

In vitro Antibacterial Activity of Organic Extracts of *Aloe barbadensis* against Multi-drug Resistant *Pseudomonas aeruginosa* Isolated from Wound Specimens

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ABSTRACT

Objectives: In order to investigate alternate therapeutic option, this study was carried out to assess the *in vitro* antibacterial activity of gel extract of *Aloe barbadensis* against multiple antibiotic resistant *Pseudomonas aeruginosa* isolated from wound specimens.

Methods: A total of 180 different wound specimens collected in a hospital, were subjected to isolate and identify *P. aeruginosa* by cultural methods. Antibiotic susceptibility testing was done by Kirby-Bauer disc diffusion method to screen multidrug resistant isolates. *A. barbadensis* extracts were prepared using aqueous and organic solvents and their *in vitro* inhibitory action was evaluated by agar well diffusion methods.

Results: Out of total, 38 (21.1%) of the wound specimens showed the occurrence of *P. aeruginosa*, among which 15 (39%) isolates were multi-drug resistant. Organic extracts of various concentrations (0.2 - 0.8 v/v %) inhibited 66.7% of MDR and all non-MDR (n=23) *P. aeruginosa* with zone of inhibition ranging from 9.5 ±1.0 to 21.3 ± 2.2 mm but not by aqueous extract. A positive Pearson's correlation (r=0.983) was found between antibacterial effect and concentrations of the extracts. The antibacterial activity of organic extracts was statistically associated with antibiotic resistance profile of the organism (p<0.05).

Conclusion: Organic extracts of *A. barbadensis* revealed variable *in vitro* inhibitory action against both MDR and non-MDR *P. aeruginosa* isolated from wound specimens. Although further confirmation is needed, aloe gel extract may be applied as an alternate treatment option.

Key words: *Pseudomonas aeruginosa*, MDR, *Aloe barbadensis*, organic extracts, antibacterial activity, wound infection

INTRODUCTION

There is an increasing incidence of *Pseudomonas aeruginosa* in wound infections (Masaadeh and Jaran 2009) because an open wound provides a moist, warm and nutritious environment perfect for microbial colonization and proliferation (Benbow 2010). *P. aeruginosa* is an opportunistic and highly resistant nosocomial pathogen. It can grow on a wide range of substrates and quickly respond to environmental alteration (Lambert 2002). It can express virulence factors and surface proteins affecting wound healing.

The development of a wound infection following elective surgery or traumatic injury remains a major cause of morbidity in patients (Kim et al. 1999).

Several studies have shown the emergence of multi-drug resistant (MDR) isolates of *P. aeruginosa* associated with community acquired and nosocomial infections (Bhandari et al. 2015; Chaudhary et al. 2016; Fan et al. 2016). Ali et al. (2015) observed higher resistance of *P. aeruginosa* to the commonly used antibiotics like ofloxacin (61.3%), cefepime (57.3%), ceftazidime (53.9%) and amikacin (53.0%). It is obvious that the

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widespread MDR *P. aeruginosa* threaten the life of the infected patients globally.

The search of newer antibacterial sources has been fostered because antibiotic alternatives may reduce the use of antibiotics. In other hand, 80% of the population in developing countries mainly relies on traditional herbal medicines for therapies against infections (WHO 2002) due to wide availability, easy use, low cost, minimal or no adverse effect on human health. During the past several decades, both *in vivo* and *in vitro* studies have demonstrated higher antibacterial potency of biologically active compounds extracted from medicinal plants (Malterud et al. 1993; Goudarzi et al. 2015). *Aloe barbadensis* a medicinal plant, contains over 75 potentially active constituents including vitamins, enzymes, minerals, sugars, lignin, saponins, lupeol, salicylic acids, amino cinnamonic acid, phenols and sulfur (Surjushe et al. 2008). Many of these compounds are bactericidal, virucidal, fungicidal and anti-inflammatory in nature (Hamman 2008). Furthermore, the inhibitory action of extracts of *A. barbasensis* against many gram negative pathogens including *P. aeruginosa* has been recognized (Ibrahim et al. 2011; Renisheya et al. 2012; Abakar et al. 2017). However, its antibacterial activity against MDR *P. aeruginosa* has rarely been evaluated in Nepal.

Therefore, in this study, we tested *in vitro* inhibitory action of different organic extracts of locally available *A. barbadensis* at different concentrations against MDR and non-MDR *P. aeruginosa* isolated from various wound specimens.

