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ANTIMICROBIAL ACTIVITY OF MEDICINAL PLANT EXTRACT AND ASSOCIATED ACTINOMYCETES AGAINST UROPATHOGENS

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Abstract - *Ethnomedicine and actinomycetes can be the potential sources of novel antimicrobial compounds to tackle increasing multidrug resistance among uropathogens. We evaluated the antimicrobial potential of methanolic extracts of three plants viz. Ocimum sanctum, Asparagus officinalis, and Tribulus terrestris, along with actinomycetes isolated from the root vicinity of the first two plants against three multi-drug resistant uropathogens. Phytochemical extraction was performed by cold maceration and actinomycetes were isolated using Actinomycetes Isolation Agar. After perpendicular streaking assay, isolated actinomycetes were subjected to submerged state fermentation followed by ethyl acetate extraction of secondary metabolites. After primary assays, MIC and MBC of both plant and associated actinomycetes extracts were figured out. All three plant extracts inhibited Escherichia coli. Only A. officinalis exhibited effect over Klebsiella oxytoca. Extract of O. sanctum and T. terrestris prevented the growth of Staphylococcus aureus. A. officinalis was more effective against Gram-negative pathogens, and O. sanctum was more effective against Gram-positive pathogen. Five of the 13 ethyl acetate extracts had an inhibiting effect over at least one Gram-negative pathogen, and ten extracts had an effect against S. aureus. Crude extract of MK1, actinomycetes, isolated from soil of T. terrestris had the lowest MIC against all three test organisms. From the results, it can be suggested that both plant extracts and actinomycetes can be potent sources of bioactive compounds.*

Key Words: Plant Extract, Uropathogens, Actinomycetes, Antimicrobial Activity, Minimum Inhibitory Concentration (MIC)

1. INTRODUCTION

Infectious diseases caused by microorganisms are among the leading causes of death worldwide, particularly in developing countries. Bacterial infections occur when the host's defense cannot withstand invading bacteria [1].

Urinary Tract Infection (UTI) is one of the most common infections caused by a range of bacterial pathogens, mainly *Escherichia coli, Klebsiella, Enterococcus,* and *Staphylococcus.* Worldwide, 150 million people are infected every year [2]. Women are more vulnerable to UTIs than men, with the risk increasing with age [3]. According to a study in Kathmandu Medical College and Teaching Hospital, Duwakot, Nepal, by Pradhan & Pradhan (2017) [4], 75% of UTI patients were female. Up to 50% of women have at least one symptomatic UTI in their lifetime, and the risk of infection increases in postmenopausal women [5]. People can acquire UTIs in a hospital or at the community level.

A Hospital-acquired UTI (HUTI) is an infection acquired by a patient during his visit to the hospital due to the hospital environment and different devices. The pathogens responsible for HUTIs are usually endogenous in origin but sometimes originate from the hospital environment [6]. *E. coli*, *Klebsiella*, *Pseudomonas aeruginosa*, *Proteus, Enterococcus, S. aureus, Candida*, are the major causative agents [7]. According to the International society for infectious diseases the frequency of HUTIs among hospital-acquired infection is 24% in developing countries. UTI causing bacteria from hospital-acquired infection were reported to be more resistant one [8].

Community-acquired UTI (CUTI) is an infection that occurs in the community. CUTIs are mostly encountered in rural areas. The most common organism causing communities to acquire UTIs are *E. coli*, *Klebsiella* and *Enterococcus*. *E. coli* is the predominant bacterial pathogen of CUTIs [9].

UTIs are treated through a diverse group of antibiotics; beta-lactams, fluoroquinolones, aminoglycosides, and others. However, uropathogens are becoming drug-resistant, which has become a hindrance to the disease's control. The reasons for drug resistance can be spontaneous mutation or acquisition of resistant genes through horizontal transfer that often occurs in organisms of different taxonomy [10, 11].

