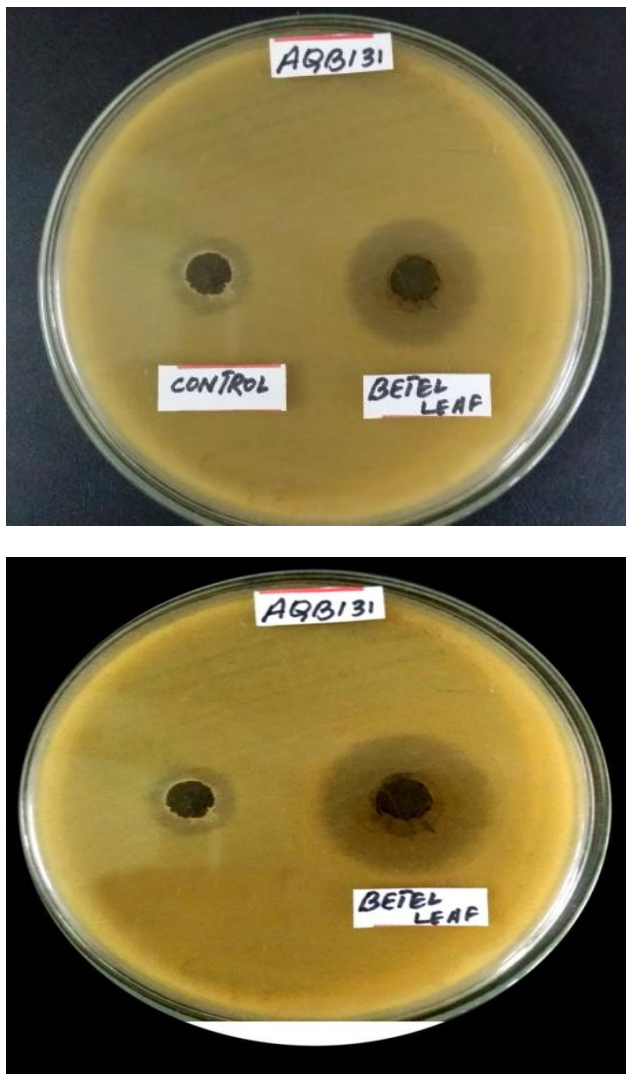


Fig. 1. Antibacterial activity of Betel leaf extract against *A. baumannii* strain AQB131 by agar well diffusion method.

Table 1. Screening for antimicrobial activity of ethanolic plant extracts by agar well diffusion method against *A. baumannii* and other clinical isolates.

Plant extract	Test strains zone of inhibition (mm)														Clinical isolates			
	AQB 24	AQB 88	AQB124	AQB 125	AQB 126	AQB 127	AQB 128	AQB 131	AQB 133	AQB 134	AQB 136	AQB 137	AQB 138	AQB 139	AQB 140	KP	SA	BS
Betel leaf	28	35	30	31	30	32	30	29	30	31	29	29	32	33	31	25	21	11
Lemon seed	10	11	9	9	NZ	NZ	NZ	NZ	10	9	NZ	NZ	NZ	NZ	NZ	11	NZ	NZ
Beal leaf	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Water melon peel	9	9	9	9	NZ	NZ	NZ	NZ	10	10	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Lemon peel	8	8	9	8	NZ	NZ	NZ	NZ	9	9	NZ	NZ	NZ	NZ	NZ	10	9	NZ
Star anise	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	9	NZ	NZ
Garlic	10	12	9	NZ	NZ	NZ	NZ	NZ	13	12	NZ	NZ	NZ	NZ	NZ	10	9	NZ
Bitter gourd pulp	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	10	9	NZ	NZ	NZ	NZ	NZ	10	11	11
Bitter gourd seed	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	10	9	NZ	NZ	NZ	NZ	NZ	11	10	10
<i>Vinca rosea</i> leaf	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	9	9	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Control (Ethanol)	5	7	7	7	7	6	8	9	8	8	7	7	8	9	7	8	8	8