MATERIALS AND METHODS

Study design and wound specimen collection: In this prospective study, 180 wound specimens including pus, postoperative wound, and burn-wound and chronic wound swabs were collected by hospital professionals from out and in-patient departments of Annapurna Neurological Institute and Allied Science Hospital, Kathmandu, Nepal in between May to November 2017.

Isolation and identification of *P. aeruginosa*: The specimens were inoculated on MacConkey agar, Blood agar and Cetrimide agar (Hi-media, India) followed by incubation at 37 °C for 24 hours. The organism was identified on the basis of the colony characteristics, pigmentation, Gram's staining, catalase, oxidase tests and standard conventional biochemical tests: indole, methyl red, Voges-Proskauer, citrate, triple sugar iron, citrate, urease, nitrate reduction and oxidative/fermentative tests.

Antibiotic susceptibility testing and screening of MDR *P. aeruginosa*: Antibiotic susceptibility test (AST) of all *P. aeruginosa* isolates was done by Kirby Bauer disc diffusion method (Figure 1A) and interpreted according to guidelines of Clinical Laboratory Standard Institute (CLSI 2016). The antibiotics used for AST were: Amikacin (AK)-30 mcg, Gentamicin (GEN)-30 mcg, Cefotaxime (CTX)-30 mcg, Ciprofloxacin (CIP)-5 mcg, Ceftriaxone (CTR)-30 mcg and Imipenem (IPM)-10 mcg. Based on the results of AST, MDR isolates of *P. aeruginosa* were screened as the isolate was resistant to two antibiotics of different groups with different mode of action.

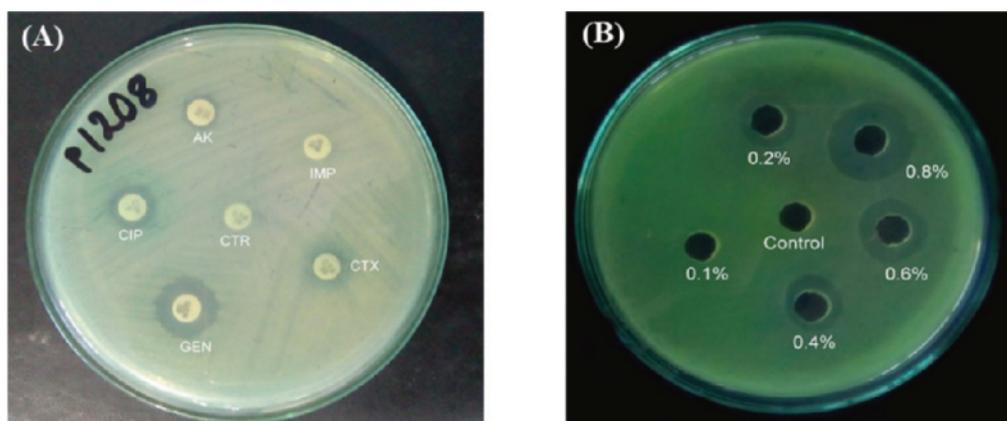


Figure 2: *P. aeruginosa* (P/1208) on Muller Hinton Agar plates showing antibiotic sensitivity testing (A) and antibacterial activity by DMSO extract of *A. barbadensis* (B). Abbreviations for antibiotics are as mentioned in the text. For *in vitro* antibacterial activity testing, 0% (control) to 0.8% (v/v) DMSO extracts was used. Zone of inhibitions were measured as mm.

Preparation of *A. barbadensis* gel extracts: Mature, healthy and fresh leaves of *A. barbadensis* were collected and washed in the running tap water for 10 minutes, rinsed with sterile distilled water and surface sterilized with 70% ethyl alcohol followed by 0.1% HgCl₂. The leaves were dissected longitudinally and the colorless parenchymatous tissue (aloe gel) was scraped out using a sterile knife. Fresh aloe gel was dried at 80 °C for 48 hours and then powdered. Ten grams of the powder was soaked in 100 ml of four different solvents (distilled water, DMSO, acetone and ethanol) separately for 24 hours. The content was filtered through Whatman filter paper (except for DMSO extract) and filtrate was evaporated to dryness. The dried powdered extract was dissolved in distilled water for further use. In case of acetone extract, extracted powder was dissolved in NaOH and neutralized with 0.15 M HCl.