Emerging multidrug-resistant bacteria has made the search for novel bioactive compounds from natural and unexplored habitat a necessity. From the ancient period, plants and their parts have been used as a source of medicine to treat different diseases. Ethnomedicine is still considered a primary source of treatment in many communities. About 60% of the world's population, with 80% in developing countries, are directly dependent on medicinal plants for treatment of different diseases [12]. *Azadirachta indica, Artemesia vulgaris, Asparagus officinals, Ocimum santum, Tribulus terrestris* are some common medicinal plants used in Nepal.

Bacteria have so far been the most promising source of antibiotics in the past decades and will undoubtedly remain an important source of novel bioactive compounds in the future. Approximately 45% of the bioactive compounds obtained from microbes are produced by actinomycetes [13]. Around 23,000 bioactive secondary metabolites produced by microorganisms have been reported, over 10,000 of these compounds are produced by actinomycetes [14]. Vancomycin, streptomycin, rifampicin, novobiocin are some antibiotics produced by actinomycetes [15].

It is necessary to manage the increasing drug resistance in microbes, so we tried two approaches to tackle MDR pathogens, i.e., medicinal plants and actinomycetes. Medicinal plants are the easiest and the most common approach for treatment. They are readily available and used since ancient times for curative purposes. Medicinal plants have been used against different infections as they have antimicrobial potential. This potential has to be studied more in this era where tackling bacterial infection is of utmost importance. As actinomycetes contribute to more than 50% of known antibiotics produced to this date, they remain at the top as the potential source of bioactive compounds. Therefore, exploring new habitats has become necessary to isolate novel actinomycetes and screen them for antimicrobial potential.

2. EXPERIMENTAL PROCEDURE

2.1 Study Design

We conducted a research from September 2019 to February 2020 to analyze the antimicrobial activity of medicinal plant extract and associated actinomycetes against uropathogens in the Department of Microbiology, National College, Kathmandu, Nepal

With the help of a literature review, we selected three different plants based on their traditional use to cure UTI. The soil sample was collected from the root vicinity of two of those three plants. Phytochemical extraction and screening were done. Bacteria isolated from urine samples were obtained from the hospital. Antibiotic susceptibility testing was conducted to evaluate their antimicrobial-susceptible profiles. An antimicrobial assay against the sample was carried out.

2.2 Plant and Soil Sample Collection

Three plants having ethno medicinal values were collected for the study. Whole plant of *Asparagus officinalis* (Kurelo) was collected from Baneshor, Kathmandu. Leaves of *Ocimum sanctum* (Kalo Tulsi) were collected from Kakkarvitta, Jhapa. Dried fruits of *Tribulus terrestris* (Gokhur Kada) were bought from Naradevi, Kathmandu.

Ten gram of soil around the root of *A. officinalis* and *O. sanctum* was collected in two separate sterile sample collection bags and transferred aseptically to the laboratory.

2.3 Phytochemical Extraction

The plant samples were shadow dried for different periods depending on the plant material (20 days for the whole plant of *A. officinalis* and 5 days for leaves of *O. sanctum*). The dried plant material was grinded into fine powder using a grinding machine. Extraction was done by cold maceration method as described by Nalin Pagi & Payal Patel (2017) [16]. 100 ml of absolute methanol was added to 50 gram of the fine powder in a beaker. Each day, the dissolved extract was filtered, filtrate was stored in a separate beaker and 100 ml of fresh methanol was added to the residue The mixture was stirred intermittently. After 3 days, the total volume of filtrate was filtered using Whatman No. 1 filter paper for final collection. Filtrate was concentrated by evaporating methanol at 50^{o}C in a water bath until semi solid mass was obtained and then percentage yield was calculated.

To perform assays that followed, 100 mg/ml (for well diffusion assay) and 128 mg/ml (for MIC and MBC determination) solutions were prepared in 50% Dimethyl Sulfoxide (DMSO).

