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Geographical variation in antibacterial activity of NepaleseAcorus calamus (Bojho) against Multi Drug Resistant Bacteria

Ronak Shrestha*, Rashmi Thimi #, Namuna Regmi #, Sushanta Sharma Poudel#, Rupa Nepal, Pratik Khanal, Ram Krishna Shrestha, Rajan Shrestha*

Department of Pharmacy, Modern Technical College, Pokhara University, Nepal
Department of Medical Lab Techonology, Modern Technical College, Pokhara University, Nepal
Department of Medical Lab Techonology, Modern Technical College, Pokhara University, Nepal
Department of Medical Lab Techonology, Modern Technical College, Pokhara University, Nepal
Department of Medical Lab Techonology, Modern Technical College, Pokhara University, Nepal
Department of Pharmacy, Modern Technical College, Pokhara University, Nepal
Department of Medical Lab Techonology, Modern Technical College, Pokhara University, Nepal
Department of Pharmacy, Kathmandu University,Nepal

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ABSTRACT

With the increasing appearance of multi-drug resistant (MDR) pathogens, plant products have been evoked as a remedy to treat the resistant microorganism.This study comparesthe phytochemical composition and theantibacterial activity of Hexane, Dichloromethane (DCM) and Water fraction of the ethanolic extract of Acorus calamus rhizome collected from Chitwan (220m), Dhading (750m) and Solukhumbu (2700m) in Nepal.

The antibacterial activity of the fractions against the MDR Staphylococcus aureus and Escherichia coliwas evaluatedby comparing zone of inhibition (ZOI), minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).The phytochemical screening of the fractionsfrom the different location showed a similar phytochemical composition and contained terpenoids, tannin, alkaloids, flavonoids, glycosides and phenolic compounds. Despite the similar phytochemical composition,Hexanefraction of Solukhumbushowed significantly higher ZOI than of Dhadingwith lowest MIC and MBC of 12.5 mg/mL and 25 mg/mL, respectively,against the MDR S. aureus.Likewise,Hexanefractionof Dhading showed significantly higher ZOI thanof Solukhumbuwith lowest MIC and MBC of 6.25 mg/mL and 12.5 mg/mL, respectively,against the MDR E. coli.This study demostrates thevariance in antibacterial activity of extract fractions of A. calamus rhizome collected from different site against the testedMDR bacteria.

Keywords: A. calamus,rhizomes**,** Plant extract,antibacterial activity, Multi Drug Resistance

I. INTRODUCTION

Microbial resistance to antibiotics and the appearance of multi drug resistant (MDR) pathogen have resulted in difficulty in treating bacterial infections with conventional antimicrobials. It has become a major global concern and threat to public health.It has been estimated that by the year 2050 A.D the problem of bacterial resistance will increase awfully and cause millions of deaths per year(O'Neil 2014). Limited therapeutic options for the MDR infectionshave resulted challenges in the combat of bacterial infections. This havemade physicians to use either relatively expensive drugs or drugswith significant side effect(s)(Boucher et al. 2009). As a result,strategies such preventive efforts to lower bacterial MDR, innovative nanoparticle-based formulations, novel plant-derived antimicrobial agentsare currently being applied globally against MDR.(Parmanik et al. 2022)

Staphylococcus aureus are spherical cocci, its infection is among the most common bacterial infections and ranges from mild to fatal. A common staphylococcal infection includes folliculitis, abscess, wound infection, osteomyelitis, tonsillitis, otitis, sinusitis, lung abscess, meningitis, bacteremia, septicemia, pyemia, endocarditisetc. Likewise, Escherichia colibelong to the family Enterobacteriaceae. They are gram negative rods and are part of intestinal tract normalflora of warm-blooded animals. The virulent strains of E.

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colihowever, act as specific pathogens and causes clinical infections includingdiarrhea, dysentery, neonatal meningitis, pneumonia,septic infections of wound, septicemia,UTI, and abscesses in various organs.