Determination of antibacterial activity of *A. barbadensis* gel extract: The antibacterial activity of *A. barbadensis* gel extract against the *P. aeruginosa* isolates

was tested by agar well diffusion technique (Figure 1B). Each of aloe extract was diluted with distilled water to prepare test solutions of 0.1%, 0.2%, 0.4%, 0.6% and 0.8% (v/v) concentrations. Exactly 100 µL of each test solution of each of aloe gel extracts were loaded into each of the 5 mm diameter wells on Mueller Hinton agar which were swabbed with an overnight broth culture of the organism and incubated at 37 °C for 24 hours. The respective pure solvent (acetone, ethanol and DMSO) was used as the control. In vitro inhibitory action was measured in terms of zone of inhibition (mm) around the test wells.

RESULTS

***P. aeruginosa* isolates from wound specimens and antibiotic resistance:** Out of total 180 wound specimens, 38 (21.1%) of the specimens showed the occurrence of *P. aeruginosa*. The highest numbers of the *P. aeruginosa* isolates were recovered from burn wound infections (n = 7, 35.0%) while the lowest number from pus specimens (n = 9, 13.9%) (Table 1).

Table 1: Distribution of *P. aeruginosa* in various wound specimens

Type of specimen	Total	Growth obtained (<i>P. aeruginosa</i>)	Percent
Burn wound swabs	20	7	35.0
Postoperative wound swabs	40	8	20.0
Chronic wound swabs	55	14	25.5
Pus specimens	65	9	13.9
Total	180	38	21.1

Antibiotic susceptibility testing revealed that out of total 38 isolates of *P. aeruginosa*, 15 (39.0%) isolates were designated as MDR *P. aeruginosa* (Figure 2) while others were regarded as non-MDR. The highest number

of MDR *P. aeruginosa* were isolated from burn wound specimens (n=6, 85.7%) followed by postoperative wound (n = 5, 62.5%).

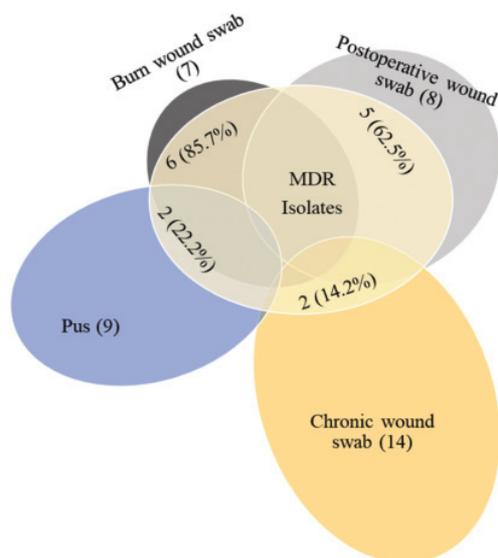


Figure 2: Venn diagram showing distribution of multidrug resistant (MDR) *P. aeruginosa* isolates in different clinical samples. Parentheses in each sample represent the number of total *P. aeruginosa* isolates (N = 38). Frequencies of MDR isolates (n = 15) from each sample are expressed as number (n/N %).

In vitro antibacterial activity of *A. barbadensis* extracts against *P. aeruginosa*: In order to evaluate the inhibitory potential of *A. barbadensis* against wound infecting *P. aeruginosa*, both the MDR (n = 15) and non-MDR (n = 23) isolates were tested. Production of clear inhibition zone around the agar well containing the extract was considered as 'sensitive' isolate, otherwise 'resistant' as no such zone was observed. While aqueous extract did not show any antibacterial effect, extracts with all three organic

solvents; acetone, ethanol and DMSO demonstrated antibacterial effect at varying concentrations.

As demonstrated in Figure 3, among total of 38 isolates of *P. aeruginosa*, 33 (86.8%) were inhibited by at least one of organic extract of *A. barbadensis*. Out of the total 15 MDR isolates, 10 (66.7%) were found to be sensitive to aloe extracts. Interestingly, all 23 (100%) non- MDR isolates of *P. aeruginosa* were found to be sensitive to aloe extracts.