2.4 Phytochemical Screening

Presence of flavonoids, tannins, glycosides, phenols, terpenoids, alkaloids and saponins in the plant extract were tested according to Gul et al. (2017) [17] and Panchal & Parvez (2019) [18]. Working solution of 10 mg/ml was used to perform the tests, however, for the test of alkaloids and saponins, crude extract was used. The detailed procedure of phytochemical screening is in Table 1.

Table 1: Phytochemical screening

Phyto-chemical Tested	Test and Procedure	Positive Indication
Flavonoids	Alkaline reagent test: 2 ml of 2% NaOH was mixed with the plant extract.	Yellow color
	Lead acetate test: Few drops of Lead Acetate solution was added to 200 µl of plant extract.	Yellow precipitate
Tannins	Bramer's test: 10 % alcoholic $FeCl_3$ was added into the 2 ml of methanolic extracts.	Blue or black or greenish grey precipitate
	Bromine water test: 1ml of Bromine water was added to the plant extract.	Discoloration of bromine water
	Gelatin test: Few drops of 1% Gelatin solution containing 10% NaCl was added to 200 µl of plant	White precipitate

	extract.	
Glycosides	Keller-Kiliani test: To 300 µl of plant extract, 200 µl of glacial acetic acid and two drops of $FeCl_3$ was added. Two drops H_2SO_4 was added to the mixture.	Brown ring between the layers
Phenols	Ferric Chloride test: Few drops of neutral $FeCl_3$ was added to 200 µl of plant extract.	Dark Green color
Terpenoids	Salkowski's test: 300 µl of plant extract was treated with few drops of chloroform. Conc. H_2SO_4 was added drop wise.	Yellow color
	Copper acetate test: To 200 µl of plant extract few drops of copper acetate solution was added	Emerald green color
Alkaloids	Wagner's test: To 1 ml of filtrate, a few drops of Wagner's reagent was added.	Reddish brown precipitate
	Mayer's test: To 1 ml of filtrate, a few drops of Mayer's reagent was added.	Yellow precipitate
Saponins	Foam test: To 0.5 gm of crude extract 2 ml of water was added and shaken.	Persistent foam for 10 minutes

2.5 Isolation and Identification of Actinomycetes

Isolation was done as by Rao et al. (2016) [19]. Ten gram of soil samples was taken and air dried. 4.5 gram of the air dried soil was taken and mixed with 0.5 gm $CaCO_3$ for chemical treatment. The mixture was left for a week. Then, the mixture was subjected to dry heat treatment (80°C for 30 minutes). 1 gram of heat treated mixture was mixed with 10 ml of sterile distilled water and diluted up to six folds. 0.1 ml aliquots from 10^{-2}, 10^{-4} and 10^{-6} dilutions were plated by spreading on Actinomycetes Isolation Agar. All plates were properly labelled and incubated at 28°C for a week.

Based on colony morphology, actinomycetes cultures were selected and sub-cultured on International Streptomyces Project Medium No. 4 (ISP4). Isolated cultures were Gram stained [20].

2.6 Bacterial Test pathogens

For the study, *E. coli*, *Klebsiella oxytoca* and *Staphylococcus aureus* were taken. The microbes had been isolated from UTI patients from one of the hospitals of Kathmandu. The antibiotic susceptibility pattern of the microbes was determined using modified Kirby-Bauer disc diffusion method as per the Clinical and Laboratory Standards Institute [21]. Various classes of antibiotics, namely; aminoglycoside, beta-lactam, penicillin, cephalosporin (2nd, 3rd and 4th generation), fluroquinolone, sulfonamide, tetracycline and oxazolidinone were used for the antibiotic susceptibility test.

2.7 Plant Antimicrobial Assay

Preliminary antimicrobial assay was done by well diffusion method similar as described by Nair et al. (2017) [22]. Stock solution of 100 mg/ml was diluted to 50 mg/ml to perform the assay. The bacterial broth was adjusted to 0.5 McFarland standard. Sterile cotton swabs were used to make a lawn culture on Mueller-Hinton agar plate. Wells were made on each plate with a 6 mm diameter sterilized cork borer. The wells were labelled and filled with 50 µl of respective extract. The extracts were left to diffuse at room temperature for 1 hour. The plates were then inverted and incubated at 37°C for 24 hours. 1 mg/ml ofloxacin was used as positive control and 50% DMSO was used as negative control. After 24 hours, the zone of inhibition (if any) including the well diameter was measured using a metric ruler.

