Isolation and identification of *Bacillus* species from soil and assessment of antimicrobial properties

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Abstract: The emergence of multi drug resistant pathogens such as Methicillin resistant *Staphylococcus aureus* (MRSA) has become a threat to public health. Thus, the development of new antibiotics has become a global concern. Many scientists are attracted towards discovery of new antimicrobial agents from microbial sources. The aim of this study is to isolate potential *Bacillus* species from soil for the production of antimicrobial substance. During the study, 20 soil samples were collected from various areas of Kathmandu valley. A total of 70 *Bacillus* isolates were identified by morphological and biochemical characteristics. Altogether 39 isolates were able to produce antibacterial extract when it was grown in Trypticase soya broth (TSB) at 37°C for 48 h. The antimicrobial activity was tested against test organisms like *Escherichia coli, Klebsiella pneumoniae, Salmonella, Pseudomonas, Staphylococcus aureus and Candida* by Agar well diffusion method. All of the extracts showed zone of inhibition against *E. coli* except 6 extracts (E39, E43, E36, E25, E45 and E46). The highest zone of inhibition was shown by extract B68 with 11 mm. Only one extract (E62) was able to inhibit the growth of *Pseudomonas*. The extract from *Bacillus* isolate (E35) showed highest zone of inhibition against *S. aureus* (6mm). The protein nature of the extracts was determined by Biuret and Ninhydrin tests. The extracts were relatively stable to heat treatment and unstable to surfactants like 70% methanol, 2-propanol and ethylacetate. The study revealed that soil is a good source for the potential antimicrobial producer bacteria.

Keywords: Antimicrobial substance; Cell free extract; *Bacillus*; Soil; Anti-bacterial activity.

Introduction

Antimicrobial substances are defined as chemotherapeutic agents produced by microorganisms as secondary metabolites. *Bacillus* species produce many types of antibiotics by ribosomal (bacteriocin) and nonribosomal (polymyxins and iturins) mechanisms. *Bacillus* species are industrially important bacteria because they are safe, grow rapidly, have short fermentation cycles and secrete large amount of protein in the medium[1]. The bacteriocins from *Bacillus* species have applications in pharmaceutical, food industry, fishery, and livestock and in agricultural sector[2].

The Bacillus is Gram-positive, rod shaped and spore-forming bacteria. It is an aerobic and catalase producing bacteria and is found in different environments such as soil, rocks, dust, marine environment, food and the gastrointestinal tract of animals[3]. This diversity is due to the presence of the endospores, their different physiological properties and growth requirements of *Bacillus* species. Members of the *Bacillus* group are known to be a good producer of antimicrobial substances, including, peptides, lipopeptides antibiotics, and polyketides[4]. Subtilin, Ericin S and ericin A, Sublancin 168, Mersacidin, etc are few examples of bacteriocins produced by *Bacillus* species[2].

The use and misuse of antibiotics had resulted in emergence of resistant strains. For example, *Staphylococcus aureus*, the most common cause of hospital acquired infections, developed resistance to methicillin and several other antibiotics. However, due to the over use of vancomycin to treat Methicillin Resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterococcus faecium* (VRE) appeared. In addition, there are many Gram negative pathogens such as *Pseudomonas aeruginosa*, *Acinetobacter baumanii* and Enterobacteriaceae which are resistant to many antibiotics[5]. Although there are many antibiotics available, there is still a need for discovery of new types of antibiotics to solve the problem of multi drug resistant (MDR) among pathogenic bacteria. There are

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a few antimicrobial compounds, such as antimicrobial peptides, probiotic bacteria, and bacteriophages which are now considered as alternatives to antibiotics. Thus, this study is aimed to isolate and identify the potential *Bacillus* species from soil that produces novel antimicrobial compound.

Material and methods

Sample collection

The soil samples were collected from agricultural fields, forests, rhizosphere and wastelands from different parts of Kathmandu, Nepal. The collection of soil samples from different environments were supposed to have possible microbial interaction.

The 20 gm soil sample was collected from 5 cm deep from the surface and kept in a sterile plastic container and sealed[6]. The samples were carried to the laboratory for further processing. Thus collected samples were stored at room temperature until processing.