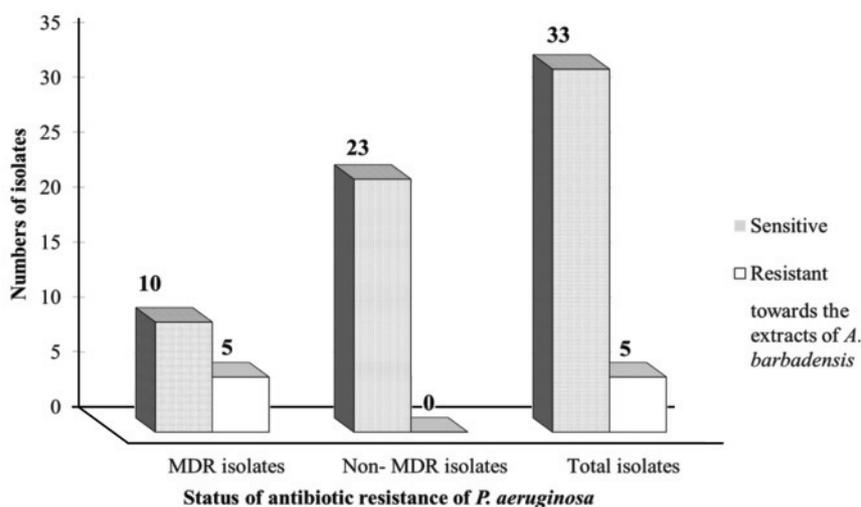


Figure 3: Bar diagram showing antibacterial activity of organic extracts of *A. barbadensis* against MDR and non MDR isolates of *P. aeruginosa*. The numbers represent the frequencies of isolates either sensitive or resistant towards at least one organic extract of *A. barbadensis*.

The maximum zone of inhibition of 21.3 ± 2.2 mm was revealed by acetone extract at concentration 0.8% against the MDR isolate of *P. aeruginosa* obtained from a burn wound while minimum zone of inhibition of 9.5 ± 1.0 mm was shown by DMSO extract at 0.2% concentration. DMSO extract of *A. barbadensis* showed increasing size of zone of inhibition as concentration was raised from 0.2% to 0.8% (Pearson's correlation coefficient, $r=0.9951$) against MDR isolates of *P. aeruginosa* and the linear curve was well fitted with R^2 of 0.9999 (Figure 4A). Comparatively, acetone extracts revealed highest in vitro inhibitory activity (larger zone of inhibition in all concentrations). Similar to the results

against MDR isolates, non-MDR *P. aeruginosa* were also inhibited by 0.4 to 0.8% concentrations of acetone and DMSO extracts and 0.6 – 0.8% concentrations of ethanol extracts (Figure 4B). The results showed comparatively similar pattern of antibacterial activity of organic extracts of *A. barbadensis* against both the MDR and non-MDR isolates.

Association between multidrug resistance and antibacterial activity of *A. barbadensis*: Despite the similar inhibitory pattern of individual aloe extracts, we attempted to determine the association between MDR status of *P. aeruginosa* and gross sensitivity towards *A. barbadensis* applying chi-square test.