MIC was determined by macro broth dilution method as described by Sayed SM (2014) [23] with slight modification. 9 test tubes with 2 ml NB each were taken. 2 ml of 128 mg/ml stock solution was re-suspended in the first test tube to dilute the concentration to 64 mg/ml. Then two-fold serial dilution was performed in the rest of the test tubes to the concentration of 32, 16, 8, 4, 2, 1, 0.5 and 0.25 mg/ml. Positive control and negative control tubes were also prepared. For Positive control tube 2 ml of NB was taken. For Negative control tube 2 ml of NB without any extract was taken. 50 µl of bacterial suspension adjusted to 0.5 McFarland standard was added to each of the test tubes except PC. All the tubes were incubated for 24 hours at 37°C. After 24 hours the turbidity of the tubes were observed. The least concentration with no turbidity was considered to be MIC of extract against the particular organism.

To determine MBC, from the two tubes with no turbidity (MIC and higher concentration) loopful of broth was streaked in the NA plate. The least concentration with no visible growth was considered to be MBC of extract against the particular organism.

2.8 Actinomycetes Antimicrobial Assay

Primary screening was done by perpendicular streaking method. Actinomycetes isolate was linearly streaked at the center of the plate and incubated at 28°C for 7 days. Then, overnight culture broth of test organisms were streaked perpendicular to actinomycetes on either side leaving about a 1 mm gap. Then the plate was incubated at 37°C for 24 hours. Inhibition of growth of test organisms was observed [24].

Isolated colony of seven days old actinomycetes that showed antimicrobial potential in the primary screening culture was inoculated in 200 ml of Tryptone Soya Broth. All the broth

flasks were incubated in a shaking incubator (150-160 rpm) at 28°C for 7 days.

After fermentation, the biomass culture was centrifuged at 5000 rpm for 15 minutes. The supernatant was collected and was filtered using a membrane filter (0.22 µm pore size) subsequently extracted with equal volume of ethyl acetate.

The antimicrobial assay of ethyl acetate extract was performed by agar well diffusion technique as in plant assay. 50 µl of ethyl acetate extract was used to perform the assay. 1 mg/ml ofloxacin was used as positive control and ethyl acetate was used as negative control for comparison. After 24 hours, the zone of inhibition (if any) including the well diameter was measured using a metric ruler.

MIC and MBC of only those extracts showing positive result in well diffusion assay was determined. For that crude extract was obtained by subjecting the extract to a rotary vacuum evaporator at 100 rpm. The water bath temperature was set at 60°C [25]. 16 mg/ml stock solution was prepared by dissolving crude extract in ethyl acetate.

MIC was determined by macro broth dilution method as described by Gebreyohannes et al. (2013) [25] with slight modification. 6 test tubes with 2 ml NB each were taken. 2 ml of 16 mg/ml stock solution was re-suspended in the first test tube to dilute the concentration to 8 mg/ml. Then two-fold serial dilution was performed in the rest of the test tubes to the concentration of 4, 2, 1, 0.5 and 0.25 mg/ml. Positive control and negative control tubes were also prepared. For Positive control tube 2 ml of NB was taken. For Negative control tube 2 ml of NB without any extract was taken. 50 µl of bacterial suspension adjusted to 0.5 McFarland standard was added to each of the test tubes except PC. All the tubes were incubated for 24 hours at 37°C. After 24 hours the turbidity of the tubes were observed. The least concentration with no turbidity was considered to be MIC of extract against the particular organism.

To determine MBC, from the two tubes with no turbidity (MIC and higher concentration) loopful of broth was streaked in the NA plate. The least concentration with no visible growth was considered to be MBC of extract against the particular organism.

