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Research Article

Elemental impact on antibacterial study of hydroalcoholic leaves extract of *Belosynapsis vivipara*

Kuntal DAS[1]*, Sravani SINGIRIKONDA[2]

[1]*Mallige College of Pharmacy, Department of Pharmacognosy, #71, Silvepura, Chikkabanavara Post, Bangalore-560090, India; drkkdsd@gmail.com (*corresponding author);*
[2]*Krupanidhi College of Pharmacy, Department of Pharmacognosy, #12/1, Chikkabelandur, Carmelaram Post, Varthur, Hobli, Bangalore-560035, India; sravanisingirikonda@gmail.com*

Abstract

Belosynapsis vivipara (Dalzell) C.E.C. Fisch. (F: Commelinaceae) is one of the rare plant species located throughout Western Ghats regions including Karnataka. Though the plant was described earlier but traditional uses and scientific evidences are still lacking. The aim of the present study was to identify the elemental content and to determine antibacterial potentiality of *Belosynapsis vivipara* (*Bv*) ethanol leaves extract. Shade dried powdered material of BV leaves was estimated for elemental content using Atomic Absorption Spectrophotometer (AAS) followed by extraction by ethanol solvent (80%) in Soxhlet apparatus for 13 hrs at 45 °C. Extract was further used for anti-bacterial screening. *In vitro* antibacterial studies on the leaf extracts (25, 50 and 100 µg/ml) were carried out on medically important micro-organisms of *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa* (Gram negative) and *Staphylococcus aureus, Bacillus subtilis* and *Streptococcus pyogenes* (Gram positive) against standard Ampicillin (25 µg/ml). The powdered drug showed the presence of zinc, and Copper in high amount less content of iron, whereas very less content of Nickel and Cobalt and absence of lead, mercury, arsenic and cadmium. The preliminary phytochemical screening revealed the presence of chemical constituents like alkaloids, flavonoids, phyto sterols, saponins and phenolics. Furthermore, ethanol leaves extract of BV showed broad spectrum antibacterial efficacy against both Gram positive and Gram-negative microorganism along with the dose dependency effects. Antibacterial activity was correlated with the elements and showed positive correlation. Finally, *Belosynapsis vivipara* (Dalzell) C.E.C. Fisch. leaves were established as an effective source against strong bacterial infection.

Keywords: antibacterial studies; elemental analysis; *Belosynapsis vivipara;* MIC; MBC; microorganisms

Introduction

A significant contributor to chronic illnesses and mortality are bacterial infections. Because of their efficacy and effectiveness, antibiotics have been the primary agent to treat for bacterial illnesses. The increasing use of antibiotics has, however, been directly linked to the emergence of bacterial strains that are multidrug resistant, according to a number of studies. In reality, the overuse of antibiotics has recently resulted in the development of super-bacteria that are resistant to almost all antibiotics. The prime classes of antibiotics are

