ORIGINAL ARTICLE

PROPOLIS AS AN ANTIBACTERIAL AGENT AGAINST CLINICAL ISOLATES OF MDR-*ACINETOBACTER BAUMANNII*

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Background: Multidrug resistant (MDR) *Acinetobacter baumannii* has emerged as an important health care problem. The organism is now identified as an important nosocomial pathogen particularly in the intensive care settings. The therapeutic options to treat this pathogen are limited; thus it needs testing for alternatives, like those of plant origin or natural products. Propolis is one of such products which have been tested against this organism. **Methods:** *A. baumannii* (n=32) were collected from Fatima Memorial Hospital, Lahore. The isolates were identified on the basis of their morphology, cultural characteristics and biochemical profile. The susceptibility of the isolates to various antimicrobials was evaluated as per Kirby-Bauer disc diffusion method according to (CLSI 2010). An ethanolic extract of propolis was prepared by the ultrasonic extraction method and its antibacterial activity was evaluated by the agar well diffusion technique. Minimum inhibitory concentration (MIC) was also determined by the agar dilution technique. **Results:** The isolates were found to be resistant to most of the commonly used anti-acinetobacter antimicrobials; doxycycline however was the exception. Propolis from Sargodha (EPS) and Lahore (EPL) showed zones of inhibition of 21.8±.29 mm and 15.66±2.18 mm respectively. MIC ranges of EPS and EPL similarly was from 1.5–2.0 mg/ml and 4.0–4.5 mg/ml respectively. **Conclusion:** It is clear that EPS has potential edge of activity as compared to EPL. Nevertheless the potential efficacy of propolis must be subjected to pharmaceutical kinetics and dynamics to precisely determine its potential antimicrobial usefulness.

Keywords: MDR *Acinetobacter baumannii*; Propolis; MIC

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INTRODUCTION

Multi-drug resistant (MDR) *Acinetobacter baumannii* are rapidly emerging pathogens in health care setting where it causes infections such as, bacteraemia, pneumonia, meningitis, urinary tract infection and wound infections.[1] These are also responsible for high morbidity and mortality particularly in immunocompromised and hospitalized patients and rank at fourth among the most frequent nosocomial pathogens causing pneumonia particularly in intensive care units.[2]

According to Infectious Diseases Society of America (IDSA), these organisms are on the hit list of six top priority dangerous drug-resistant organisms due to its propensity to develop drug-resistance.[3] During the last decade these have emerged as multi-drug resistant (MDR) and threatening to become a pan-drug resistant.[4] Centres for Disease Control and Prevention (CDC) has defined MDR-Acinetobacter spp., as those organisms that produce resistance to at least one agent in three or more antimicrobial classes, namely β-lactams, aminoglycosides, carbapenems and fluoroquinolones.[5]

The increasing incidence of MDR-*A. baumannii* is a prime example of disparity between unmet medical needs and the current antimicrobial research. Therefore, there is an urgent need for new antimicrobial agents or natural products which can be effective against highly resistant pathogens. Propolis (bee glue) is one of the compounds that are dark colour natural resinous material collected by honeybees (*Apis mellifera*). It has been reported to exert a broad spectrum of biological functions, including anticancer, anti-inflammatory, antioxidant, antifungal and as antibacterial.[6]

The most significant active constituents of propolis are aromatic acids; phenolic compounds especially flavonoids (flavones, flavonols, and flavonones) and phenolic acids.[7]

The antimicrobial properties of propolis are mainly due to the flavononones pinocembrin, flavonoles galangin and the caffeic acid phenethyl ester. Studies have demonstrated that inhibitory effect of propolis on organisms depends on synergism of these compounds.[8].

In Pakistan, propolis is being produced alongside honey in commercial apiaries. According to our knowledge no data has been published regarding the antimicrobial activity of propolis against Gram-negative organisms so far. The present study was conducted to determine the antibacterial activity of Pakistani propolis against clinical isolates of MDR-*A. baumannii*.

MATERIAL AND METHODS

Prior to start this study, approval was obtained from the Ethical Committee, University of Health Sciences, Lahore, Pakistan. Thirty-two clinical isolates of *A. baumannii*; tracheal aspiration n=20, endotracheal tubes n=09, wound swabs n=03 were obtained from the Department of Microbiology, Fatima Memorial Hospital, Lahore.