3. RESULTS

3.1 Phytochemical Extraction

Extract of all three plants were semi solid in consistency. The highest yield was found to be of *Asparagus officinalis.* The percentage yield of extracts are listed in Table 2.

Table 2: Percentage yield of plant extract

Plant	Part used	Dry weight (gm)	Percentage yield
A. officinalis	Whole plant	50	31.06
O. sanctum	Leaves	35	15.11
T. terrestris	Fruit	50	7.33

3.2 Phytochemical Screening

The phytochemical screening of *Asparagus officinalis* showed the presence of flavonoids, phenols, tannins and terpenoids. Out of seven classes of phytochemicals tested, *Ocimum sanctum* extract showed positive result for six constituents except alkaloids. Extract of *Tribulus terrestris* showed the presence of all seven phytochemical classes that were tested. The phytochemical screening result is in Table 3.

Table 3: Phytochemical screening of plant extract

Compound Tested	Name of the test	Plant extract		
		Asparagus officinalis	*Ocimum sanctum*	*Tribulus terrestris*
Flavonoids	Alkaline reagent	+	+	+
	Lead acetate	+	+	+
Tannins	Braemer	+	+	+
	Bromine water	+	+	+
	Gelatin	-	+	+
Glycosides	Keller Killiani	-	+	+
Phenols	Ferric Chloride	+	+	+
Terpenoids	Salkowski	+	+	+
	Copper acetate	+	+	+
Alkaloids	Wagner	-	-	+
	Mayer	-	-	+
Saponins	Foam	-	+	+

3.3 Isolation and Identification of Actinomycetes

Based on the colony morphology, a total of 19 actinomycetes were isolated. Nine of them were isolated from soil samples of root vicinity of *A. officinalis* and ten were isolated from soil samples of root vicinity of *O. sanctum.*

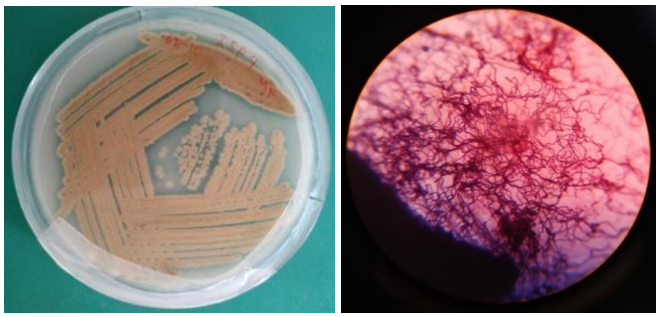

Fig- 1, 2: Actinomycetes isolate (MK1) sub-cultured on ISP4 media (Left) and Gram staining of actinomycetes isolate (MSC4) under oil immersion (Right)

3.4 Antibiotic Susceptibility Test

All three test organisms were found to be resistance against more than one classes of antibiotics.

3.5 Plant Extract Antimicrobial Assay

Extracts of all three plants had shown effect against *E. coli*. The largest zone of inhibition was shown by *Ocimum sanctum* extract (10.67±1.155 mm). *K. oxytoca* was the most resistant pathogen to plant extracts showing inhibition to *A. officinalis* only. The result of well diffusion assay is in Table 4.

Table 4: Antimicrobial activity of medicinal plant extracts

Plant extracts	Zone of inhibition against test organisms (in mm, excluding well diameter)		
	E. coli	*S. aureus*	*K. oxytoca*
A. officinalis	9.67±0.577	-	5.33±0.577
O. sanctum	5.67±0.577	10.67±1.155	-
T. terrestris	6.33±1.155	5.33±0.577	-
Ofloxacin (1 mg/ml)	24.33±1.528	22.33±0.577	19.33±0.577

[(-): No zone of inhibition]