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mainly work under the mechanism of the production of cell walls, translational machinery, and DNA replication machinery against the bacterial activities. Unfortunately, each of these mechanisms is susceptible to development of bacterial resistance. The mechanisms of resistance include the expression of enzymes that alter or degrade antibiotics, such as beta-lactamases and aminoglycosides (Poole, 2002), the modification of cellular components, such as the cell wall in the case of vancomycin resistance and ribosomes in the case of tetracycline resistance (Jayaraman, 2009),and the expression of efflux pumps, which offer concurrent resistance against a number of antibiotics (Knetsch and Koole, 2011).The main cause of endangering public health are those related to antibiotic resistance and as a result microbial infections millions of deaths occur in each year Worldwide. Various strategies have been proposed in recent years to combat antibiotic resistance. Combining failed antibiotics with other molecules has been one of the suggested methods to accomplish this, which appears to restore the desired antibacterial activity (Brown, 2015). These compounds could be non-antibiotic medications with possible antibacterial capabilities that could open up new therapeutic possibilities (Vandevelde *et al*., 2016). Henceforth, in numerous studies natural products have been used to combat bacterial resistance in this instance, and phytochemicals have demonstrated strong effects (Fazly Bazzaz *et al*., 2018; Shaker*iet al*., 2018). Plant bioactive compounds can act alone or in combination with antibiotics to produce the synergistic antibacterial activity against a wide range of bacteria (Fazly Bazzaz *et al*., 2010; Betts and Wareham; 2014). Thereafter, elements in the plant also play great role in formation of secondary metabolites and other physiological functions. Many repots revealed the significant role of elements in physiological function and proper growth of the plant's vis a vis improvement of the bioactive components in the plant body which further enhances the therapeutic or medicinal properties (Kheyrkhah *et al*., 2018; Lo Piccolo *et al*., 2021). Based on the concept, the present study was carried out with the herbal plant. There are many herbals reported with antimicrobial efficacy with their important bioactive compounds. Due to the resistance towards antibiotics as well as unwanted health issues, the application of synthetic drugs are reduced and people inclined towards natural way of treatment using herbal plants though the responses are delayed but cures from root level and effective against broad spectrum microbes based on various mechanisms viz. potent free radical scavenger, by acts on destruction of cell membranes and cell walls, or blocking of nucleic acid and protein synthesis, or by enhancement of intracellular osmotic pressure (Scheepmaker*et al*., 2019; Parham *et al*., 2020; Liang *et al*., 2022).

Oflate, *Belosynapsis vivipara* (Dalzell) C.E.C. Fisch. (F: Commelinaceae) is one of the rare plant species in India which distributed mainly in Western Ghats Region especially Maharashtra (Sangali), Karnataka (Chikmagalur, Coorg, Hassan, Mysore, Shimoga), Kerala (Wayanad) and Tamil Nadu (Anamalai Hills). The leaves are look like spider legs and hence it is also known as Spiderwort (Kavade *et al*., 2012). The bioactive compounds and the therapeutic efficacies are not explored so far but currently the plant got immense attention due to its rarity. Very recently, an anti-inflammatory and potent antioxidant activities were carried out for the said plant and revealed the presence of plant constituents like anthraquinones derivatives, phenolic nucleus, proteins, saponosides and flavonoids, saponin, alkaloids, steroids, tannins, proteins, coumarins (Sasane*et al*., 2021). There are many research activities still need to be carried out. Based on the phytoconstituents present and antioxidant potentiality of the plant, first time the research extensively was carried out to establish element content in the plant, their impact on phytochemicals vis a vis potent antimicrobial efficacy.

Materials and Methods

Plant collection

The *Belosynapsis vivipara* (BV) plants were collected from Wayanad, Kerala, India (Latitude: N 11° 42.5668'. Longitude: E 76° 5.7322') (Figure 1) and were identified and authenticated by Dr. P.E. Rajshekharan, Principal Scientist, Indian Institute of Horticultural Research, Bengaluru, India. The plant was kept as

herbarium in Department of Pharmacognosy, Krupanidhi College of Pharmacy, Bengaluru (Herbarium No: PCOG/BV-436/Leaves/KCP/2022-2023) for future reference.