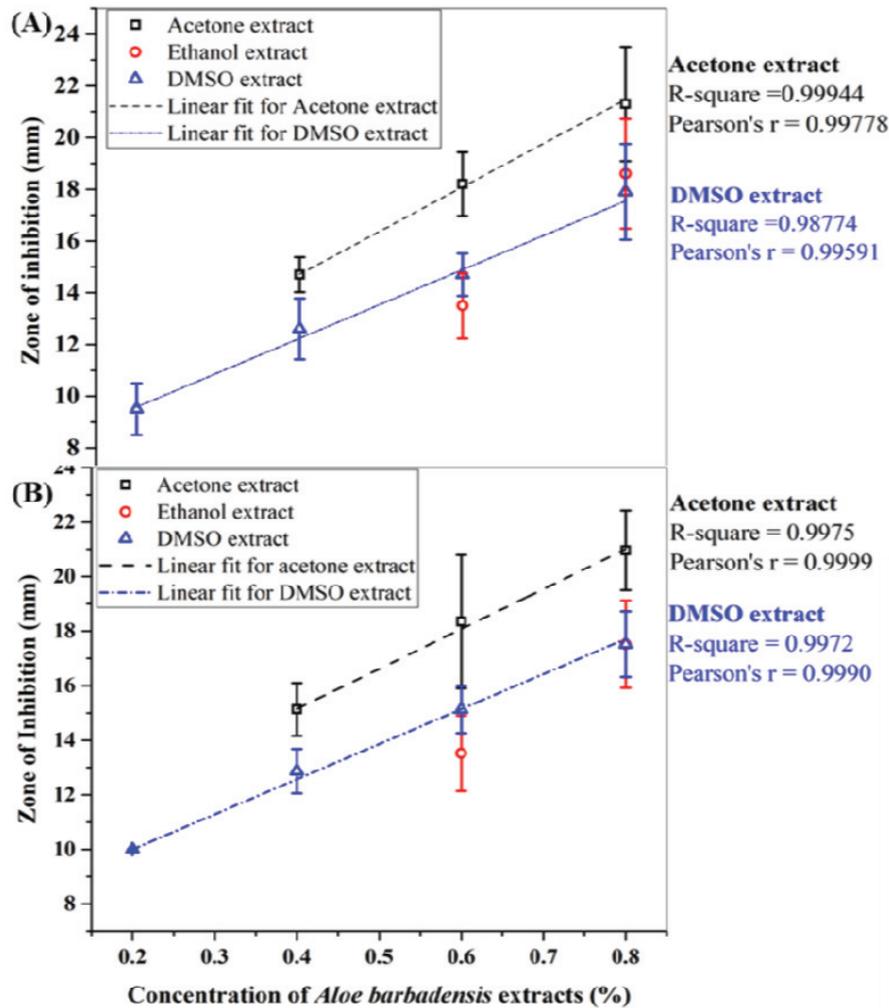


Figure 4: Antibacterial activity of *Aloe barbadensis* extracts at different concentration against multidrug resistant (A) and non-multidrug resistant (B) isolates of *P. aeruginosa*. Error bars represent the standard deviation. Pearson's r is correlation coefficient between concentration of extracts and ZOI produced by respective extracts. Since ethanol extract showed inhibition only at two concentrations, statistical analysis was not valid.

Compared to all non-MDR isolates, only two-third of MDR isolates (n = 5) of *P. aeruginosa* were sensitive towards *Aloe* extracts. A significant statistical

association ($p < 0.05$) was revealed between MDR status and the sensitivity towards *A. barbadensis* (Table 2).

Table 2: Sensitivity of MDR isolates to *A. barbadensis*

Status of antibiotic resistance	Sensitivity to <i>A. barbadensis</i> extract		Total (%)	p-value*
	Sensitive (%)	Resistant (%)		
MDR	10 (66.7)	5 (33.3)	15 (100.0)	0.006
Non- MDR	23 (100.0)	0 (0.00)	23 (100.0)	
Total	33 (86.8)	5 (13.2)	38 (100.0)	

*Chi-square test

In order to understand which organic solvent extracts of *A. barbadensis* produce maximum inhibitory activity against *P. aeruginosa* isolates, the sizes of zone of inhibition (mm) revealed by each organic extracts at different concentrations were compared for MDR and non-MDR isolates. Though acetone extracts at all

concentrations (0.4 - 0.8%) revealed higher magnitude of inhibition than DMSO and ethanol extracts, there was no significant difference in diameter of the zone of inhibition produced by different extracts for MDR and non-MDR isolates ($p > 0.05$) for all concentrations (Table 3).

Table 3: Association between drug resistance and antibacterial activity of organic extracts of *A. barbadensis* at different concentrations

Extract	Concentration	Diameter of zone of inhibition (mm) against		p-value*
		MDR	Non-MDR	
Acetone	0.8 %	21.3 ± 2.2	21.0 ± 1.5	0.129
Ethanol		18.6 ± 2.1	17.5 ± 1.6	
DMSO		17.9 ± 1.9	17.5 ± 1.2	
Acetone	0.6 %	18.2 ± 1.2	18.4 ± 2.4	0.299
Ethanol		13.5 ± 1.3	13.5 ± 1.4	
DMSO		14.7 ± 0.8	15.1 ± 0.9	
Acetone	0.4 %	14.7 ± 0.7	15.1 ± 1.0	0.191
Ethanol		0	0	
DMSO		12.6 ± 1.2	12.9 ± 0.8	
Acetone	0.2 %	0	0	
Ethanol		0	0	
DMSO		9.5 ± 1.0	0	

*ANOVA

DISCUSSION

In order to investigate an alternate therapeutic option, this study was carried out to assess the antibacterial activity of *A. barbadensis* against MDR *P. aeruginosa* isolated from wound specimens. Since *P. aeruginosa* is generally a part of wound infection etiology, the target pathogen was isolated from different types of wound specimens. In this study, *P. aeruginosa* was recovered from 21.1% as key etiological agent of wound infections. However, previous studies reported the prevalence of *P. aeruginosa* as 5.6% to 44.0% (Bhatt and Lakhey 2007; Bangerla et al. 2015; Rai et al. 2017). Considering variable prevalence of *P. aeruginosa* in different types of wound infections (Rajbahak et al. 2012; Bastola et al. 2017; Serra et al. 2015; Kshetry et al. 2015), we selected burn wound swab, postoperative wound swab, chronic wound swab and pus specimens for this study where the burn wound specimens contributed maximum of *P. aeruginosa* isolates (35%).