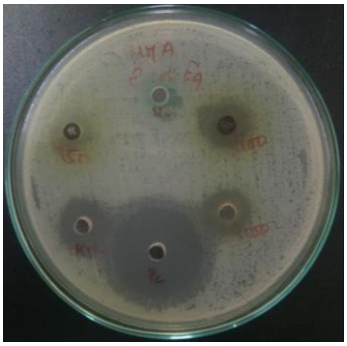

Fig- 3: Antimicrobial screening of all three medicinal plant extracts against *E. coli*

Table 5*:* MIC and MBC of medicinal plant extracts

Plant Extracts	Organisms					
	E. coli		*S. aureus*		*K. oxytoca*	
	MIC	MBC	MIC	MBC	MIC	MBC
A. officinalis	4	4	-	-	16	16
O. sanctum	8	8	4	8	-	-
T. terrestris	8	8	8	16	-	-
Ofloxacin (Positive control)	0.064	0.064	0.032	0.032	0.064	0.128

[MIC and MBC are expressed in mg/ml, (-): Not tested]

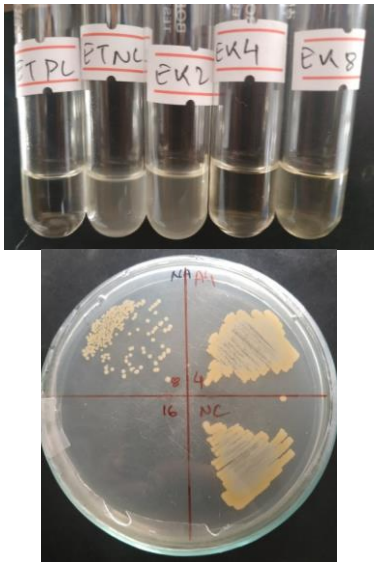

Fig- 4, 5 : MIC determination of extract from *A. officinalis* against *E. coli* MBC determination of extract of *T. terrestris* against *S. aureus*

3.6 Actinomycetes Antimicrobial Assay

After primary screening, 13 of the 19 isolates showed inhibition against at least one test organism. Five of the isolates inhibited *E. coli*. 13 isolates inhibited *S. aureus*. Four isolates inhibited *K. oxytoca*. The result suggested that isolates were more effective against Gram-positive bacteria.

As the 13 isolates could be potential antibiotics' source, they were subjected to submerged state fermentation followed by ethyl acetate extraction and extract's antimicrobial assay. *E. coli* was inhibited by 3 ethyl acetate extracts (FC2, MSC4 and MK1). MK1 had the largest zone of inhibition (9.33±0.577 mm). Only MK1 and MK5 inhibited *K. oxytoca,* the latter having the highest zone of inhibition (6±1 mm). Ten extracts were exhibited against *S. aureus* (FC1, FC2. FC4, MSC4, MK1, MK2, MK4, MK5, MK9, and MK10). MK4 had the highest zone of inhibition (5±1 mm). While MK1 had shown effect against all 3 test organisms, FC5, MK3, and MK6 did not affect any of the test organisms. The well diffusion assay of ethyl acetate extract is in Table 6. The MIC and MBC of crude extract is shown in Table 7.

Table 6: Antimicrobial activity of ethyl acetate extract

Extract sample	Zone of inhibition against test organism (in mm, excluding diameter of well)		
	E. coli	*S. aureus*	*K. oxytoca*
FC1	-	2±0	-
FC2	4.67±0.577	4.67±0.577	-
FC4	-	3.67±0.577	-
FC5	-	-	-
MSC4	5±1	4±0	-
MK1	9.33±0.577	3.33±0.577	5.67±0.577
MK2	-	3.33±0.577	-
MK3	-	-	-
MK4	-	5±1	-

MK5	-	2.67±0.577	6±1
MK6	-	-	-
MK9	-	4.67±0.577	-
MK10	-	3.67±0.577	-
Ofloxacin (1 mg/ml)	24.67±0.577	22.67±1.155	18.67±0.577

[(-)**:** Activity not detected, Amount of extract: 50 µl]