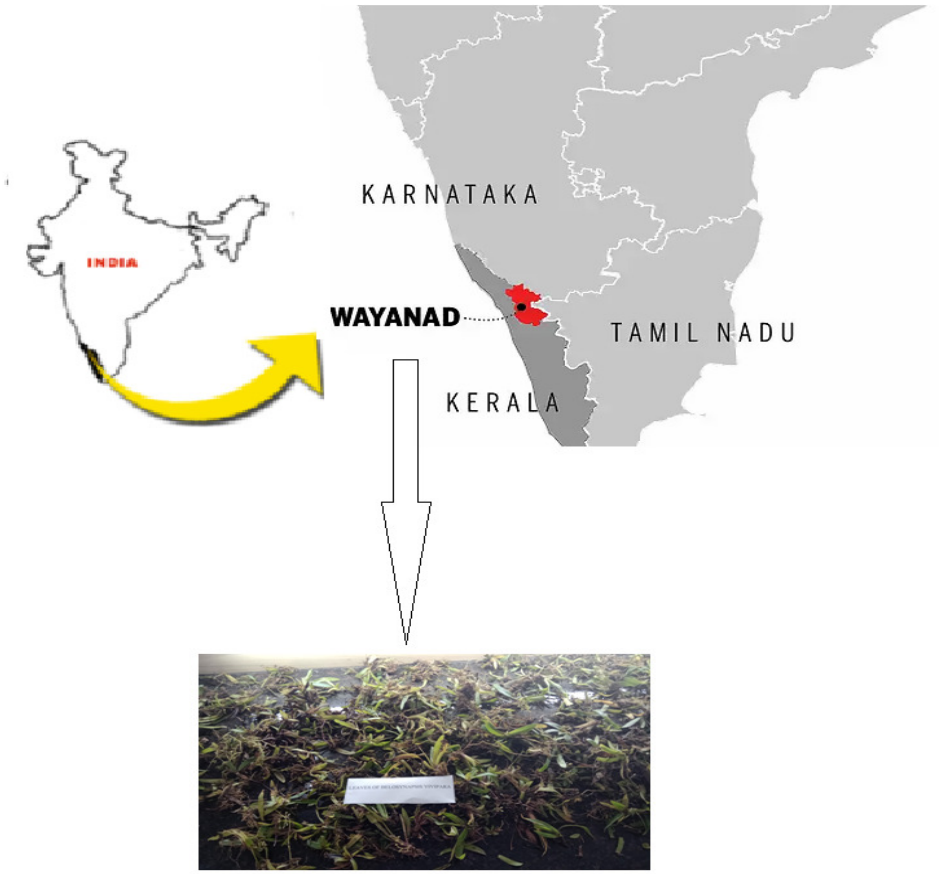

Figure 1. Collection of BV leaves from Wayanad, Kerala, India

Preparation of plant material for extraction and for elemental content:

The leaves were cleaned with running tap water followed by distilled water and spread on newspaper for drying. The leaves were separated from the root part and further dried under shade for 20 days and then powdered by mixer grinder to form coarse powder (Sieve No. 44) (Figure 2).

Figure 2. BV leaves after cleaning with water

About 500 g of powdered drug was separated for the extraction and 100 g of dried powder was kept aside for the elemental analysis. Soxhlet extraction was performed using 80% ethanol solvent for extraction of the BV leaves for 9 hrs. Further, the extracted solvent was filtered using muslin cloth and dried the solvent using rotary flash evaporator at 45 °C to procure viscous semi solid extract. The yield was calculated and kept for further investigation.

In other hand, remaining 100 g of dried powder was used for determination of elements especially few heavy metals and some essential elements present in the leaves using Atomic Absorption Spectrophotometer (AAS). The powdered leaves were digested with three strong acid mixtures. Triacid mixture was prepared by mixed with concentrated nitric acid, concentrated sulphuric acid, and 60% perchloric acid (100: 10: 40). Fe, Mn, Mg, Cu, Zn, Pb, Cd, Ni, As and Cr were determined. 5 g of powdered plant samples were mixed with 15 ml of ternary acid mixture (previously prepared with three concentrated acids) and digested at 180 °C to 200 °C until dense white fumes evolved and formed residue. The residue was further diluted with glass distilled water and made up to definite volume in a volumetric flask. Then the solution was ready for the analysis of Fe, Cu, Mn, Mg and Zn and toxic heavy metals like Cd, Cr, Pb, As and Ni.

Phytochemical screening

The crude extract was screened for presence of group of bioactive constituents present in BV extract through various chemical tests. All the tests were performed as per the standard method described in the previous literature (Devi *et al*., 2014; Das *et al*., 2018).

Antimicrobial activity

Microorganism used: *Escherichia coli* ATCC 8739*, Klebsiella pneumonia* ATCC43816*, Pseudomonas aeruginosa* ATCC 25619 (Gram negative) and *Staphylococcus aureus* ATCC 29726*, Bacillus subtilis* ATCC 6633 and *Streptococcus pyogenes* ATCC 13813 (Gram positive) were used as study organisms for the present investigation. All the organisms were procured from department of Microbiology, Bangalore University, Bengaluru, India. Broad spectrum antibiotic Ampicillin (25 µg/ml) was used as standard and compared the result against the selected antibiotic in this study (Das *et al*., 2011). All the organisms were maintained by sub cultured on nutrient agar medium in Department of Pharmaceutical microbiology, Krupanidhi College of Pharmacy, Bengaluru, India. Ampicillin stock was prepared as 25 µg/ml (w/v) concentration in sterile distilled water and used in the present study.