Researchers has been screening medicinal plant in thequestof new and more effective antimicrobial compounds, (Adhikary et al. 2011; Gyawali et al. 2014; Rahamouz-Haghighi et al. 2014). Acorus calamus(commonly known as Bojho in Nepal) belongs to the Araceae family and is also one type of medicinal plants with many benefits. In Nepal, Bojho is found in the wild as well as are cultivated up to 2700 m altitude. Among the various parts of the plant, the rhizomes are extensively used by the people. The medicinal properties of rhizomes include but is not limited to antispasmodic, anthelmintic, aromatic, sedative and stimulant effects.It is being used in treatment of various health conditions such as bronchial congestion,chronic diarrhea, dysentery,epilepsy, intermittent fever,mental illnesses, and tumors(Imam et al. 2013).The rhizomes are also considered to have antifungal and antibacterial properties(Souwalak et al. 2005). Earlier studies on A. calamus have revealed the presence of various phytochemical compounds which are regarded as a responsible factor for its medicinal use(Muchtaromah et al. 2019; Rahamouz-Haghighi et al. 2014).In the study conducted byGyawali etal. the essential oil of A. calamus showed antibacterial activity against S. aureus and E. coli(Gyawali et al. 2013).The ethanolic extract of A. calamus was found to be effective against S. aureus, E. coli and C. albicans while Water extract showed ineffectiveness against both bacteria(Rita et al. 2019). Likewise, chloroform extract of A. calamus was found to be effective against S. aureus (Pokharel et al. 2023).Maharjan et al. (2012) evaluated the antibacterial activities of medicinal plants and found that the minimum bactericidal concentration (MBC) of hexane extract of A. calamus was 25mg/mL for S. aureus (ATCC 25923).Rita et al. (2019) found a positive correlation between antimicrobial property of A. calamus and itsflavonoid and phenolic content. This indicates flavonoid and phenolic contents of A. calamus are responsible for its antimicrobial property. The major volatile phytochemical components in A Calamus L includes asarone, trans-β-Ocimene, Isocalamendiol, Methyleugenol, 3-Carene, β-asarone, Cetene, β-Guainene, β-copaene, Tridecane and α-Pinene.(Atalar and Türkan 2018)

Although many studies have been carried out previously on the screening of antimicrobial properties of A. calamus, there has been paucity ofdataon its activity against MDR pathogen and on the minimum inhibitory concentration (MIC) and MBC value. This study aimed to find the effect of geographical variation onin vitro antibacterial activity of A. calamus rhizome extracts against MDR bacteria.

II. MATERIALS AND METHODS

Collection, identificationand extraction of plant materials

Plant samples were collected from 3 different altitude of Nepal,220 m from Chitwan, 750 m from Dhading, and 2700m fromSolukhumbu. The collected plants were identified as A. calamus from National Herbarium and Plant Laboratories, Godawari,Nepal (Regd.no 079-80-336).

The rhizome of the plant was shade dried and powdered using knife mill. The milled 80 gm powder wassoakedin 800 mL ethanol for 24hours at room temperature.Following the maceration,it was filtrated using filter paper. The obtained filtrate was concentrated using a water bath. The hexane fractionof the ethanolic extract was collected with 100 mL of a 1:1 mixture of Water and hexane followed by the DCM fraction with 100 mL of a 1:1 mixture of Water and DCM using a separating funnel.Finally, 3 fractions viz. hexane, DCM and Water were concentrated using awater bath.The percentage yield of each solvent fraction was calculated using the formula "Percentage yield = (Weight of solvent fraction / Weight of sample)× 100". The stock solution of100 mg/mLfor each fraction was made in DMSOand kept in cleaned capped glass tubes. These tubes were then stored in the refrigerator (2-8^{o}C) until further use.

Phytochemical screening

Phytochemical screening ofA. calamus extracts of each location wasdone using detection reagents for distinct groups of compoundsas described by Muchtaromah et al (2019).