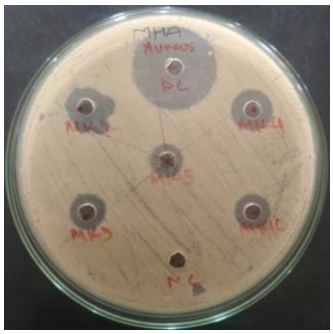

Fig- 6: Antimicrobial screening of ethyl acetate extracts (MK2, MK4, MK5, MK9 and MK10) against S. aureus

Table 7: MIC and MBC of crude actinomycetes extract

Extract sample	Organisms					
	E. coli		*S. aureus*		*K. oxytoca*	
	MIC	MBC	MIC	MBC	MIC	MBC
FC1	-	-	4	4	-	-
FC2	2	4	2	2	-	-
FC3	-	-	-	-	-	-
FC4	-	-	4	4	-	-
MSC4	2	2	2	2	-	-
MK1	1	2	2	2	1	2
MK2	-	-	4	4	-	-
MK4	-	-	4	4	-	-
MK5	-	-	2	2	2	2
MK9	-	-	4	4	-	-
MK10	-	-	4	4	-	-
Ofloxacin (Positive control)	0.064	0.064	0.032	0.032	0.064	0.128

[MIC and MBC are expressed in mg/ml. (-): Not tested]

4. DISCUSSION

Methanol was taken as solvent for extraction. Its high polarity enables it to extract polar phytochemicals that have antimicrobial properties efficiently. Also, methanol is efficient to extract polyphenols that are commonly stored in vacuoles [26]. A comparative study also shows that methanol as a solvent for extraction provides better yield, more phytochemicals in the extract and antimicrobial effect against more test organisms [27]. Also, due to its comparatively low boiling point, it evaporates quickly without degrading the extract's active components.

According to Alferova & Terekhova (1988) [28], enrichment of soil samples with calcium carbonate greatly enhanced the number of actinomycetes. A similar result was obtained by the study of Ng & Amsaveni (2012) [29]. Natsume et al. (1989) [30] has stated that calcium ions enhance the aerial mycelium growth of actinomycetes. Thus, the soil sample was pretreated with $CaCO_3$. Rough, tough, and dry colonies are the characteristic of Actinomycetes colonies. Some sub-cultured plates of isolates had an earthy smell due to the production of volatile compound geosmin [31]. Gram staining revealed that the isolates were Gram-positive and had a filamentous and hair-like structure, the mycelium. FC1 had a distinct silvery black colony, while MSC4 had a pinkish colony. The earthy smell, mycelium, and color pigmentation further confirmed that isolates were actinomycetes [32].

A. Officinalis showed activity against only Gram-negative organisms. The zone of inhibition was highest against *E. coli* (9.67±0.577 mm), and it subsequently had the lowest MIC against *E. coli* (4 mg/ml). Not much study on antimicrobial activity of *A. officinalis* was found while searching for research papers that relate to our research. However, most of the research is done on *A. racemosus*, which is the wild variety of *Asparagus*. In a study by Chouhan & Pande (2014) [33], the methanolic extract of *A. racemosus* showed inhibiting activity against Gram-positive and Gram-negative bacteria while ethanolic extract showed effect against only Gram-negative bacteria.

O. sanctum inhibited *E. coli* and *S. aureus* but not *K. oxytoca*. The extract shown more zone of inhibition against *S. aureus* (10.67±1.155 mm) compared to *E. coli* (5.33±0.577 mm). Its MIC for *E. coli* and *S. aureus* were 8 mg/ml and 4 mg/ml respectively. In a study by Srinivas Naik et al. (2015) [34], the zone of inhibition of methanolic extract of *Ocimum sanctum* against *E. coli* and *S. aureus* was 7 mm and 8 mm, the concentration and amount of extract taken was 30mg/ml and 100µl respectively. Our results correspond to Khair-ul-Bariyah (2013)[35] study, where it was stated that extract of *O. sanctum* showed more activity against Gram negative bacteria. The interaction of plant extract with cytoplasmic components and nucleic acids of bacteria interferes with membrane permeability and respiratory chain enzyme [34].