Minimum inhibitory concentration (MIC) determination: The bacterium was injected into Muller Hinton culture medium for a 24-hour culture, and the result was a colony (Hi Media Lab, India). Extract concentrations of 100, 50, and 25 µg/ml were obtained for each of the two distinct plants that were gathered from various zones by adding 1.0 ml of the extract solution at concentrations of 200 g/ml to 1 mL of nutrient broth (Das, 2014). 9 ml of Nutrient Broth containing standardized test organisms of bacterial cells were mixed with 0.1 ml of each concentration, and 0.5 McFarland turbidity standard (1.0×10^8 CFU/ml) was inoculated into each test tube using the serial dilution procedure (Das, 2014). After 24 hours of incubation at 37 degrees C, growth was found in the tubes, and depending on the MIC value, a further dose was determined for the said activity.

Antibacterial activity of the BV extract was assessed by the agar well diffusion method (Hussein *et al*., 2020) where each isolated microbe was subculture on the recommended specific media for each microorganism at 35-37 °C for 25 h. 100 mg of the BV extract were sterilized by filtration through a membrane filter. 6 mm discs were impregnated with sterile cock borer and 30 µl of BV extract was placed in the wells of agar plates inoculated with microbial culture (after dilution) including standard and then incubated all the plates 37 °C for 16 hours. The zone of inhibition (mm) was measured by using sliding calipers from the back of the inverted Petri dishes (Kaur *et al*., 2013). Triplicate readings were taken to minimize the error.

Correlation study

Antimicrobial study was further correlated with the metal ion contents and determined the effect of metals on the activity.

Statistical analysis

M-Stat software was used for the elemental content in the BV leaves extract by taken mean values of three replicated set of data. Further, antibacterial activities were expressed as the mean ± standard error of mean (SEM) where values of ***P< 0.01 and * P < 0.05 were considered statistically significant. The plots for MIC and antimicrobial activity determination were tabulated using Microsoft Excel and Graph Pad Prism 5, respectively.

Results

BV leaves were extracted using ethanol solvent and yielded about 53.72 g of the extract. The per cent yield was calculated as 10.74 per cent.

In case of elemental content, it was observed that leaves content Mg, Mn, Fe, Cu and Zn with satisfactory amount. Table-2, indicated that content of Zn, Fe and Cu were higher whereas, As, Cr and Pb were not detected as specified condition (Table-1). In the present study Atomic Absorption Spectrophotometer (AAS) was used with selected various wavelength for the detection of elements in the BV plant (Tables 1 and 2).

Table 1. Conditions of AAS for BV leaves analysis

Elements	Wavelength (nm)	Intensity (mA)	Slit used (nm)
Mn	279.5	5	0.2
Mg	285.2	4	0.5
Fe	248.3	5	0.2
Zn	213.9	5	1.0
Cu	324.8	4	0.5
Ni	232.0	4	0.2
Cd	228.8	4	0.5
As	193.7	31	2.0
Cr	357.9	7	0.2
Pb	217.3	10	1.0

Table 2. Concentration of elements (mg/kg) in ethanolic leaves extract of BV plant

Plant extract	Content of heavy metals (mg/ kg)									
	Fe	Ni	Cu	Zn	Cd	As	Cr	Pb	Mg	Mn
BV leaves ethanol extract	2.08 ± 0.21	0.02 ± 0.14	3.73 ± 0.16	4.04 ± 0.11	0.01 ± 0.23	ND	ND	ND	3.21± 0.21	2.03± 0.40

Mean ± SEm (n = 3); ND = Not detected

Simultaneously, various chemical tests were performed for the BV ethanol plant extract and revealed the presence of alkaloids, flavonoids, phyto sterols, saponins and phenolics (Table 3). Further, confirmed with the TLC for the extract based on the LCMS study, showed the presence of particular alkaloids namely, Betanine, Betanidin, flavonoid like quercetin, and plant sterol like beta sitosterol.