Isolation and Identification of *Bacillus* from soil sample

For isolation of *Bacillus*, first of all, 1 gm of soil was mixed in 10 ml of distilled water and it was heated at 80°C followed by serial dilution up to 10^{-5} dilution. The aliquots from dilution tubes were inoculated on Nutrient Agar (NA, HiMedia, India) medium by spread plate technique[6]. The plates were incubated at 37°C for 24 hours. The following day, the media plates were observed for visible growth of colonies. For the identification of *Bacillus* species, following examinations were done: Colony characteristics, Gram staining, spore staining, biochemical tests (catalase, oxidase, O/F, motility) and other tests (starch hydrolysis, gelatin hydrolysis, blood haemolysis, glucose and mannitol fermentation tests).

Test microorganisms

The *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa*, *Candida species*, *Salmonella* species and *Klebsiella pneumoniae* (ESBL positive) were used as test microorganisms in the study. All organisms were cultured in NA medium and incubated at 37°C for 24 hrs and stored at 4°C for further use.

Antibiotic susceptibility test

The antibiotic susceptibility test was performed for indicator strains by Kirby´s Bauer disc diffusion method. Different standard antibiotic discs were kept on the Mueller Hinton Agar (MHA, HiMedia, India) plate seeded with indicator strains. The plates were incubated at 37 °C for 24 hours. After incubation, the plates were observed for zone of inhibition. ZOI was measured using scale in mm.

Growth of *Bacillus* and its antimicrobial activity

For the growth of *Bacillus*, Nutrient Broth (NB, HiMedia, India) and Trypticase Soya Broth (TSB, HiMedia, India) media were used. A loopful of the *Bacillus* isolates was inoculated in 100 ml of above mentioned broths in 250 ml Erlenmeyer flask and was incubated at 37°C for 2 days. After incubation, every 24 h, and 48 h culture was centrifuged at 10,000 rpm, 4°C for 30 min. The cell free supernatant (extract) was used for antimicrobial activity assay.

The cell pellet was then dissolved in 70% methanol (Emplura, Germany) and was again centrifuged at 10,000 rpm and 4°C for 30 min. The cell wash extract was taken and again tested for its antimicrobial activity against indicator strains.

Cell free supernatant was used for antimicrobial activity by agar well diffusion assay. MHA plates were seeded with the indicator strains and incubated at room temperature until the plates were dried. The concentration of indicator test organism was standardized by comparing with the standard MacFarland solution (0.5%). The well of 7 mm diameter were made on the agar with the help of cork borer.100 µl of culture filtrate was pipetted into the agar well. The plates were incubated at 37°C for 24 h and were observed for zone of inhibition. The diameter of ZOI was measured in mm[7]. All assays were carried out in triplicates (3 plates per isolate per test organism). Similarly, antimicrobial assay was performed for cell wash extracts against indicator strains.

Characterization of cell free extracts:

Biuret and Ninhydrin test

Biuret and ninhydrin tests were performed to determine the proteinaceous nature of cell free extract.

Biuret test was performed in sterile test tubes. The 1 ml of the extract was transferred into one test tube. Then, 2-3 drops of biuret reagent was added and shook well. The mixture was allowed to stand for 5 min and observed for color change to purple. Similarly, 1 ml of distilled water taken as negative control and test was performed as above.

For ninhydrin test, 1ml of supernatant and 1ml of distilled water (negative control) was taken in test tube. Then, 2-3 drops of ninhydrin solution was added to each test tube, and was gently warmed in a water bath for 5 min.

Thermal Stability test:

To determine the effect of temperatures and surfactants in the cell free extract, a stability assay was performed. The thermal stability of the extract was tested by incubating 2 ml of extract at 37 °C, for 1h. The residual antimicrobial activity was determined by agar well diffusion assay against test organism. Similarly, the effect of surfactants like 70% methanol, 2-propanol (Emplura, Germany) and ethylacetate (Emplura, Germany) were done by adding equal volume of solvent to the extract and incubated at 37 °C, for 1h. Then the activity was determined by agar well diffusion assay against test microorganism. The untreated one was taken as positive control.

Results and Discussion

Isolation and Identification of *Bacillus*

A total 20 soil samples were collected from different parts of Lalitpur, Kathmandu and Bhaktapur districts of Nepal. From these 20 soil samples, altogether 70 *Bacillus* species were isolated. Isolated *Bacillus* species were coded as B1, B2, and so on. The distribution of *Bacillus* isolates from different sample sites are shown in Table 1.

Table 1: Distribution of *Bacillus* spp among different sites.