Collection of microbial samples

The clinical isolates of MDRS. aureus and E. coli were collected from STAR Hospital Pvt. Lt (Lalitpur, Nepal). The MDR S. aureus was resistant to Ceftriaxone, Cefixime, Cefpodoxime, Ciprofloxacin, Cloxacillin, Clotrimazole, Gentamicin, Norfloxacin, Novobiocin and Ofloxacin but sensitive to Cefatrizine, Meropenem, Nitrofurantoin and Piperacillin. The MDR E. coli was resistant to Amoxicillin, Ceftriaxone, Cefixime, Cefotaxime, Ciprofloxacin, Clotrimazole, Gentamicin, Meropenem, Ofloxacin and Piperacilline.

Evaluation of antibacterial activity

For the study of the zone of inhibition (ZOI) produced by the fractions against the test organisms, the agar well diffusion methodwas used as described byMaharjan et al. (2012).Briefly, sterile plates of about 4 mm thick Mueller-Hinton agar (MHA) were inoculated with fresh bacterial inoculum comparable with 0.5 McFarland Standard using sterile cotton swab. After adequate drying at room temperature,with the help of sterile cork-borer no.8, four wells of 8 mm diameter were made in the plates. In each well 100 µl of hexane or DCM orWater fractionor DMSO as negative control were added and incubated for 18 to 24 hrat 37°C. The plates were then observedfor a clearinhibition zone of bacterial growth around the wells. The diameter of inhibition zone was measured and recorded as ZOI. The triplicate study for each sample was performed.

For the determination ofMIC, the serial two-fold macro dilution method was used as described byBaron et al. (1994).Briefly, a set of 8 test tubes holding1mL of 50mg/mL, 25mg/mL, 12.5mg/mL, 6.25mg/mL, 3.125mg/mL, 1.563 mg/mL, 0.781mg/mL, and 0.39mg/mLof the fractions in Mueller-Hinton broth (MHB) were prepared. 100mg/mL was used as negative control and 0mg/mL was used as positive control. To all the tubesexcept in negative control, 50 µL of inoculums with turbidity equal to 0.5 McFarland standard were added. The tubes were observed for developed turbidityafter incubating for 24 hrat 37°C.The first tube with the least concentration of thefractionin a series with no sign of apparent growth as detected by lack of visible turbidity wasconsidered as the MIC. The triplicate study for each sample was performed.

For the determination of MBC, one loop full of inoculum from 24 hours culture of test organism innegative control, positive control, 50mg/mL, 25mg/mL, 12.5mg/mL, 6.25mg/mL, and 3.125mg/mLsolution of the fractions in MHB that was used in MIC study were taken and subculturein MHA.The plates were then incubated for 24 hrat 37^{o}C andobserved for the growth of bacteria. The fraction concentration resulting in a 99.9% reduction in CFU/mL was taken as MBC. The triplicate study for each sample was performed.

Ethical consideration

Ethical clearance, Ref no 327/078/079 was taken from Star Hospital Research Center-Institutional Review Committee, an Institutional Review Committee of Star Hospital Pvt Ltd, Lalitpur, Nepal.

III. RESULTS

Fractions yield increased with altitude

The percentage yield of different solvent fractions of ethanol extract of A. calamusrhizome from different region of Nepal is presented in Table 1. The results showed that the cumulative percentage yield of fractions increased with altitude showing the highesttotal yieldof 9.15% in Solukhumbu followed by 6.40% in Dhading and 5.00% in Chitwan. Similarly, the hexanefraction gave the highest yield followed by Water and DCM. The highest yield was obtained in hexane fraction of Solukhumbu with a yield percentage of 7.00% (w/w). The lowest yield was obtained in the DCM fractionof Dhading with a yield percentage of 0.60% (w/w).

Table 1Percentage yield of solvent fractions of ethanol extract of A. calamus from different region of Nepal

Types of Solvent	Yield % (w/w)		
	Chitwan	Dhading	Solukhumbu
Hexane	2.40	3.00	7.00
DCM	1.00	0.60	0.75
Water	1.60	2.80	1.40
Cumulative yield	5.00	6.40	9.15

Fractions from different location showed similar phytochemical composition

Phytochemical screening forhexane, DCM, and Waterfractionof ethanolic extract of A. calamus collected from all locationsshowed similar phytochemical composition (Table 2). Terpenoids, alkaloids and phenolic compounds were present in hexane, DCM, and Waterfractions. Tannin and flavonoids were absent in the DCM fraction. Glycosides were absent in Hexane fraction and

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volatile oils were absent in Water fraction. Nonetheless,Saponins were absent in all the fractions.