NZ= no zone of inhibition; AQB=*A. baumannii* strains
 Clinical isolates: KP= *Klebsiella pneumoniae*; SA= *Staphylococcus aureus*; BS= *Bacillus subtilis*

Results

In this study, 10 different plant materials were evaluated for their antimicrobial activity against 15 clinical isolates of MDR *A. baumannii* using the agar well diffusion method. The plants tested include: betel leaves, lemon peel, lemon seeds, beal leaf, watermelon peel, star anise, garlic, bitter gourd pulp, bitter gourd seeds and vinca rosea leaves. We observed that betel leaf ethanolic extract showed promising effect on *A. baumannii* isolates (Fig. 1). The maximum zone of inhibition was 35 mm against AQB88 followed by 31 mm against AQB125 and AQB140. The average zone of inhibition was 31.33 mm±1.799. Other plant extracts like water melon peel, lemon seeds, garlic, bitter gourd, bitter gourd seeds and vinca rosea leaf showed weak activity against only a few strains (Table 1).

MIC was determined by broth macro-dilution method. Colistin was used as positive control. The MIC₅₀ and MIC₉₀ of betel leaf was 16 µg/ml and the MIC₅₀ and MIC₉₀ of garlic (*Allium sativum*) extract was >64 µg/ml and 32 µg/ml respectively while all other plant extracts showed MIC value >64 µg/ml (Table 2).

Discussion

The foundation of traditional herbal medicine across the world is based on the curative herbs and medicinal food plants (Taukoorah *et al.*, 2016). The emergence of MDR and PDR has directed the efforts of the scientific community towards the use of plants as potential source of antimicrobial agents. We observed high rate of antibiotic resistance in clinical isolates of *A. baumannii* which prompted us to search some natural compounds as alternative to antibiotics. For this purpose we examined ten plant materials for their antibacterial activity and found that betel leaf extract was most effective against all the tested isolates of MDR *A. baumannii* with MIC₉₀ of 16 µg/ml, followed by the garlic extract with MIC₉₀ of 32 µg/ml. We also included a colistin resistant strain AQB24 in this study and betel leaf extract showed strong activity against this isolate as well while a moderate activity of garlic extract was also noted (Table 1). These results indicate that betel leaf has strong antibacterial activity against MDR *A. baumannii* whereas, other plant extracts i.e., lemon peel, lemon seeds, watermelon peel and bitter gourd exhibited a weak activity. While beal leaf and star anise extracts

failed to show any effect on the tested organism. In order to determine the spectrum of antibacterial activity of these plant extracts, Gram negative *K. pneumoniae*, Gram positive non-sporing *S. aureus* and Gram positive sporing *B. subtilis* were also included. These isolates were obtained from the Department of Microbiology strain collection. Betel leaf ethanol extract showed antibacterial activity against other pathogenic bacteria i.e., *K. pneumoniae* and *S. aureus* however the activity was weak against *B. subtilis* which could be attributed to the spore forming nature of this organism.

Betel leaves have been used for the treatment of different diseases in traditional medicine for a long time (Ali *et al.*, 2010). They are rich in a number of phytochemicals including sterols and hydroxychavicol, which possess antimicrobial activity (Ali *et al.*, 2010; Chakraborty & Shah, 2011). Hoque and co-workers (2011) have reported significant antibacterial activity of ethanol extract of betel leaves against food borne pathogens i.e., a zone of inhibition of 14.67±1.15 mm against *E. coli* and 14.67±0.57 mm against *S. aureus* and MIC value in the range of 0.625-0.75 mg/ml. Similar results were also reported by Datta *et al.*, (2011) who tested activity of betel leaf against clinical bacterial isolates.