Districts	Location	Total no. of samples	Total no. of *Bacillus spp*
Lalitpur	Gwarko	3	12
	Godawari	1	3
	Bhaisepati	1	5
	Hatiban	1	5
	Lubhu	2	3
Bhaktapur	Chyamasingh	1	4
	Changunarayan	2	5
	Bode	2	4
Kathmandu	Jorpati	3	13
	Samakhusi	2	6
	Balaju	1	4
	Kirtipur	1	5
	Grand total	**20**	**70**

Bacillus strains were found to be Gram positive rods with centrally located spores (**Figure 1**). Biochemical characteristics of only *Bacillus* isolates that exhibited activity against test strains are presented in **Table 2**.

All the isolates were motile and able to hydrolyze starch as well as gelatin. Yahya et al (2021) carried out amylolytic, esterases, proteolytic and haemolytic activity for biochemical characterization of *Bacillus*. They found strong amylolytic and proteolytic and beta haemolytic activity while no esterase activity[8]. In this study–positive haemolytic activity was also exhibited by isolated *Bacillus species*. Here, all the *Bacillus* isolates were able to ferment mannitol while glucose fermentation was shown by only some of the isolates Table 2.

Likewise, all the isolates were tested for susceptibility pattern against antibiotic Penicillin (Table 2) and all the isolates were found to be Penicillin resistant which indicates that isolated *Bacillus* strains were other than *B. anthracis*. Salgado et al (2020) also performed Penicillin susceptibility test to identify *B. anthracis*. Also, all isolates were found to be motile and were able to hydrolyze starch and gelatin[9]. The study done by Sambhavi et al (2020) showed that the *Bacillus* isolates were screened for amylase activity on starch hydrolysis test. They found, out of ten isolates, seven bacteria showed the zone of clearance on starch agar media[10]. Similar study was also performed by Singh et al (2016) in which they reported that, out of ten isolates, five showed clear zone of hydrolysis on starch agar media[11].

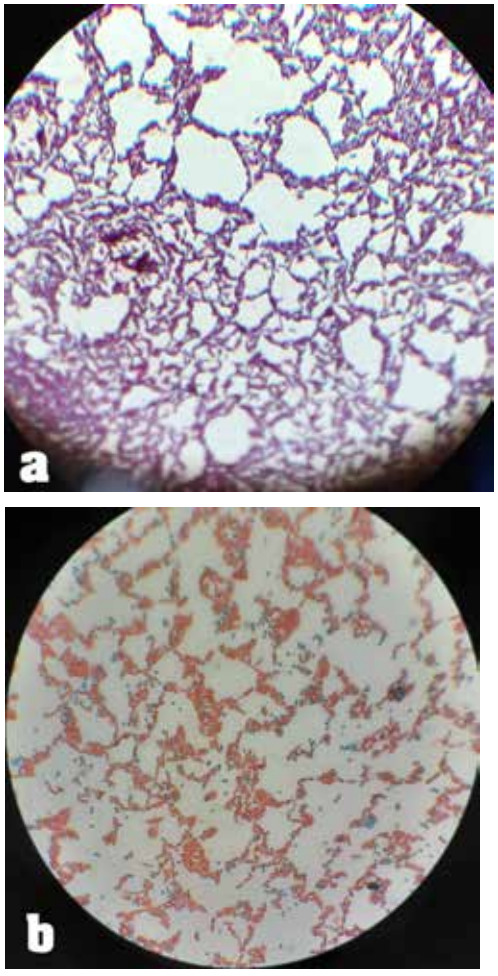

Figure 1: (a) Gram positive rod and (b) spores of *Bacillus* under 100 X microscope.

Table 2: Characteristics of Isolated *Bacillus* species that showed activity against test strains.