Table 2. Phytoconstituents of solvent fractions of ethanol extract of A. calamus

Phytoconstituent	Chitwan			Dhading			Solukhumbu		
	Hexane	DCM	Water	Hexane	DCM	Water	Hexane	DCM	Water
Terpenoids	+	+	+	+	+	+	+	+	+
Tannin	+	-	+	+	-	+	+	-	+
Alkaloids	+	+	+	+	+	+	+	+	+
Flavonoids	+	-	+	+	-	+	+	-	+
Glycosides	-	+	+	-	+	+	-	+	+
Phenolic	+	+	+	+	+	+	+	+	+
Saponins	-	-	-	-	-	-	-	-	-
Volatile oil	+	+	-	+	+	-	+	+	-

+ **Presence**, **- Absence**

Hexane and DCM fractionsfrom Solukhumbu showedthe highest antibacterial activity against MDR S. aureus

Antibacterial activity ofthe three fractionsof ethanolic extract of A. calamus rhizome collected from the threelocationswere performedagainst MDR S. aureusby determiningZOI, MIC and MBCstudies.The results of the studies are presented in Figure 1. All fractions showed antibacterial activity against tested MDR S. aureus. The ZOI ranged from 20.7±1.2 mm for DCM fraction fromSolukhumbu to 11.0±1.0 mm for Water fraction from Chitwan. The MIC value ranges from 12.5 mg/mL to 50 mg/mL and the MBC value ranges from 25 mg/mL to >50 mg/mL.

Comparing the ZOI betweenHexane, DCM and Water fractions from the three locations, DCM fraction consistently showed the highest ZOI followed by Hexane and then by Water fractions from all locations.Among the locations,all the fractions from Solukhumbu showed the highest ZOI with DCM fractionof 20.7±1.2followed byHexane fraction of20.3±0.6 mm, and Waterfraction of15.0±0.1mm.The difference in ZOI was significant between Hexane-Water and DCM-Waterfractionbut was not significant between Hexane-DCM fraction.Similarto the resultsof Solukhumbu, fractions of Chitwan as well as Dhading also showed significant difference in ZOI between Hexane-Water and DCM-Waterfractions against MDR S. aureus (Figure 1a).

Likewise, comparing the ZOI within Hexane, DCM and Water fractions from the three locations, Solukhumbu showed significantly higher ZOIagainst MDR S. aureusfor Hexane, DCM and Watercompare to that of Chitwan and Dhading.The difference in ZOI between the locations for the same fractionswere not significant for DCM fractions but it was significant between Solukhumbu-Dhading and Solukhumbu-Chitwan for Water fractions and between Solukhumbu-Dhading for Hexane fractions. (Figure 1b).

Next, MIC and MBC studies were performed against multi drug resistant S. aureus. The results of the studies are shown in Figure 1c and 1d respectively.The Hexane and DCM fraction from Solukhumbu and DCM fraction of Chitwanshowed the lowest MIC of 12.5 mg/mL. Similarly, Hexane and DCM fraction from Solukhumbushowed the lowest MBC of 25 mg/mL against MDR S. aureus.All other fractions showed 50 mg/mL or higherMBC.