T. terrestris like *O. Sanctum* exhibited inhibition against *E. coli* and *S. aureus* only. Larger zone of inhibition was seen against *E. coli* (6.33±1.155 mm). Its MIC was the same for both organisms (8 mg/ml) while MBC against *S. aureus* was greater (16 mg/ml) compared to *E. coli* (8 mg/ml). Batoei et al. (2016) [36] demonstrated that the MIC and MBC for clinical *E. coli* isolates were 3.5±0.27 mg/ml and 7.4±0.5 mg/ml, respectively. While the MBC from our study matches their findings, the MIC is quite high. The MBC of *T. terrestris* against *E. coli* and *S. aureus* was 6.25 mg/ml and 12.5 mg/ml, respectively, according to Usman et al. (2007) [37] but their MIC was relatively lower (1.563 mg/ml and 3.125 mg/ml respectively). Batoei et al. (2016) [36] and Hussain et al. (2009) [38] states that the saponins in *T. terrestris* play a crucial role in antimicrobial property of the plant. While not all saponins are antimicrobial, the one found in *T. terrestris* is found to disturb bacteria's cell membrane [39].

The production of secondary metabolites in actinomycetes is regulated by the joint effect of environmental and physiological setup. Usually, secondary metabolites are produced when nutrients start depleting [40]. Production is triggered only when required and is generally coordinated with morphological differentiation [41]. Actinomycetes undergo physiological and morphological differentiation that culminates in the production of secondary metabolites and the dispersal of spores. During starvation, aerial hyphae can differentiate into spores that is accompanied by secondary metabolite production [42].

Out of 19 total isolated actinomycetes, 13 had shown inhibition in the perpendicular streaking method. Out of those 13, only the ethyl acetate extract of 10 could show inhibition to at least one of the test organisms. Some actinomycetes fail to reproduce the bioactivity in liquid culture that they are capable of showing in agar medium. Loss of this activity could be due to the fragile morphology of some actinomycetes that is prone to physical damage due to flask shaking, which can be sufficient to cause fragmentation preventing antibiotic production [43]. Morphology of actinomycetes on agar and liquid medium are also reported to be different, filamentous mycelia in solid and fragmenting mycelium in liquid media [44].

MIC of the crude extract ranged from 1 mg/ml to 4 mg/ml and the MBC ranged from 2 mg/ml to 4 mg/ml. The crude extract from MK1 had the lowest MIC for both the gram-negative test organisms (1 mg/ml). For gram-positive *S. aureus*, FC2, MSC4, MK1, and MK5 exhibited the lowest MIC (2 mg/ml). The crude extract of these actinomycetes could be a potential source of the antimicrobial compound.

5. CONCLUSIONS

The phytochemical screening analysis showed that the extract of *T. terrestris* contained all seven classes of phytochemicals tested while the other two extracts contained at least four phytochemicals. Plant extract of *A. officinalis* was more effective against Gram-negative pathogens, and the extract of *O. sanctum* was more effective against Gram positive pathogens. *A. officinalis* showed the lowest MIC against *E. coli* (4 mg/ml). Actinomycetes isolated from the root vicinity of *O. sanctum* had a better antimicrobial effect. Ethyl acetate extract of MK1, actinomycetes isolated from the root vicinity of *O. sanctum,* had an inhibiting effect against all three test organisms with the lowest MIC. The results suggested that both medicinal plants and actinomycetes can be potent sources of bioactive compounds with promising antimicrobial effects against MDR pathogens.

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AUTHORS' CONTRIBUTION

Dikshanta Vinaya Luitel, Bidhan Gautam and Saroj Maharjan share the first authorship. DVL, BG and SM carried out experiment. Santosh Khanal and Saurav Ranjitkar designed the experiment. DVL, BG, SM and Barsha Khadka wrote the experiment report. SK and SR supervised the experiment. DVL, BG, SM and SK analyzed the results. DVL and BG wrote the manuscript.

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