These isolates were confirmed on the basis of their morphology, cultural characteristics and API 20NE (Biomeurix France). Antibiotic susceptibility profile was performed using Kirby-Bauer disc diffusion method according to Clinical Laboratory Standard Institute (CLSI) 2010 guidelines. Antibiotics used were piperacillin (100µg), piperacillin-tazobactam (100/10µg), tetracycline (30µg), amikacin (30µg), cefotaxime (30µg), imipenem (10µg), ciprofloxacin (5µg), co-trimoxazole (25µg), tigecycline (30µg) and doxycycline (30µg) were tested. Interpretation of was done according to CLSI guidelines. Statistical analysis was done using SPSS 16.0.

Two varieties of Apis mellifera bee propolis; one propolis from Sargodha (EPS) and other from Lahore (EPL) were procured from NARC Islamabad, Pakistan. Both were dark brown colour had hard consistency. The plant origin of EPS was from Shisham (Dalbergia sissoo) and Sumbul (Ferula moschata) while EPL was from Litchi chinensis. The crude propolis was obtained in pieces. These pieces were further dehydrated at 45^{o}C for 5 minutes. The Ultrasonic Extraction (UE) was carried out using a 300 W ultrasonic bath. Propolis was placed in an Erlenmayer flask with the corresponding amount of solvent, i.e., 95% ethanol. It was treated with ultrasound at 25^{o}C for 30 minutes.

After extraction, the mixture was centrifuged at 8000g to obtain the supernatant. The supernatant was named the EPS and EPL. The extracts thus were stored in amber coloured bottles at 4^{o}C till use.[9]

EEPs were screened against isolates of MDR-*A. baumannii* by agar well diffusion assay. *A. baumannii* (ATCC 19606) was used as the quality control. The isolates were adjusted to 0.5 McFarland standards and lawned on Mueller Hinton (MH) agar. The EEPs were separately diluted in ethanol to achieve concentrations of 30%, 15%, 7.5%, 3.75% and 1.875%. Agar plates with 20ml of MH were prepared and wells were cut with a 9 mm sterile borer.

The wells were filled with undiluted and serial dilutions in quantities of 120 µl. The plates were incubated overnight at 35°C. Clear zone ≥12 mm was considered as inhibition. Phenol 6% and ethanol 95% was used as positive control and negative control respectively. Duplicate plates were prepared in this way. This procedure was performed in duplicate.[10]

MIC was determined by agar dilution method using multi-inoculator (MAST, UK). EEPs were mixed separately in MH agar at 50°C to achieve the desired gradient concentrations from 0.5 mg/ml to 1.0mg/ml through 30 mg/ml. The grids of multi-inoculator were filled with 500 µl of each 0.5 McFarland standard bacterial suspensions. Two control plates were also set up in parallel. The positive control plate contained the inoculation of bacteria without any extract while the sterility control contained un-inoculated MH agar plate. All the plates were incubated overnight at 35°C. Triplicate plates were prepared in this way.

RESULTS

All the 32 MDR-A baumannii showed 100% resistance to the commonly used antibiotics including imipenem; most effective drug was doxycycline (Figure-1).

Zone size of inhibition was inversely proportional to the increase in the dilution of EEPs. Overall the EPS showed a higher sensitivity as compared to EPL. At 30% concentration of EPS zone of inhibition was 21.8±.29 mm while at 15% concentration it was 19.5±0.5 mm. At 30% EPL concentration demonstrated 15.66±2.18 mm zone of inhibition while at 15% concentration it was 14.5±0.84 mm (Table-1).

Over all MIC of EPS had better antibacterial activity as compared to EPL (*p*-value <0.001). All the MDR-*A. baumannii* were inhibited at the concentration of 2.0 mg/ml and 4.5 mg/ml of EPS and EPL respectively. Table-2 shows the MIC of EPS; MIC_{50} was 1.5 mg/ml, MIC_{90} and MIC_{100} was 2.0 mg/ml. Whereas the MIC of EPL; MIC_{50} was 4.0 mg/ml, MIC_{90} and MIC_{100} was 4.5 mg/ml.