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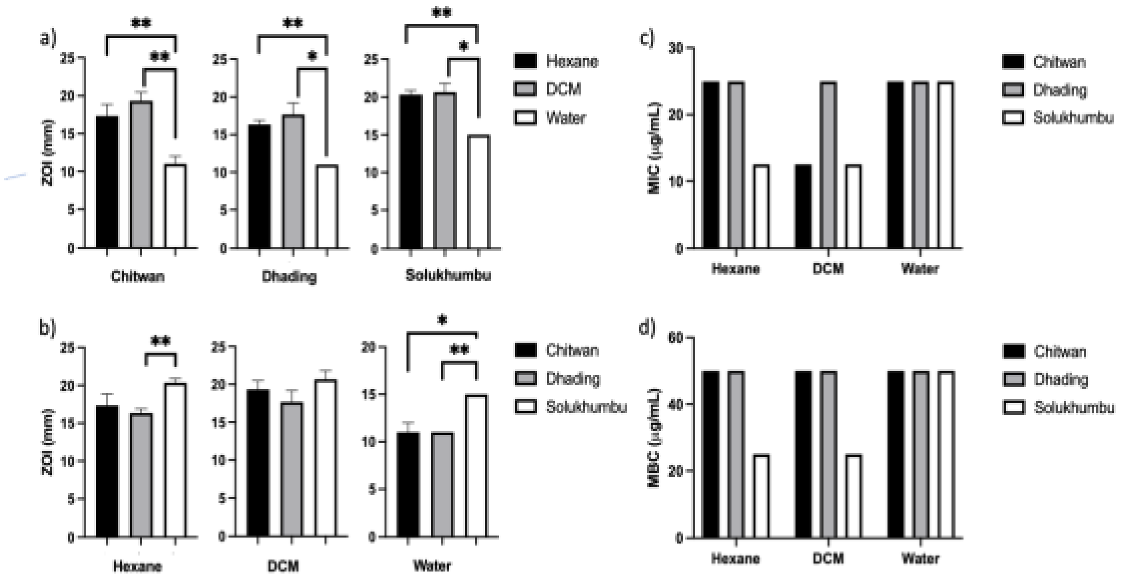

Figure 1: a) Fraction wise ZOI (control: 8 mm)b) Location wise ZOI (control: 8 mm) c) MIC and d) MBC of Hexane, DCM, and Water fraction of A. calamus rhizome collected from Chitwan, Dhading, and Solukhumbuagainst MDRS. aureus (n=3, *P<0.05, **P<0.01)

Hexane fraction from Dhading showed highest antibacterial activity against MDR E. coli

Antibacterial activity of all the fractions were performed against E. coliby determiningZOI, MIC and MBCof the fractions and the results are presented in Figure 2.Similar to S.aureus, all the fractionsshowed antibacterial activity against E. colitoo. The ZOI ranged from 21.7±1.5 mm for Hexane fraction from Dhading to 14.0±1.7 mm for DCM fraction fromSolukhumbu.The MIC value ranged from 6.25 mg/mL to 25 mg/ml andthe MBC value ranged from12.5 mg/mL to 50 mg/mL for E.coli.

Comparing the ZOI. between Hexane, DCM and Water fractions from the three locationsagainst MDR E. coli, Hexane and Water fraction showedsimilar ZOI but higher than DCM fractions for Chitwan and Solukhumbu.However, Hexane fraction showed higher ZOI than DCM followed by Water fraction for Solukhumbu.The difference in ZOI between Hexane-DCM, Hexane-Water, and Water-DCM fraction for all locations was not significant except for Water-DCM fraction for Solukhumbu.(Figure 2a).Likewise, comparing the ZOI within Hexane, DCM and Water fractions from the three locations, Dhading showed significantly higher ZOI than Solukhumbuagainst MDR E. coli. for Hexane and DCM fractions.All other difference in ZOI between other locations for the same fraction was not significant. (Figure 2b).

The results of MIC and MBC studiesagainstMDRE. coli. are shown in Figure 2c and 2d respectively. The DCM fraction of Chitwan, and Hexane fraction of Dhading and Solukhumbushowed the lowest MIC of 6.25 mg/mL. Similarly, Hexane fraction from all location and DCM fraction from Chitwan and Solukhumbushowed the lowest MBC of12.5 mg/mL against MDR E. coli.All other fractions showed 25 mg/mL or higherMBC.