Table 3. Phytochemical screening of BV leaves extract

Chemical tests	Ethanol BV leaves extract
Alkaloids	++
Glycoside	+
Flavonoids	++
Phenolics	++
Tannins	+
Steroidal compounds	+
Phytosterols	++
Saponins	++
Proteins	+
Resins	--
Terpenoids	+
Gum	--

(++) = prominently present; (+) = Present; (--) = Absent

Antimicrobial activity further investigated with the extract of BV plant and showed broad spectrum antimicrobial efficacy when compared with the standard antibiotics. Initially, MIC flowed by MBC were determined. The extract's MIC value was established as the lowest concentration that, after 48 hours at 37 °C, totally inhibited bacterial growth. A quantity of liquid (5 µl) was removed from each plate well that showed no growth in order to determine the MBC, which was subsequently incubated at 37 °C for 24 hours. MBC was determined to be the lowest concentration at which there was no discernible bacterial growth during sub-culturing.

MIC determination: The result showed that the MIC values varied from 10.6 to 24.2 µg/ml against all three positive organisms and 12.4 to 26.4 µg/ml against all three negative organisms for the ethanol BV leaves extract (Figure 3). The MIC value 12.4 µg/ml was obtained with extract against *Escherichia coli,* and 10.6 µg/ml against *Staphylococcus aureus.*

MBC determination: Minimum bactericidal effects were exhibited with various degrees for all the organisms. The lowest MBC (2.41 µg/ml and 2.33 µg/ml respectively) were obtained in this study with *Staphylococcus aureus,* and *Escherichia coli* respectively.

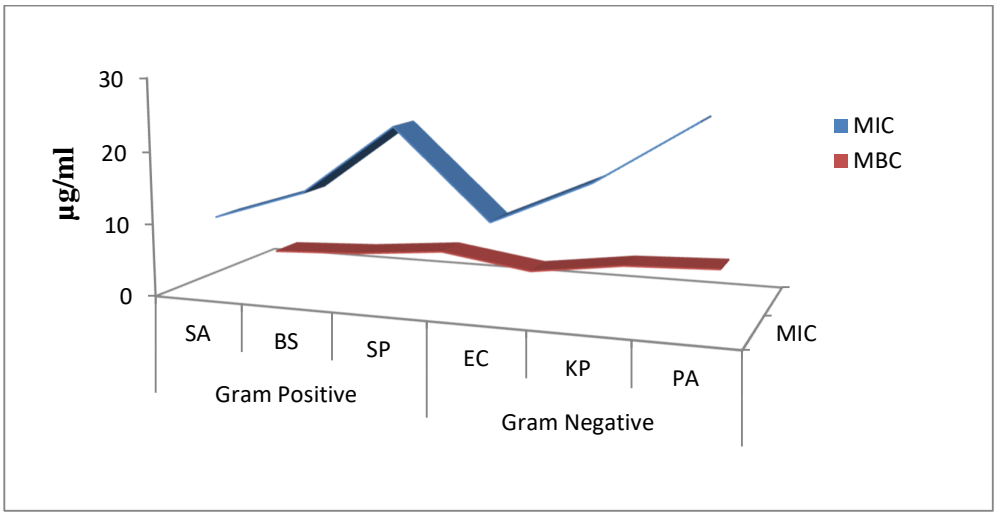

Figure 3. MIC and MBC of ethanol BV leaves extract

EC = Escherichia coli, KP = Klebsiella pneumonia, PA = Pseudomonas aeruginosa;
SA = Staphylococcus aureus, BS = Bacillus subtilis, SP =Streptococcus pyogenes

BV ethanol leaves extract was studied for antibacterial activity and resulted significant dose dependent inhibition ($p<0.01$) against all the microorganisms and as compared standard Ampicillin the results were lesser. Ethanol leaves extract showed maximum inhibition against gram positive organisms than gram negative organisms. Maximum inhibition showed for gram positive organisms *Straphyloccus aureus* (18.5±0.04**) followed by *Bacillus subtilis* (14.3±0.01**) and lowest activity showed for gram negative organisms *Escherichia coli* (11.3±0.12*) followed by *Pseudomonas aeruginosa* (9.7±0.31*) at the concentration of 100 µl/ml (Figure 4; Table 4).