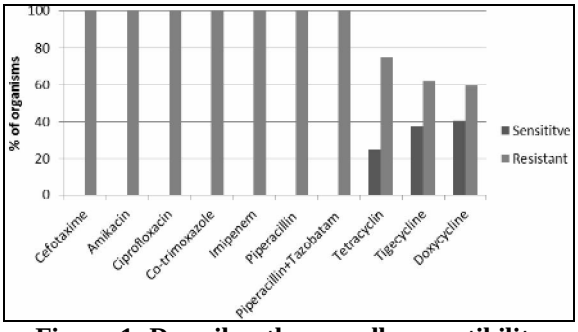

Figure-1: Describes the overall susceptibility pattern of MDR- *A. baumannii* that shows resistance against commonly used antibiotics

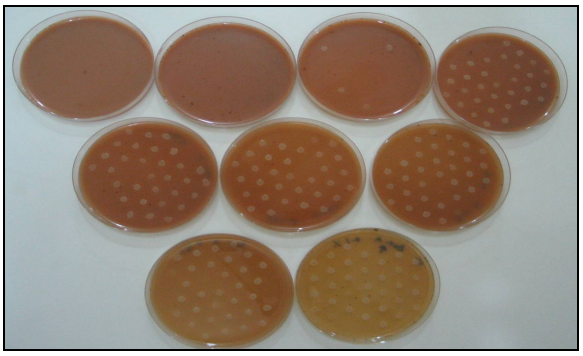

Figure-2: Minimum inhibitory concentrations of propolis extract from Lahore (EPL) against 32 isolates of *A. baumannii*

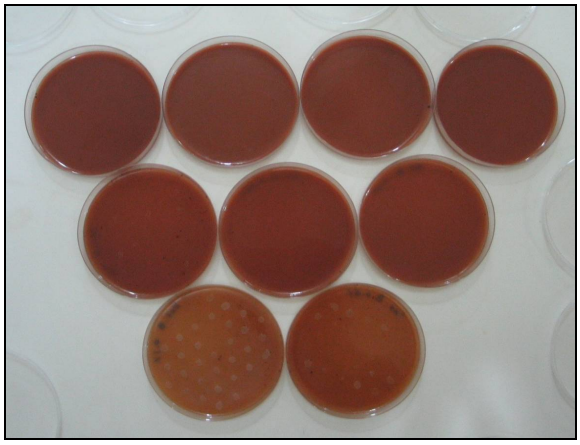

Figure-3: Minimum inhibitory concentrations of propolis extract from Sargodha (EPS) against 32 isolates of *A. baumannii*

Table-1: EPS and EPL effect against MDR *A. baumannii* in agar well diffusion assay

Zone of inhibition (mm)		
Con. of extracts (%)	**EPS Mean±SD**	**EPL Mean±SD**
30	21.8±0.09	15.66±2.8
15	19.5±0.5	14.5±0.84
7.5	17.8±0.29	13.83±1.93
3.75	16.1±0.29	12.9±2.43
1.875	15.0±0.5	11.33±2.43

EPS; ethanolic extract of propolis from Sargodha, EPL; ethanolic extract of propolis from Lahore

Table-2: MIC of EPS and EPL against MDR- *A. baumannii* (n=32)

EPS (MIC range 1.5–2.0)			
	MIC_{50} (mg/ml)	MIC_{90} (mg/ml)	MIC_{100} (mg/ml)
MDR-*A. baumannii*	1.5	2.0	2.0
A. baumannii (ATCC 19606)	1.5	1.5	1.5
EPL (MIC range 2.0–-4.5)			
	MIC_{50} (mg/ml)	MIC_{90} (mg/ml)	MIC_{100} (mg/ml)
MDR-*A. baumannii*	4.0	4.5	4.5
A. baumannii (ATCC 19606)	4.0	4.0	4.0

EPS; ethanolic extract of propolis from Sargodha, EPL; ethanolic extract of propolis from Lahore, ATCC; American Type Culture Collection

DISCUSSION

Emergence and spread of MDR-*A. baumannii* is a matter of great concern and now is in fact becoming a global public health problem. Most of the MDR-*A. baumannii* demonstrated resistance against broad range of antibiotics in this study. These findings are in accordance with the previous studies from Malaysia[11], Saudi Arabia[12], Iran[13] and Pakistan[14]. The high rate of resistance in our setup could be due to the irrational use of antibiotics, broad range of empirical therapy and substandard infection control practices.[15]