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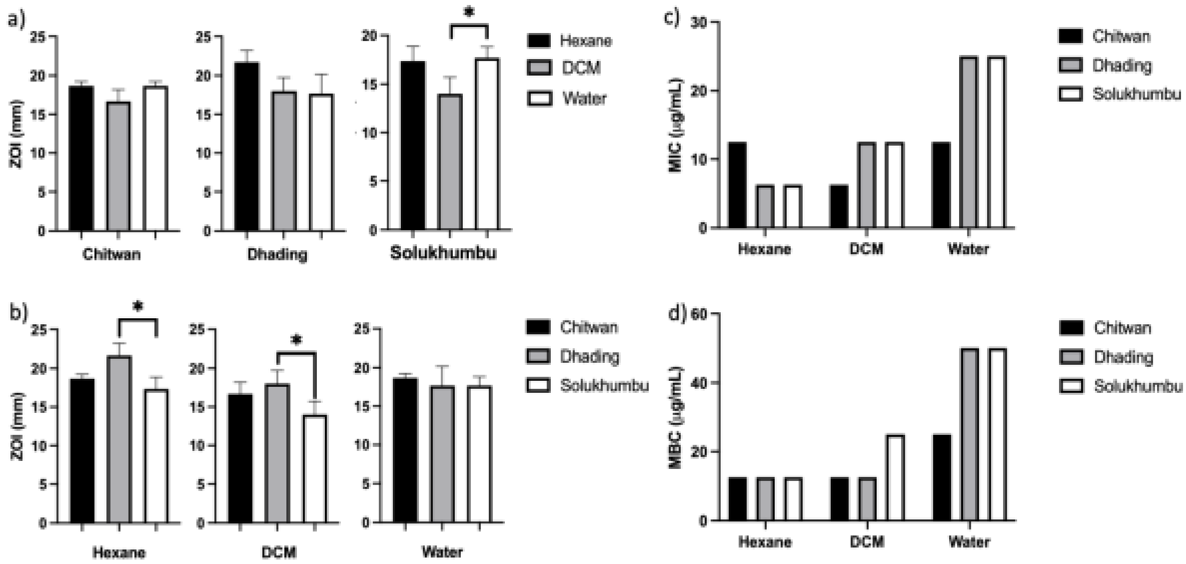

Figure 2:a) Fraction wise ZOI (control: 8 mm) b) Location wise ZOI (control: 8 mm) c) MIC and d) MBC of Hexane, DCM, and Water fraction of A. calamus rhizome collected from Chitwan, Dhading, and Solukhumbu against MDR E. coli(n=3, *P<0.05, **P<0.01)

Next, an MIC study was performed on multi drug resistant S. aureus and E. coli. The results of the MIC study are presented in Figure 3.The MIC value ranges from 12.5 mg/mL to 50 mg/mL for S. aureus and 6.25 mg/mL to 25 mg/ml for E.coli. The Hexane fraction from Solukhumbu,DCM fractions from Chitwan and Solukhumbu showed the lowest MIC of 12.5 mg/mL against MDR S. aureus. Likewise, the DCM fraction from Chitwan and the DCM fraction from Solukhumbu showed the lowest MIC of 6.25 mg/mL against MDRE. coli.

IV. DISCUSSION

The plant samples from three different elevation of Nepal were collected to evaluate the geographical variation in antibacterial activity of A.calamus. The samples were macerated using ethanol, then Hexane, DCM and Water fractions of the extracts were obtainedand used forantibacterial evaluation of non-polar, semi-polar and polar phytocomponents of the plant. It was observed that, the cumulative percentage yield increased with altitudeand was highest for the Solukhumbu. Likewise,the amount of non-polar constituents in the fractions remainedrelatively higher than polar constituents followed by semi-polar constituents regardlessthe sample location. Interestingly, when comparing the yield of individual fractions, the amount of non-polar constituents was found highestin the highest altitude samples, semi-polar constituents was highestin the lowest altitude sample,and polar constituents was highestin the intermediate altitude sample.To evaluate theeffect of this variance in phytoconstituents, next phytochemical screening of the fractions was performed. It was observed that the phytoconstituents was similar within same type of solvent fraction collected from different locations. This indicates that amount of the phytoconstituent in each fraction of A. calamusmay have changed depending on the geographic location of the samplesbut possibly without altering its constituents.