Bacillus code	Glucose fermentation	Manitol fermentation	Motility	Starch hydrolysis	Gelatin hydrolysis	Penicillin susceptibility
B10	-	+, G-	+	+	+	Resistant
B13	-	+, G-	+	+	+	Resistant
B15	-	+, G-	+	+	+	Resistant
B23	-	+, G-	+	+	+	Resistant
B28	-	+, G-	+	+	+	Resistant
B35	-	+, G+	+	+	+	Resistant
B37	-	+, G-	+	+	+	Resistant
B38	-	+, G+	+	+	+	Resistant
B39	+	+, G-	+	+	+	Resistant
B40	-	+, G-	+	+	+	Resistant
B41	+	+, G-	+	+	+	Resistant
B42	-	+, G-	+	+	+	Resistant
B43	+	+, G+	+	+	+	Resistant
B45	+	+, G-	+	+	+	Resistant
B46	+	+, G-	+	+	+	Resistant
B47	+	+, G-	+	+	+	Resistant
B48	+	+, G-	+	+	+	Resistant
B49	+	+, G-	+	+	+	Resistant
B50	-	+, G-	+	+	+	Resistant
B51	-	+, G-	+	+	+	Resistant
B52	-	+, G-	+	+	+	Resistant
B53	-	+, G-	+	+	+	Resistant
B54	-	+, G-	+	+	+	Resistant
B55	-	+, G+	+	+	+	Resistant
B56	-	+, G-	+	+	+	Resistant
B57	-	+, G-	+	+	+	Resistant
B58	-	+, G-	+	+	+	Resistant
B59	-	+, G-	+	+	+	Resistant
B60	-	+, G-	+	+	+	Resistant
B61	-	+, G-	+	+	+	Resistant
B62	+	+, G-	+	+	+	Resistant
B63	+	+, G-	+	+	+	Resistant
B64	+	+, G-	+	+	+	Resistant
B65	-	+, G-	+	+	+	Resistant
B66	+	+, G-	+	+	+	Resistant
B67	+	+, G-	+	+	+	Resistant
B68	-	+, G-	+	+	+	Resistant
B69	+	+, G-	+	+	+	Resistant
B70	-	+, G-	+	+	+	Resistant

(+) indicates positive; (-) indicates negative; (G+) indicates presence of gas; (G-) indicates absence of gas

Growth of *Bacillus* and detection of antimicrobial activity

For the growth of *Bacillus*, NB and TSB were used. The cell free extract (E) was obtained by centrifuging the bacterial culture at 10,000 rpm for 15 min. In the present study, both of the media used supported the growth of *Bacillus*. However, the cell free extract from TSB only inhibited the growth of test organisms. Thus, TSB was used as culture medium to produce the crude cell free extract which were named as E1, E2, and so on. Singh et al (2016) also centrifuged bacterial culture at 10,000 rpm for 15 min to obtain crude extract as supernatant[11]. Similar results were found in some other

research works, such as, Tabbene et al (2009) showed that *B. subtilis* B38 strain grown in nutrient broth did not inhibited test organisms[12]. In the study done by Kumar et al (2009), extract from TSB produced better inhibition zones than nutrient broth[13]. Moreover, Thubiani et at (2018), prepared Man Rogosa and Sharpe (MRS) broth for the culture of *Bacillus megaterium*. To extract the antimicrobial compound, the culture medium was centrifuged at 10,000 rpm, 4°C for 15 min and filtered on sterile nitrocellulose membrane filter with pore size 0.2µm. Then the filtrate was mixed with n-butanol and supernatant was separated and concentrated[14]. Furthermore, Barale and colleagues compared different extraction methods for extraction of antimicrobial lipopeptide from *Bacillus velezensis* SK. They used organic solvents like chloroform and ethylacetate in equal volume to extract active compound from cell free broth[15]. However, in this study, only cell free supernatant was extracted and tested against different strains. Therefore, further experiments are required to extract the active compound by using different solvents like butanol, ethylacetate, chloroform, etc and test them against pathogenic organisms. It is necessary to purify the active compound and analyze it by chromatographic techniques like thin layer chromatography as shown by Thubiani and colleagues in 2018[14].

Among 70 *Bacillus* isolates, only 39 isolates were found to exhibit antimicrobial activity against indicator strains. Antimicrobial activity was measured as zone of inhibition in mm. All the test strains were tested for susceptibility pattern against standard antibiotic disc and results are shown in Table 3. In this study, extracts were able to inhibit the growth of both Gram positive and Gram negative bacteria while inactive against *Candida*. It indicates the antibacterial compound is of broad spectrum. In the present study, all the extracts (100%) exhibited activity against *E. coli* followed by *S. aureus* (44.7%) and *Salmonella* (34.21%). The Cell free extract E10 and E15 showed activity against *S. aureus* (3mm) and *E. coli* (5mm) while it was unable to inhibit the growth of *K. pneumoniae*, *Pseudomonas*, *Salmonella* and *Candida* (Figure 2). The extract E35 demonstrated the highest zone of inhibition against *S. aureus*, *E. coli* (ATCC) and *E.coli* which is 6mm, 10 mm and 6mm respectively (Figure 2) while did not exhibit inhibition against *K. pneumoniae*, *Pseudomonas*, *Salmonella* and *Candida*. Similarly, extract of E68 showed activity against *E. coli* with the highest zone of inhibition of 11 mm and was also able to inhibit *Pseudomonas* with inhibition zone of 7mm. Likewise, only 13 extracts (E47-E58, E60) exhibited activity against *Salmonella* spp (Figure 3) with the highest inhibition zone of 5mm by extract E52 (Figure 2). However, only one extract E42 inhibited *K. pneumoniae* (ESBL) with inhibition zone of 6 mm. Here only antimicrobial activity of Extracts (E10, E15, E23, E35, E42, E52, E56 and E68) is presented in Figure 2. In contrary to this study, the research done by Adamu et al (2009), demonstrated higher inhibitory effect against *K. pneumoniae* (83%) followed by *Salmonella* species (66.7%) and *P. aeruginosa* (16.7%), whereas lower inhibitory effect against *S. aureus* (41.7%) and *E. coli* (25%)[16].