Table 4. Antimicrobial activity of ethanol BV leaves extract against various organisms

Plant extract	Conc (µg/ml)	Zone of inhibition (mm)					
		SA	BS	SP	EC	KP	PA
BV extract	25	14.21±0.11**	12.7± 0.31**	9.21± 0.24**	9.36± 0.33**	7.32± 1.32**	8.62±0.36**
	50	16.23±0.22**	13.4±0.10**	10.33±0.51**	10.3±0.52**	8.52± 1.10**	9.33±0.05**
	100	18.5±0.04**	14.3±0.01**	12.08±0.10**	11.3±0.20**	8.73± 2.11**	9.7±0.33**
Standard	25	22.3±0.11					

Statistically significant: **$p<0.01$; *$p<0.05$ (One way ANOVA); (n =3)

EC = Escherichia coli, KP = Klebsiella pneumonia, PA = Pseudomonas aeruginosa;

SA = Staphylococcus aureus, BS = Bacillus subtilis, SP = Streptococcus pyogenes

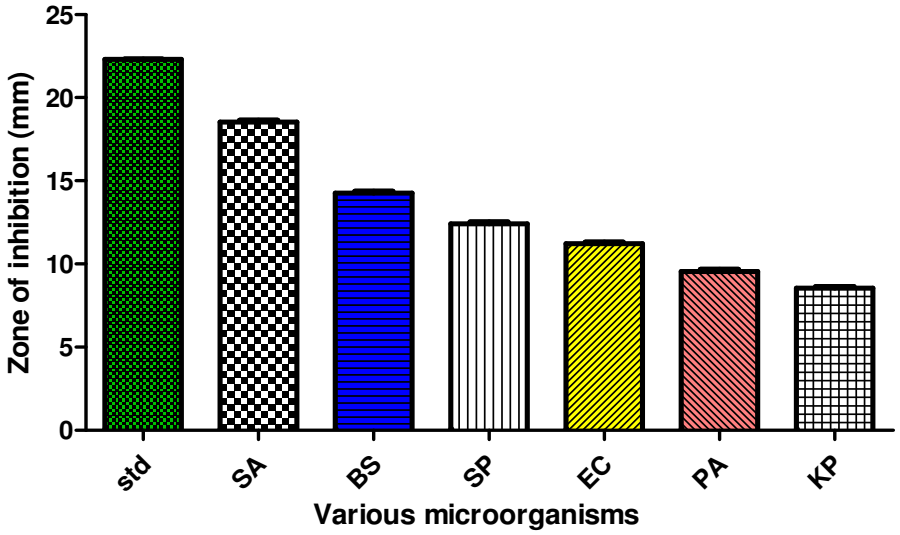

Figure 4. Antimicrobial activity of the BV ethanol leaves at conc. 100 µg/ml

EC = Escherichia coli, KP = Klebsiella pneumonia, PA = Pseudomonas aeruginosa;

SA = Staphylococcus aureus, BS = Bacillus subtilis, SP = Streptococcus pyogenes

Data represented three times n =3; (Mean ± SEm).

Correlation study of the metal ions with the plant extract and the antimicrobial activity was carried out and the positive correlation was observed and revealed the impact of elements on antimicrobial activity (Table 5). Thereafter, it was recorded that non-essential heavy metals were not detected which indicated the BV plant was safe and content no toxic metals.