Next, antibacterial activity ofthe three fractions of ethanolic extract of A. calamus rhizome collected from the threelocationswere performed against MDR S. aureusand E. coliby determining ZOI, MIC and MBC. The effectiveness of the A. calamus acetone extractshave been previously reported against Tetracycline resistant E.coli, P. aeruginosa, K. pneumonia and Chloramphenicol resistant S. aureus, K. pneumonia and A. baumanii(Vakayil et al. 2021). It was found that all three fractions of A. calamus rhizome from each locationhad some degree ofantibacterial effect against both MDR S. aureus and MDRE. coli that are resistant to at least ten common antibiotics. The antibacterial activity was significantly higher in non-polar Hexane fractions and semi-polar DCM fractions than polar Water fractions against MDR S. aureus (Figure 1a) for all locations but the hexane fraction of Solukhumbu showed siginificantly higher ZOI than that of Dhading.Similarly, higher antibacterial activity was observed in non-polar Hexane fractions and polar Water fractions than semi-polar DCM fractions against MDR E coli but the difference was statistically significant for Water-DCM fractions of Solukhumbu only. The highest ZOI in Hexane fractions can be related to yield in Hexane fractions which is highest among the fractions, but interestingly lowest yield DCM fractions too showed similar degree of antibacterial activity (Table 1) at least against MDR S. aureus. Theantibacterial activity for non-polar and semi-polar fractions that was observed in this study is similar to that of earlier reported results, where the author reported that the methanolic extract effectively inhibited the gram-negative bacteria, the gram-positive bacteriaand fungi. However, the antibacterial effect observed for the Water fraction in this study is different from the earlier reports where aqueous extract of A. calamus was found inactive against the gram-negative bacteria includingE. coli, P. mirabilis,P. aeruginosa but with moderate antibacterial activity against the gram-positive bacteria B. subtilis and S. Aureus. (Muchtaromah et al. 2019, Sharma et al. 2022).

Likewise,it was also observed that A. calamus extracts were more effective against gram-negative bacteria than gram-positive bacteria, as the extract showed the lowest MIC at 6.25 mg/mL for MDR E. coli while for MDR S. aureus the lowest MIC was 12.5 mg/mL. This might be due to the differences in morphology and constitution of cell walls of microorganisms which will affect the sensitivity to extract (Balakumbahan et al. 2010). Hexane and DCM fractions of Solukhumbu showed lowest MIC, MBC and highest ZOI than other place fractions for MDR S. aureus (Figure 1b, 1c and 1d) but Hexane fractions of Dhading showed lowest MIC, MBC and highest ZOI than other place fractions for E coli (Figure 2b, 2c and 2d). This variation in antimicrobial activity of same solvent fraction from different geographical location suggest that thedifferences in factors such as geography of plants, quantity of secondary metabolites in extract and the bacterial strain can significantly affect antibacterial activity(Gyawali et al., 2022).

V. CONCLUSION

This study evaluated the antibacterial activity of three different fraction of ethanolic extracts of A. calamus collectedfrom three different locationsin Nepal (Chitwan, Dhading and Solukhumbu).This study showed that all three fractions of ethanolic extracts of A. calamus rhizome has antibacterial activity against MDR S. aureus and MDR E. coli.This study also highlighted that the antibacterial property depends upon the plant locations, solvents used for extraction and its fractions, and bacterial strains against which it is being tested. Hence, sources of plants, extraction methods and types of solvent used for extraction of plant material must be carefully studied to optimize the antibacterial properties of plant.

Authors' contribution statement

Ronak Shrestha, Pratik Khanal and Rajan Shrestha conceptualized the work. Rashmi Thimi Namuna, Regmi Sushanta, and Sharma Poudel collected the samples and did the lab work. Ronak shrestha supervised the whole project, Pratik Khanal supervised the extraction process,. Rupa Nepal and Ram Krishna Shrestha supervised the antimicrobial studies. Ronak Shrestha and Rajan Shrestha analyzed and interprete data and prepared the final manuscript.

Conflict of Interest: The authors do not have any conflict of interest pertinent to this work.

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