In this study, all the cell pellets were again dissolved in 70 % methanol and centrifuged to get cell wash extract. When cell wash extract was subjected to antibacterial test, none of the extracts showed activity against indicator strains. The antimicrobial property of cell wash extract may depend on the solvents used to dissolve the cell pellet. In the similar experiment performed by Basi-Chipalu et al (2014), they dissolved cell pellet in 70 % isopropanol and the cell wash extract exhibited activity against indicator strain *Micrococcus luteus* and other Gram positive bacteria[17]. Likewise, Dischinger et al (2009) also did similar study, in which isopropanol cell extract were active against Gram positive bacteria[18].

Pang et al (2019) suggested that Gram negative bacteria such as *Pseudomonas* spp. is usually resistant to a wide range of antibiotics [19]. It is similar to this study because only one extract E68 inhibited *Pseudomonas* spp., with inhibition zone of 5 mm (Figure 2). Moreover, Motta et al (2004) suggested that the Gram positive bacteria produced antimicrobial substances which inhibited only other Gram positive bacteria[20]. In contrary, according to Aslim et al (2002), the antimicrobial agents from *B. subtilis* MIR 15 inhibited mostly Gram negative bacteria including *E. coli* and *P. aeruginosa* [21].

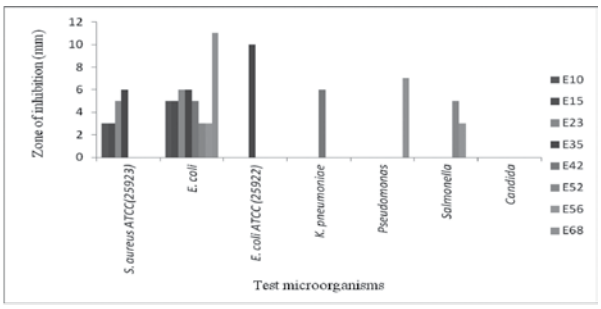

Figure 2: Antimicrobial activity of Cell free extracts against test organisms.

Figure 3: Zone of inhibition exhibited by cell free extracts (E10, E35, E68, E23 and E37) against test strain *Salmonella* sps.

Table 3: Antibiotic susceptibility pattern of test strains against standard antibiotics.

Bacteria	Antibiotic	Zone of inhibition	Inferences
E.coli ATCC 25922	Amoxicillin	-	Resistant
	Ciprofloxacin	14 mm	Resistant
	Nalidixic acid	5mm	Resistant
	Co-trimoxazole	7mm	Resistant
	Nitrofurantoin	6mm	Resistant
	Ceftriaxone	2mm	Resistant
E.coli	Amoxicillin	3mm	N/A
	Ciprofloxacin	17 mm	Resistant
	Nalidixic acid	13 mm	Resistant
	Co-trimoxazole	17 mm	Susceptible
	Nitrofurantoin	12 mm	Resistant
	Ceftriaxone	11 mm	Resistant
Klebsiella pneumoniae	Amoxicillin	-	Resistant
	Ciprofloxacin	13 mm	Resistant
	Co-trimoxazole	10 mm	Resistant
	Nitrofurantoin	4 mm	Resistant
	Ceftriaxone	10 mm	Resistant
	Penicillin	-	Resistant
Salmonella	Amoxicillin	4 mm	N/A
	Ciprofloxacin	18 mm	Resistant
	Co-trimoxazole	8 mm	Resistant
	Nitrofurantoin	7 mm	Resistant
	Nalidixic acid	No ZOI	Resistant
Pseudomonas	Gentamycin	No ZOI	Resistant
	Ciprofloxacin	16 mm	Resistant
	Co-trimoxazole	4 mm	Resistant
	Nitrofurantoin	No ZOI	Resistant
	Ceftazidime	8 mm	Resistant
S. aureus ATCC 25923	Penicillin	3 mm	Resistant
	Amoxicillin	7 mm	N/A
	Ciprofloxacin	10 mm	Resistant
	Nitrofurantoin	11 mm	Resistant
	Cotrimoxazole	16 mm	Susceptible
Candida	Penicillin	6 mm	N/A
	Amoxicillin	8 mm	N/A
	Ciprofloxacin	19 mm	N/A
	Nitrofurantoin	9 mm	N/A
	Cotrimoxazole	10 mm	N/A
	Gentamycin	13 mm	N/A