Garlic has also been used since ancient times for the treatment of different infections around the world. Louis Pasteur was the first to recognize the antibacterial activity of garlic (Whitemore, 2000). The active ingredient of garlic is Allicin which is believed to have a variety of potential target sites to control the bacteria (Cutler and Wilson, 2004). Khashan (2014) have reported anti-bacterial effect of ethanolic extract of garlic against *Staphylococcus aureus*, and demonstrated that the sensitivity of the *S. aureus* gradually increased with the increasing concentrations of the extract. In another study by Shah *et al.*, (2016), significant antibacterial activity of aqueous garlic extract against beta-lactamase producing *A. baumannii* with MIC in the range of 2.5-10 mg/ml was demonstrated.

A number of studies have been conducted to explore the therapeutic potential of Betel leaves and Garlic but to the best of our knowledge, this is the first report of antibacterial activity of ethanolic extract of betel leaves and garlic on *A. baumannii*. Further detailed studies to understand the mechanism of action and to identify the chemical components responsible for antibacterial activity of *Piper betle* L. are required.

Table 2. Minimum inhibitory concentration of *Acinetobacter baumannii* against various plants extract (N= 15).

Plant extract	Range (µg/ml)	MIC (µg/ml)	MIC50 (µg/ml)	MIC90 (µg/ml)
Betel leaf	64-0.25	16	16	16
Lemon seed	64-0.25	>64	>64	>64
Beal leaf	64-0.25	>64	>64	>64
Water melon peel	64-0.25	>64	>64	>64
Lemon peel	64-0.25	>64	>64	>64
Star anise	64-0.25	>64	>64	>64
Garlic	64-0.25	>64	>64	>32
Bitter groud	64-0.25	>64	>64	>64
Bitter groud seed	64-0.25	>64	>64	>64
Vinca rosea leaf	64-0.25	>64	>64	>64
Colistin	16-0.625	2	2	2

