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Original Article

Diversity and biological activities of endophytic fungi from Nepalese Woodfordia Fruticosa (Linn.) Kurz

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ABSTRACT

Endophytic fungi were isolated from leaf, stem and root of *Woodfordia fruticosa* through surface sterilization. 141 isolates were obtained from 220 segments. *Alternaria* species, *Penicellium* species, *Fusarium* species and *Aspergillus* species were identified up to generic level. The colonization frequency varied from 40% to 89%. Maximum colonizing 26 isolates were selected and their extracts were examined for antibacterial activity against 11 human pathogenic bacteria. 77% extracts inhibited the growth of *Staphylococcus aureus* followed by 70% to *Bacillus subtilis*, *Salmonella typhi*, *Salmonella paratyphi* each. Only, 30% extracts inhibited the growth of *Citrobacter frendii*. The present research findings tend to support the ethnomedicinal uses of *W. fruticosa* and may facilitate the natural product discovery process.

Keywords: antibacterial activity, endophytic fungi, ethnobotany, *W. fruticosa*

INTRODUCTION

Endophytic fungi are the microorganisms inhabiting inside the plant tissues. Endophytes produce many pharmaceutical subtances that are originally characteristic to the host. Most of the chemical compounds produced by the endophytes have high economic values and used for the treatment of several severe diseases such as cancer, inflammation, diabetes including bacterial and fungal infections.

In Nepal, *W. fruticosa* is commonly called 'Dhainyaro', which is distributed between 200--1800 m above mean sea level [1]. The leaf and flower have been reported for their diverse ethnomedicinal value in Nepal. Flowers, leaves, stems and barks have exceptionally wide diversity of medicinal uses to treat more than 60 ailments [2]. The plant has been used in the course of drug formulation and that has encouraged many researchers to find its usefulness to treat several diseases. Flowers and leaves have been scientifically investigated [3-8], and experimentally proved to possess immunomodulatory, anti-inflammatory, anti-leucorrhoeic, anti-tumor activities. The use of flower, leaf and stem of *W. fruticosa* in managing of various sicknesses is due to the presence of copious bioactive compounds including tannins, flavonoids, anthraquinone glycosoides and polyphenols. The flavonoides present in the plants are known for their immunomodulatory and anti-inflammatory activities [9-10]. Several studies have been conducted for the isolation and identification of endophytic fungi from the medicinal plants [11-13].

However, very few researches have been carried out in Nepal using Nepalese medicinal plants. To the best of our knowledge, there is no precise detail about the endophytic fungi of Nepalese *W. fruticosa*. In recent years, study on endophytes from medicinal plants has received much attention because they are believed to be an excellent source of biologically active compounds. Because of its long ethnomedicinal use history combined with biologically promising results of *in-vitro* activities, the chances of getting novel microorganisms and in turn novel compounds from the study of its endophytic fungus are very high. Hence, we selected *W. fruticosa* and isolated endophytic fungi from leaf, stem and root samples. Furthermore, the selected endophytes isolated extracts were assessed for their antibacterial activity against different human pathogenic bacteria.

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MATERIALS AND METHODS

***Collection of plant materials*:** The healthy mature leaf, stem and root samples were collected from Naghdhunga Forest, Kathmandu district, Nepal. Kathmandu district lies in the Central Development Region of Nepal, which is about 1600 m above mean sea level. The sampling plot wa s located approximately 200 m far from the Prithivi Highway in Naghdhunga. In each sampling plots, *W. fruticosa* shrub was naturally established and among them five healthy plants were selected for sampling. *Pinus roxburghii* was the dominant tree species in the sampling plots and in the understory seedlings of the *Pinus roxburghii*, *Asparagus* species, Fern species, etc were growing.

***Isolation, culture and identification of endophytic fungi*:** To kill unwanted fungal propagules adhering to the plant materials, samples were surface sterilized by following the standard protocol [14]. Leaf, stem and root samples collected from the sampling plots were washed under running water and cut into 5 x 5 mm size pieces from the upper, middle and lower portion with the aid of a flame-sterilized blade. The excess moisture was blotted in a sterile filter paper. The surface sterilized materials were evenly spaced in petridishes (6 cm diameter) containing Potato Dextrose Agar (PDA) medium (amended with streptomycine 150 mg l^{-1}). The petridishes were sealed using Parafilm and incubated at 27 ^{0}C in an incubator for 4 weeks. The petridishes were monitored everyday for the growth of endophytic fungus colonies from the segments. The hyphal tips, which grew out from the segments, were isolated and sub-cultured onto PDA and brought into pure culture. All the endophytic fungal isolates were documented and maintained in PDA slants.