Table 5. Correlation study of elements with the antimicrobial activity

	Fe	Mn	Mg	Zn	Cu	SA	BS	SP	EC	KP	PA
Fe	1										
Mn	0.981*	1									
Mg	0.980*	0.990**	1								
Zn	0.998**	0.988*	0.990**	1							
Cu	0.988*	0.962	0.983*	0.990**	1						
SA	0.920	0.886	0.939	0.930	0.970	1					
BS	0.998**	0.988*	0.990**	1.00***	0.990**	0.930	1				
SP	0.810	0.789	0.868	0.830	0.891	0.971	0.830	1			
EC	0.981*	1.00***	0.990**	0.988*	0.962	0.886	0.988*	0.789	1		
KP	0.951	0.906	0.873	0.932	0.902	0.784	0.932	0.614	0.906	1	
PA	0.996**	0.966*	0.976	0.994**	0.996**	0.947	0.994**	0.847	0.966*	0.938	1

Statistically significant: ***p<0.001; **p<0.01; *p<0.05 (One way ANOVA)

EC = Escherichia coli, KP = Klebsiella pneumonia, PA = Pseudomonas aeruginosa;

SA = Staphylococcus aureus, BS = Bacillus subtilis, SP = Streptococcus

Discussion

In the present study ethanol was used as the solvent is nontoxic with polarity index value of 5.2 (Abarca-Vargas *et al*., 2016). Thereafter, ethanol has high dielectric constant of 24.55 which capable to extract maximum bioactive components of the plant such as polyphenols, tannins, flavonols, terpenoids, and alkaloids (Azmir *et al*., 2013). Therefore, in the present study ethanol was used as solvent.

Detection and analysis of elements through Atomic Absorption Spectrophotometer is most essential because the elements are responsible for the production, accumulation and enhancement of the bioactive component in plants (Aziz *et al*., 2016). Very few reports explained about role of elements on plant chemicals enhancement (Singh *et al*., 2022) and productivity and thereafter, it was also required to know about the non-essential heavy metal content in the leaves because the plants were collected from the forest zone and there was chanced for contamination by the climatic conditions and variation of constituents.

AAS is a flexible, non-destructive analytical technology that is frequently used to identify minor and trace components in intricate biological materials (El-Mesery *et al*., 2019). The results were compared with the standard limits of the nonessential heavy metals as per WHO guidelines, and was found to be within safe limits.

Various chemical constituents like Zn, Fe and Cu were present prominently may be due to the presence of metals like Mn, Zn, Cu and Fe content in plant. Previous literature reported that Zn, Cu and other metals influence the enhanced bioactive compounds in plant part (Das and Tribedi, 2015) and the same result reported in the present experiment.

It was reported that the value of the lowest MBC obtained was not more four times higher than that of MIC's on the corresponding pathogens, It seems possible that the sample tested was possessed the antimicrobial activity (Kowalska-Krochmal and Dudek-Wicher, 2021). This value is not more than four times greater than that of the MIC's of the corresponding microorganisms. This data was supported by the previous literatures *(*Meyer and Lall, 2007; Kuete *et al*., 2008).

Conclusions

Microbiological resistance is becoming a bigger issue, and it is unclear how antimicrobial medications will be used in the future. As a result, the study also revealed that plant-based extracts might suppress bacteria. The result of the present investigation revealed the ethanol extract of BV leaves is significantly active against

three gram positive and three-gram negative bacterial strains and suggested that BV leaves can be used for treatment of infection caused by broad spectrum microorganisms. Further, content of elements were determined and resulted essential elements such as Fe, Mg, Mn, Cu and Zn were present whereas toxic non-essential elements vis. Cr, Cd, Ni, Co, Pb and As were not detected or may be present in very low limits which indicated the leaves of BV is safe for further applications. Thereafter, when metal ions were correlated with the said activity, it showed positive correlation and established potential role in accumulation and availability of the bioactive constituents which causes potent antimicrobial activity. Some of the observations have helped in identifying the active principle responsible for such said activity and first time reported as a developing plant source as drug for the therapeutic use in human beings. Finally, in conclusion, the result clearly elaborated that *Belosynapsis vivipara* has great potential to invade human pathogenic bacteria.

Authors' Contributions

KD has designed the work and written the whole manuscript. SS was performing the antimicrobial test along with design of graphs. Both authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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