Infections caused by *P. aeruginosa* are often severe and life threatening (Ahmed et al. 2008) and are difficult to treat because of its limited susceptibility to antimicrobial agents and the high frequency of the emergence of antibiotic resistance during therapy (Niederman, 2001; Paladino et al. 2002). Because we found high prevalence of MDR *P. aeruginosa* in wound infections (39.5%) which is higher than the study of Bangerla et al. (2015) with resistance against the most of aminoglycoside, fluoroquinolone and beta-lactams (Rajbahak et al. 2012), we argue that there may be increasing trend of antibiotic resistance of *P. aeruginosa* causing wound infection. The high resistance of *P. aeruginosa* may be due to the intrinsic resistance

like chromosomally encoded β -lactamase, biofilm formation, energy dependent efflux (Hancock 1997) and acquired resistance by mutational events leading to over-expression of endogenous β -lactamases or efflux pumps, diminished expression of specific porins and target site modifications. Acquired resistance is also due to acquisition of resistance genes which mainly refers to transferable β -lactamases and aminoglycoside-modifying enzymes.

In order to assess the in vitro inhibitory activity of *A. barbadensis*, we analyzed if there is relation among concentration, types of extraction solvents and antibacterial activity of the *A. barbadensis*. We found that organic extracts with acetone, ethanol and DMSO extracts gave zone of inhibition in certain concentrations but not by aqueous extract. It may be due to partial solubility or insolubility of active compound in aqueous solution (non-polar nature and active compound). Broadly, the magnitude of inhibition was obtained in the pattern of acetone > DMSO > ethanol extracts (0.4 - 0.8%). The results of this study corroborated with Ibrahim et al. (2011) that reported better antimicrobial activity of the acetone extract as compared to ethanol and aqueous extracts. In this study, the maximum zone of inhibition (21.3 ± 2.2 mm) by acetone extract at 0.8% concentration was found which was to be similar (21 mm by acetone extract) to the study of Abakar et al. (2017) for *P. aeruginosa* suggesting that acetone could be the best organic solvent for extraction. Various extracts showed different zone of inhibition which may be attributed to different solubility of various compounds found in *Aloe barbadensis*. More polar substances extract more of the compounds embedded within the plant cells

(Karpagam and Devaraj 2011). Our experiment showed that, the inhibitory action increases proportionately with the increment of concentration of the extracts corroborating with results of Renisheya et al. (2012).

Among MDR isolates, two-thirds of the isolates were sensitive to aloe extract while all non-MDR isolates were found sensitive. The antibacterial activity of extract was found statistically associated with antibiotic resistance profile of the organism ($p < 0.05$). This indicates the multidrug resistant *P. aeruginosa* were less susceptible to aloe extract. Irrespective of the different wound specimens, the zone of inhibition was nearly similar for all isolates of *P. aeruginosa*. However, in a particular concentration of all extracts, there was no significant difference in diameter of the zone of inhibition for MDR and non-MDR isolates ($p > 0.05$).

From this study it is clear that *A. barbadensis* demonstrates inhibitory activity against *P. aeruginosa* indicating its therapeutic potential. Lawrence et al. (2009) identified the compounds with maximum antibacterial activity against *P. aeruginosa* as p-coumaric acid, pyrocatechol and cinnamic acid. Pyrocatechol denatures proteins and disrupts cell membranes (Cowan 1999). Cinnamic acid inhibits glucose uptake and ATP production in the resting cells of bacteria (Kouassi and Shelef 1998). Similarly, p-coumaric compound is reported to increase the lag phase of the microorganism (Baranowski et al. 1980) and is also able to inhibit the enzymatic activity of the microorganisms (Weir et al. 2004). However, in this study the active compounds were not identified. So it is recommended for further research that isolation of key bioactive compound of Nepalese variety of *A. barbadensis* would be useful asset in this regard.

CONCLUSION

Both the MDR and non-MDR isolates of *P. aeruginosa* recovered from wound specimens were inhibited in vitro by organic extracts of *A. barbadensis*. However, comparison study showed slightly higher frequency of non-MDR isolates was sensitive towards organic extracts of *A. barbadensis*. Acetone extract had comparatively better inhibitory activity and magnitude of *in vitro* inhibition was positively correlated with concentration of extracts. Though further confirmation is necessary, in the light of the multiple antibiotic resistance problems, this study showed that *A. barbadensis* may be used as an alternate therapeutic option for the treatment of *Pseudomonas* caused infections more particularly wound infections.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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