***Antibacterial assay*:** The bioassay used was the standard disc diffusion assay [16]. The selected endophytic fungi were grown in PDA medium and incubated for four weeks at 27 ^{0}C. After incubation period, the fungal cultures were harvested and filtered through two layers of cheese cloth. The liquid culture filtrate was collected and extracted with methanol. The extracts were completely evaporated under reduced pressure using a rotary evaporator. The crude evaporated extracts were dried at room temperature for 15-30 days. The dried samples were collected in vials and tested for its antibacterial activity [16]. 50 mg of each crude extract was dissolved in 1 mL (1,000 microlitres) of the methanol to give a final concentration of crude extract in solvent of 50 mg/mL. Paper discs (6 mm diameter) were impregnated with 20 µL (the maximum capacity of the disk) to give a final concentration equivalent to 1 mg dried material per mL solvent.

Negative control discs were prepared by dipping the discs into methanol solution or impregnating the discs with 20 µL of the methanol. Ciprofloxacine and erythromycine discs were used as positive controls. Paper discs were impregnated with 10 µL of 0.25 mg/mL erythromycin and ciprofloxacin with MeOH [17]. The prepared disks were allowed to dry at room temperature. The petriplates containing PDA medium were spread with 100 µL of actively growing broth culture of the test bacteria using sterile cotton swab and allowed to dry for 10 minutes. Then the impregnated dried discs were placed on the surface of inoculated Agar medium and incubated at 3 7 ^{0}C for 24 h. Results were recorded as presence or absence of zone of inhibition and the experiment was repeated three times to ensure reliability of the results.

***Microorganisms used*:** Eleven strains of bacteria were used in the screening process including Gram-positive *Staphylococcus aureus*, *Bacillus subtilis* and Gram-negative *Salmonella typhi*, *Salmonella paratyphi*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Citrobacter frendii*, *Enterococcus faecalis* and *Acenitobacter* spp. These microorganisms were obtained from Department of Clinical Microbiology, Teaching Hospital, Maharajgunj, Tribhuvan University and Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu.

RESULTS AND DISCUSSION

***Endophytic fungi*:** Altogether, 220 segments, from leaf (100) stem (60) and root (60) samples were processed for the isolation of endophytic fungi. 141 cultures were obtained from these segments. The results showed that the colonization frequency varied widely. The frequency of colonization was highest in leaf samples (89%) followed by in stem samples (47%). The lowest frequency of colonization was 40% in root samples (Table 1).

Four endophytic fungi namely; *Alternaria* species, *Penicellium* species, *Fusarium* species and *Aspergillus* species were identified up to the generic level. Among 100 segments analyzed from the leaf samples, *Alternaria* species and *Penicellium* species were produced and identified. Of 60 stem samples, *Fusarium* species and *Penicellium* species were produced and identified. Likewise, *Aspergillus* species was produced and identified from the 60 root samples processed during this research (Table 2).

Endophytic fungal colonies in tropical plant leaves are exceptionally frequent [18]. The fact is also supported by present research findings. The present results showed that the frequent colonization of endophytic fungus occurs in leaves than stems and followed by roots. Similar observation has also been obtained in other related studies [19]. The main possible effective factors of the isolation rate of endophytic fungi for this study depend upon the plant species used, the amount of sample used and medium used for the isolation process.

In this study, we used small number of samples and only one isolation media which may be responsible in limiting the endophytes isolation. So, more endophytic fungi may be recovered with the increase of sample quantity and isolation media along with the enlargement of the investigation scope and reasons [20].

***Antibacterial a*ssay:** The antibacterial activity of endophytic fungi extracts isolated from the leaf, stem and root samples were screened by Agar disc diffusion method against 11 human pathogenic bacteria. The inhibition zones determined by the 11 strains of bacteria were presented in Table 2.

Antibacterial activity was determined by observing an inhibition zone. The extract containing discs were placed on a freshly seeded lawn of bacteria and then allowed to grow until the bacteria created an easily visualized lawn. A positive result is obtained when the extract inhibited the growth of the bacteria and thus produced zone of inhibition. This zone indicates that the extract has antibacterial activity. A negative result is obtained when the extract did not inhibit the growth of bacteria *in-vitro* and did not produce a zone of inhibition. Out of 141 isolates, 26 extracts were examined for their antibacterial activity since they produced maximum colonization. Among 26 extracts examined, 77% inhibited the growth of *Staphylococcus aureus* followed by 70% to *Bacillus subtilis*, *Salmonella typhi*, *Salmonella paratyphi* each. 66% extracts inhibited the growth of *Escherichia coli*, 62% to *Enterococcus* species, 58% to *Proteaus mirabilis*, *Pseudomonas aeruginosa* each. Similarly, 54% extracts inhibited the growth of *Acinetobacter frendii* and 35% to *Klebsiella pneumonia.* Only, 30% extracts inhibited the growth of *Citrobacter frendii* (Figure 1).