As per our knowledge there is no such data published on the antibacterial activity of propolis against MDR- *A. baumannii* so far. In this study all the tested MDR-*A. baumannii* isolates were susceptible to EEPs on agar well diffusion plate. Comparing these two extracts, EPS had better antibacterial activity than EPL. However, there are certain studies conducted on EEP activity against Gram-positive as well as other Gram-negative bacteria around the world.[16] Studies from Brazil[17] and Bulgaria[18] documented that even low concentration EEP had a better activity. Whereas Malaysian propolis is effective at higher concentration[19] These variations could be due to the difference in quality or types, chemical composition and geographical location of the propolis.

In this study, MDR isolates were inhibited at 2.0 mg/ml of EPS and at 4.5mg/ml of EPL as compared to ATCC strain, illustrating that some type of resistance may exist in MDR isolates. But in contrast to this observation both MDR isolates and ATCC strain was inhibited within the same range (1.5–4.5 mg/ml). It might be due to the difference in mechanism of action of propolis because antibiotics have a single mode of action and it is 1000-fold easier to develop resistance against antibacterial drugs. On the other hand EEP has multiple mechanisms due to its various constituents that give their effects simultaneously.[20,21] This showed that EEP was equally effective against MDR and ATCC strain.

In the present study, the MIC range of 50, 90 and 100 isolates was different to EPS (1.5–2.0 mg/ml) and EPL (4.0–4.5 mg/ml). Overall EPS has a better MIC as compared to EPL. According to our knowledge there is no data available on MIC of these EEP against MDR- *A. baumannii* so far. However a Turkish study reported the MIC of EEP was 3.7–281 µg/ml against Acinetobacter lowffi, P. aeruginosa and C. albicans.[22] Similarly an Iranian study also reported the MIC of EEP as 0.75 mg/ml against P. aeruginosa.[23]

The most probable explanation to this is in the differences in composition of propolis, methodology adopted for determination of MIC, other variables such as pH, components of medium, size of inoculum, and length of incubation. One of the disadvantages in

assessing antibacterial activity of unknown substance is lack of standardization in techniques being used giving unreliable results. It is very important to develop guidelines for all procedures adopted in evaluating antibacterial activity of propolis and analyse extracts of propolis of different regions for the actual ingredient which is responsible for their antibacterial activity. Since this organism is MDR, in fact becoming PDR, so the reported antimicrobial activity is of relevance. The present study regarding susceptibility of *A. baumannii* to EEP demonstrates the potential antibacterial activity of propolis on this pathogen with a possibility of its addition to the armamentarium against MDR- *A. baumannii*.

CONCLUSION

We conclude that the EPS was found to be a better inhibitory agent against the isolates of MDR- *A. baumannii* as compared to EPL. It is worth describing that EEP might be utilized as anti *A. baumannii* agent after determining its pharmacokinetics and pharmacodynamics.