Characterization of cell free extracts

Biuret test and ninhydrin test

All the extracts with antimicrobial activity were tested for its proteinaceous nature by biuret test and ninhydrin test. All of the extracts showed positive result in both the tests. This indicates that the extracts may contain amino acids or peptide residues. Yahya et al (2021) also performed Ninhydrin, Sudan III, Molisch test, for determination of amino acid, lipid and carbohydrate in cell free extract. They reported the presence of peptide residues, carbohydrate while absence of lipids in the purified extracts[8]. In the present study only crude extract was used for chemical analysis, however, purification of extract could result in better characterization.

Stability tests

For stability assay, only extracts exhibiting ZOI of > or

= to 2mm against 2 or more than 2 indicator strains were selected. In heat stability assay, extracts were treated to 37°C for 1 hr. All the extracts treated showed activity against test strains even after heat treatment (Table 4). Ramachandran et al (2014), also performed heat stability assay and they found that the antimicrobial substance was heat stable at all temperatures tested. They reported that 100% activity was retained at 37°C for 5h, 95% and 88% at 50°C and 60°C for 3h, respectively, 85% at 70°C and 80°C for 2h and 1h, respectively, 82% at 90°C and 100°C for 1h, respectively, and 72% activity at 121°C for 40 min[22]. Cavalini et al (2021) assessed stability against temperature and reported that 100% activity was seen when treated at 100 °C for up to 5 min, but complete inactivation after treated for 10 min at the same temperature, and also at 121 °C/15 min[23].

In stability test against 70% methanol , all of the extracts were found to be unstable except (B23 and B37) as the extracts were failed to show activity against test organism after treated with solvents (*data not shown*). Additionally, when treated with 2-propanol, all the extracts were inactive against test organism except 3 extracts (B10, B23 and B68) which showed activity against *E. coli* and *K. pneumoniae (data not shown)*. In contrast to this result, Cavalini et al (2021) found 100 % residual activity of extract after treatment with methanol. Likewise, the activity of extract was relatively decreased when treated with ethylacetate[23].

The use of antimicrobial peptides in medicine is limited due to their susceptibility towards different factors like heat, pH, proteases, and in case of lantibiotic nisin low stability at physiological pH level[24]. Thus, the stability of a peptide towards the proteases and physiological pH would be an advantage for its application.

Table 4: Thermal stability test of extracts (37°C).

Zone of inhibition against test organisms (mm)

Cell free extracts	*S. aureus ATCC(25923)*		*E. coli*		*E. coli* ATCC (25922)	
	Before	After	Before	After	Before	After
E10	3	0	5	5	0	0
E23	5	0	6	3	0	0
E35	6	4	6	4	10	4
E37	0	0	6	5	0	0
E48	0	0	2	3	0	0
E49	0	0	2	2	0	0
E52	0	0	3	3	0	0
E57	0	0	2	0	0	0

Conclusions

Bacillus species were isolated and identified from soil samples collected from various areas of Kathmandu valley. Among 70 *Bacillus* isolates, cell free supernatant of 39 isolates exhibited antibacterial activity against both Gram positive and Gram negative bacteria. All of the extracts showed zone of inhibition against *E. coli* except 6 extracts (B39, B43, B36, B25, B45 and B46), highest zone of inhibition was shown by extract B68 with 11 mm. When compared to standard antibiotic discs, it was found that the extract E68 exhibited comparable zone diameter against *E. coli*. The Biuret and ninhydrin tests indicated that the extract were proteinaceous in nature (mixture of peptides). This study showed most of the extracts was stable to heat treatment while extracts were inactivated by surfactants like 2-propanol, 70 % methanol and ethylacetate.

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