Out of eight different fungal extracts (that included *Alternaria* species and *Penicellium* species) obtained from the leaf samples were examined for their antibacterial activity, one extract did not show any zone of inhibition with the tested bacteria. Among 11 different extracts (that included *Fusarium* species and *Penicellium* species) obtained from the stem samples were examined for their antibacterial activity, three extracts did not show any zone of inhibition and one extract (that included *Aspergillus* species) did not show any zone of inhibition among seven different root extracts tested. However, s ome extracts were effective against all the bacterial strains tested in the study (Table 2).

In Nepal, very f ew researches have been intended to validate the traditional knowledge and the use of medicinal plants [21-22]. The ethanol extracts of the leaves of *W. fruticosa* against *Escherichia coli, Salmonella typhi, Salmonella paratyphi, Proteus vulgaris, P. mirabilis, Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp., *Shigella* spp., and *Pseudomonas* spp. explained the antimicrobial activity [23]. T he antimicrobial activity of the methanol extracts of leaves was observed with *Bacillus subtilis, Candida albicans, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhi, Salmonella paratyphi, Shigella dysenteriae, Staphylococcus aureus* and *Vibrio cholera* but not detected with *Escherichia coli* [24].

In general, the action of extracts with Gram-positive bacteria compared to Gram-negative bacteria obtained in this research was not an astonish result because prior research results showed that greater number of extracts were active against Gram-positive bacteria than Gram-negative bacteria. The results obtained in this research may possibly be due to the more complex cell wall/membrane structure of Gram-negative bacteria in comparison to the Gram-positive bacteria [25-27].

CONCLUSION

Endophytes are an excellent source of bioactive natural products because there are so many of them occupying with unique biological niches growing in harsh environmental conditions. The medicinal properties of *W. fruticosa* could also be recognized to their endophytic fungi. Hence, present research was commenced to find out endophytic flora associated inside this medicinal plant since isolated fungi may be used in the development of potential drugs. This plant has long been proved to be the source of several chemical compounds. Therefore, we commended this additional investigation. We hope that the results of this research may play a noteworthy role in the conservation of traditional medicine knowledge of Nepalese *W. fruticosa* and persuade the scientific community for further investigations by extracting and identifying the active chemical compounds responsible for the antibacterial effect observed.

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Table 1. Colonizing frequency of endophytic fungi isolated from *W. fruticosa*

Site of Isolation	Total number of segments analyzed	Number of segments colonized by endophytes	Colonization frequency (%)
Leaf	100	89	89
Stem	60	28	47
Root	60	24	40
Total	**220**	**141**	

Table 2. Antibacterial activity of selected endophytic fungi isolated from *W. fruticosa*

Sample number	Isolated fungi	SA	BS	EC	Aspp.	ST	SP	Espp.	CF	KP	PM	PA
Leaf-1	NI	++	++	++	+	++	++	++	-	-	++	++
Leaf-2	NI	+++	-	-	++	-	-	-	-	-	-	-
Leaf-3	NI	+++	+++	+++	+++	++	+++	-	-	++	+++	+++
Leaf-4	NI	+++	++	+	+	+	++	+	+	+	++	+
Leaf-5	*Alternaria* spp.	+++	+	+	-	++	++	+	-	-	-	-
Leaf-6	NI	++	++	+	-	+	+	++	-	-	-	-
Leaf-7	*Penicellium* sp.	+++	++	+++	+	+++	+++	++	-	-	++	+
Leaf-8	NI	-	-	-	-	-	-	-	-	-	-	-
Stem-1	NI	++	-	-	-	-	-	-	-	-	-	++
Stem-2	NI	+++	+++	+++	+	+++	+++	+++	-	+++	+++	+++
Stem-3	NI	+++	-	-	+++	+++	+++	+++	++	++	+++	+++
Stem-4	NI	-	-	-	-	-	-	-	-	-	-	-
Stem-5	*Fusarium* spp.	+++	+++	++	+	++	++	++	-	-	++	+
Stem-6	NI	+++	+	+++	+++	+++	+++	+++	+++	+++	+	-
Stem-7	NI	-	-	-	-	-	-	-	-	-	-	-
Stem-8	NI	-	-	-	-	-	-	-	-	-	-	-
Stem-9	*Penicellium* spp.	+++	++	++	++	++	+	+	+	+	++	++
Stem-10	NI	+	+	+	-	-	++	+	-	-	-	-
Stem-11	NI	+++	+++	+	+	+	+	+	-	-	++	+
Root-1	NI	+++	++	++	-	++	-	-	-	-	-	+
Root-2	NI	+++	+++	+++	++	++	++	++	+	+	++	++
Root-3	*Aspergillus* spp.	+++	+++	+++	-	+++	+++	+++	-	-	+++	+++
Root-4	NI	-	-	-	-	+	+	-	-	-	++	-
Root-5	NI	-	-	-	-	-	-	-	-	-	-	-
Root-6	NI	+++	+++	++	+++	++	++	++	++	++	++	++
Root-7	NI	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