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REFERENCES

1. Towner KJ. Acinetobacter: an old friend, but a new enemy. J Hosp Infect 2009;73(4):355–63.
2. Dauner DG, May JR, Steele JC. Assessing antibiotic therapy for *ACINETOBACTER BAUMANNII* infections in an academic medical center. Eur J Clin Microbiol Infect Dis 2008;27(11):1021–4.
3. Talbot GH, Bradley J, Edwards JE Jr, Gilbert D, Scheld M, Bartlett JG, *et al*. Bad bugs need drugs: an update on the development pipeline from the Antimicrobial Availability Task Force of the Infectious Diseases Society of America. Clin Infect Dis 2006;42(5):657–68.
4. Abbo A, Carmeli Y, Navon-Venezia S, Siegman-Igra Y, Schwaber MJ. Impact of multi-drug-resistant *acinetobacter baumannii* on clinical outcomes. Eur J Clin Microbiol Infect Dis 2007;26(11):793–800.
5. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, *et al*. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect 2012;18(3):268–81.
6. Syamsudin, Wiryowidagdo S, Simanjuntak P, Heffen WL. Chemical Composition of Propolis from Different Regions in Java and their Cytotoxic Activity. Am J Biochem Biotech 2009;5(4):180–3.
7. Koru O, Toksoy F, Acikel CH, Tunca YM, Baysallar M, Uskudar Guclu A, *et al*. In vitro antimicrobial activity of propolis samples from different geographical origins against certain oral pathogens. Anaerobe 2007;13(3-4):140–5.
8. Boyanova L, Kolarov R, Gergova G, Mitov I. In vitro activity of Bulgarian propolis against 94 clinical isolates of anaerobic bacteria. Anaerobe 2006;12(4):173–7.
9. Trusheva, B, Trunkova D Bankova V. Different extraction methods of biologically active components from propolis: a preliminary study. Chem Cent J 2007;1:13.
10. Najmadeen HH, Kakamand FAK. Antimicrobial Activity of Propolis Collected in Different Regions of Sulaimani Province-Kurdistanm Region/Iraq. J Duhok Univ 2008;12(1):233–9.
11. Kong BH, Hanifah YA, Yusof MY, Thong KL. Antimicrobial susceptibility profiling and genomic diversity of multidrug-resistant *Acinetobacter baumannii* isolates from a teaching hospital in Malaysia. Jpn J Infect Dis 2011;64(4):337–40.
12. Somily AM, Absar MM, Arshad MZ, Al Aska AI, Shakoor ZA, Fatani AJ, *et al*. Antimicrobial susceptibility patterns of multidrug-resistant Pseudomonas aeruginosa and *Acinetobacter baumannii* against carbapenems, colistin, and tigecycline. Saudi Med J 2012;33(7):750–5.
13. Rahbar M, Mehrgan H, Aliakbari NH. Prevalence of antibiotic-resistant *Acinetobacter baumannii* in a 1000-bed tertiary care hospital in Tehran, Iran. Indian J Pathol Microbiol 2010;53(2):290–3.
14. Hasan, Perveen K, Olsen B, Zahra R. Emergence of carbapenem-resistant *Acinetobacter baumannii* in hospitals in Pakistan. J Med Microbiol 2014;63(Pt 1):50–5.
15. Hannan A, Qamar MU, Usman M, Ahmad K, Waheed I, Rauf K. Multidrug resistant microorganisms causing neonatal septicemia: In a tertiary care hospital Lahore, Pakistan. Afr J Microbiol Res 2013;7(19):1896–1902.
16. Yaghoubi SMJ, Ghorbani GR, Soleimanian ZS, Satari R. Antimicrobial activity of Iranian propolis and its chemical composition. DARU 2007;15(1):45–8.
17. Gebara ECE, Lima LA, Mayer MPA. Propolis antimicrobial activity againstperiodontopathic bacteria. Braz J Microbiol 2002 33(4):365–9.
18. Boyanova L, Gergova G, Nikolov R, Derejian S, Lazarova E, Katsarov N, *et al*. Activity of Bulgarian propolis against 94 Helicobacter pylori strains in vitro by agar-well diffusion, agar dilution and disc diffusion methods. J Med Microbiol 2005;54(Pt 5):481–3.
19. Rahman MM, Richardson A, Sofian-Azirun MF. Antibacterial activity of propolis and honey against Staphylococcus aureus and Escherichia coli. Afr J Microbiol Res 2010;4(16):1872–8
20. Al-Waili N, Al-Ghamdi A, Ansari MJ, Al-Attal Y, Salom K. Synergistic effects of honey and propolis toward drug multi-resistant Staphylococcus aureus, Escherichia coli and Candida albicans isolates in single and polymicrobial cultures. Int J Med Sci 2012;9(9):793–800.
21. Salomao K, Pereira PR, Campos LC, Borba CM, Cabello PH, Marcucci MC, *et al*. Brazilian propolis: correlation between chemical composition and antimicrobial activity. Evid Based Complement Alternat Med 2008;5(3):317–24.
22. Kilic A, Ucar M, Baysallar M, Salih B, Sorkun K, Yildiran ST, *et al*. Antimicrobial Effects of Propolis and Honey Samples Collected from some Regions of Turkey. AJCI 2007;1(4):213–8.
23. Zeighampour, F, Mohammadi-Sichani M, Shams E, Naghavi NS. Antibacterial Activity of Propolis Ethanol Extract against Antibiotic Resistance Bacteria Isolated from Burn Wound Infections. Zahedan J Res Med Sci 2014;16(3):25–30.

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