References

- Adams, M.D., G.C. Nickel, S. Bajaksouzian, H. Lavender, A.R. Murthy, M.R. Jacobs and R.A. Bonomo. 2009. Resistance to colistin in *Acinetobacter baumannii* associated with mutations in the pmrAB two-component system. *Antimicrob. Agents Chemother.*, 53(9): 3628-3634.
- Adwan, G., B. Abu-Shana, K. Adwan and F. Abu-Shanab. 2006. Antibacterial effects of nutraceutical plants growing in Palestine on *Pseudomonas aeruginosa*. *Turk. J. Biol.*, 30: 239-42.
- Ain, U.Q., A. Naim and A. Saeed. 2019. Prevalence and resistance profile of clinical isolates of *Acinetobacter* species from Karachi, Pakistan. *R.A.D.S.J. Biol. Res. Appl. Sci.*, 10(1):1-8.
- Ali, I., F. G. Khan, K.A. Suri, B.D. Gupta, N.K. Satti, P. Dutt, F. Afrin, G.N. Qazi and I.A. Khan. 2010. *In vitro* antifungal activity of hydroxychavicol isolated from *Piper betle* L. *Ann. Clin. Microbiol. Antimicrob.*, 9: 7.
- Anonymous. 2012. Performance standards for antimicrobial susceptibility testing; Twenty-second informational supplement. CLSI document M100-S22. Wayne PA: Clinical and Laboratory Standards Institute.
- Atrouni, A. A., M. Hamzeand K. Marie. 2016. Reservoirs of non-baumannii *Acinetobacter* species. *Front. In Microbiology.*, 7(49):1-12.
- Balouiri, M., M. Sadiki and S.K. Ibensouda. 2016. Methods for *In vitro* evaluating antimicrobial activity: A review. *J. Pharm. Anal.*, 6(2): 71-79.
- Begum, S., F. Hasan, S. Hussain and A.A. Shah. 2013. Prevalence of multi drug resistant *Acinetobacter baumannii* in the clinical samples from tertiary care hospital in Islamabad, Pakistan. *Pak. J. Med. Sci.*, 29(5):1253-1258.
- Chakraborty, D. and B. Shah. 2011. Antimicrobial, antioxidative and antihemolytic activity of *Piper betel* leaf extracts. *Int. J. Pharm. Pharm. Sci.*, 3: 192-199.
- Cutler, R.R. and P. Wilson. 2004. Antibacterial activity of a new, stable, aqueous extract of allicin against methicillin-resistant *Staphylococcus aureus*. *Br. J. Biomed. Sci.*, 61(2): 71-74.
- Datta A., A. Ghoshdastidar and M. Singh. 2011. Antimicrobial property of *Piper betel* leaf against clinical isolates of bacteria. *Int. J. Pharm. Sci. Res.*, 2: 104-109.
- Djeussi, D.E., J.A. Noumedem, J.A. Seukep, A.G. Fankam, I.K. Voukeng, S.B. Tankeo, A.H. Nkuete and V. Kuate. 2013. Antibacterial activities of selected edible plants extracts against multidrug-resistant Gram-negative bacteria. *B.M.C. Complement. Altern. Med.*, 13: 164.
- Ehlers, M.M., J.M. Hughes and M.M. Kock. 2012. Prevalence of Carbapenemases in *Acinetobacter baumannii*, Antibiotic Resistant Bacteria - A Continuous Challenge in the New Millennium, Marina Pana, IntechOpen, DOI: 10.5772/30379.
- Hoque, M.M., S. Rattila, M.A. Shishir, M.L. Bari, Y. Inatsu and S. Kawamoto. 2011. Antibacterial activity of ethanol extract of Betel leaf (*Piper betel* L.) against some food borne pathogens. *Bangl. J. Microbiol.*, 28(2): 58-63.
- Khameneh, B., M. Iranshahy, V. Soheili and B.S.F. Bazzaz. 2019. Review on plant antimicrobials: A mechanistic viewpoint. *Antimicrob. Resistance and Infec. Control*, 8:118.
- Khashan, A. A. 2014. Antibacterial activity of garlic extract (*Allium sativum*) against *Staphylococcus aureus* *In vitro*. 3(4): 346-348.
- Longo, F., C. Vuotto and G. Donelli. 2014. Biofilm formation in *Acinetobacter baumannii*. *New Microbiol.*, 37: 119-127.
- McConnell, M.J., J.D. Herrera, Y. Smani, R.L. Rojas, F.D. Pérez and J. Pachón. 2011. Vaccination with outer membrane complexes elicits rapid protective immunity to multidrug-resistant *Acinetobacter baumannii*. *Infect. Immun.*, 79(1): 518-526.
- Oskay, M., D. Oskay and F. Kalyoncu. 2009. Activity of some plants extract against Multi- drug resistant human pathogens. *Iran. J. Pharm. Res.*, 8(4): 293-300.
- Rado, J., E. Kaszab, T. Benedek, B. Kriszt and S. Szoboszlai. 2019. First isolation of carbapenem-resistant *Acinetobacter beijerinckii* from an environmental sample. *Acta Microbiologica et Immunologica Hungarica*. 66(1): 133-130.
- Shah, P.J., M.T. Williamson and S.J. Rathod. 2016. Antibacterial activity of aqueous garlic extract on beta-lactamase producing *Acinetobacter* isolates from skin and soft tissue infections. *Asian J. Pharm. Clin. Res.*, 9(6):152-156.
- Taukoora, U., N. Lall and F. Mahomoodally. 2016. *Piper betel* L. (betel quid) shows bacteriostatic, additive, and synergistic antimicrobial action when combined with conventional antibiotics. *S. Afr. J. Bot.*, 105: 133-140.
- Trajkovska, B.K. 2009. *Acinetobacter* spp.—A serious enemy threatening hospitals worldwide. *Maced. J. Med. Sci.*, 2(2):1857-5773.
- Whitemore, B.B. and A.S. Naidu. 2000. Thiosulfates. In: Naidu A.S. (1st Ed.) Natural food antimicrobial systems. Boca Raton, FL: CRC Press, pp. 265-380.
- Yang, H., L. Liang, S. Lin and S. Jia. 2010. Isolation and characterization of a virulent bacteriophage AB1 of *Acinetobacter baumannii*. *Bio. Med. Central*, 10(131): 1471-2180.

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