Key: +++: >20mm; ++: 10-20; +: <10mm; -: indicated no zone of inhibition; ***Bacteria tested*:** SA: *Staphylococcus aureus*; BS: *Bacillus subtilis*; EC: *Escherichia coli*; PA: *Pseudomonas aeruginosa*; PM: *Proteus mirabilis*; KP: *Klebsiella pneumonia*; SD: *Shigella dysentriae*; SP: *Salmonella paratyphii*; ST: Salmonella Typhi; CF: *Citrobacter frendii*; Espp.: *Enterococcus* species; Aspp: *Acenitobacter* species; ***Solvent control:*** MeOH is negative, producing no zone of inhibition; Positive controls were erythromycin & ciprofloxacin; ***NI***: Not identified; ***spp***.: species

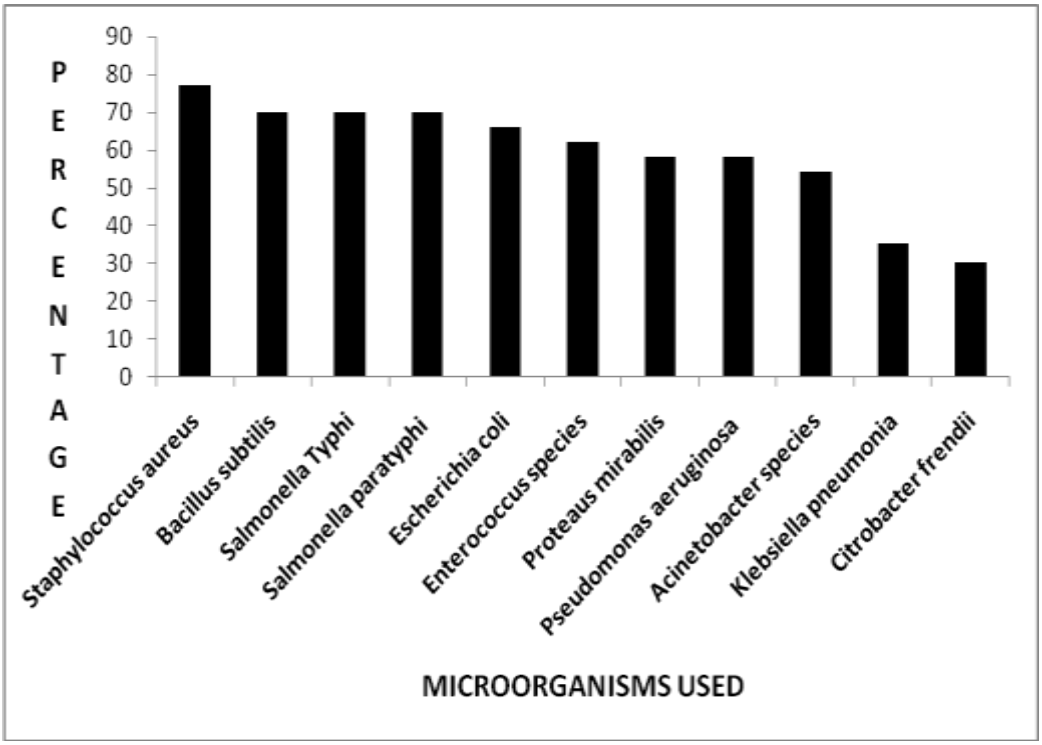

Figure 1: **Antibacterial activity of endophytic fungi with variety of microorganisms**

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