ISOLATION OF ENDOPHYTIC FUNGI FROM *ANDROGRAPHIS PANICULATA* WALL.*ex* NEES AND THEIR ANTIMICROBIAL ACTIVITIES

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Abstract

In this research work, *Andrographis paniculata* plant samples were collected from Magway University Campus, Magway Region. The isolation of endophytic fungi was undertaken by the surface sterilization method and Baiting method. Fourteen fungi was isolated from leaves, stem and root of *Andrographis paniculata.* All isolated fungi were tested by five kinds of test organisms, *Escherichia coli, Pseudomonas fluorescence, Bacillus subtilis, Saccharomyces cerevisiae* and *Candida albicans* by paper disc diffusion assay method. Among them, Fungus TF-02 exhibited only selective antimicrobial activity against *Escherichia coli* and the highest activity. Therefore, this fungus TF-2 was selected for further investigations such as fermentation for the production of antimicrobial metabolite, identification. In the study of microbial growth phase of TF-2 was observed that between 66hrs and 96 hrs ages of inoculums and 5%, 10%, 15%, 20%, 25%, 30% size of inoculums. In the investigation of ages and sizes of culture, 84hrs cultivation and 25% seed culture could be optimized for fermentation. The highest activity reached at 84hrs and 25% seed culture by using five kinds of fermentation medium, FM-4 at 25ºC showed the best activity on test organism. The extraction of compound was extracted from the fermented broth. The antimicrobial metabolite TF-02 was conducted with eluting solvents, Chloroform ($CHCL_3$), Dicholoromethane (CH2CL2), Toluene and Hexane. The eluting solvent, Dichloromethane (CH2CL2) was chosen by antimicrobial fungus TF-02. This fungus TF-02 was identified as *Aspergillus versicolar.*

Keyword Endophytic Fungi, Metabolite, Bioactive

Introduction

The microorganisms that isolated from plant parts are endophytes. Tough the meaning of the term "endophytes" varies depending on the researchers, it can be defined the endophytes as microorganisms living inside the healthy plants. (Scott and Lori 1998). their role in plants growth stimulation, protection, against biotic and abiotic stresses and pest via modulation of growth hormone signalling, higher seed yield and plant hormones. (Miliute *et al*., 2015).

Fungi produce a wide range of secondary metabolites with high therapeutic value as antibiotics, cytotoxic substance metabolites, insecticides compound that promote inhibit growth, attractor, repellent etc, secondary metabolites produced from fungi in production, function and specify to a particular fungus. These metabolites were being exploited in different fields of medicine and industry (Kishare *et al.*, 2007).

The research work, endophytic fungi were isolated from *Andrographis paniculata* (Say-Kha-Gyi) plant part collected Magway University Campus. Endophytic fungus TF-02 for the production of antibacterial secondary metabolite against *Escherichia coli*. In the investigation of fermentation of TF-02 showed the ages and sizes of inoculum for the production of antibacterial activity the best for fermentation. Medium optimization studies are usually carried out in the chemical, food and pharmaceutical industries with respect to increase the yield and activity of the desired product. (Shih *et al.*, 2002, Singh and Rai 2012).

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Four kinds of solvent were used for the extraction of bioactive from fungus TF-02. And then bioactive compound was extracted from the fermentation broth of fungus TF-02 using best solvent Dichloromethane. Identification of organisms was an important step in understanding and analyzing biological process. Traditional methods of classification and identification of the organisms were based on morphological, physiological, biochemical developmental and nutritional characteristics (Singh and Rai 2012).

In this study, aim and objectives are to know the knowledge that endophytic fungi can isolated from (Say-Kha-Gyi) plant parts, to find out the antibacterial activity of secondary metabolite from fungus TF-02, to know different ages and sizes of inoculum to optimize the fermentation, to share the suitable fermentation medium for the production of secondary metabolite, to know how extract bio-active compound. From the fermented broth by which solvent systems. To give knowledge of identical the isolated endophytic fungi.

Materials and Methods

Isolation of Endophytic Fungi from *Andrographis paniculata* Parts

Plant parts sample were collected from Magway University campus. Plant parts sample were carried out from 5 to 12 January, 2020. Plant parts sample were transported to the laboratory and processed immediately for the isolation and cultivation of fungi. Plant parts samples used for the isolation of endrophytic fungi and their location, collected data are shown in Table.

Table 1 Plant Parts Sample Collected at Magway University Campus

Plant	Family	Place	Location	Collected Date
Andrographis paniculata Wall ex. Part Use- Leave, stem and root	Acanthaceae	Magway university campus	20º8'13.12"N 94º56'12.66"E	29.12.2019

Figure 1 Habit of *Andrographis paniculata* NEES

Scientific Name - *Andrographis paniculata* Wall.ex Nees. Pl. Asiant Rar 3:116. 1832 *Justici paniculata* Burman, f.FL. Ind.9.1768
Myanmar Name - Say-kha-gyi
English Name - King of Bitter
Family - Acanthaceae

Annual herbs, stems quadragular, glabrous. Leaves simple, opposite and decussate; exstipulate; leaf blades lanceolate. Inflorescences terminal, leafy panicles of second racemose. Flowers bisexual, zygomorphic, pentamerous, hypogynous, white, with purplish spot; bracteate; bracteolate. Calyx 5-partite. Corolla bilabiate, infundibuliform. Stamens 2, anther basifixed. Ovary bilocular, superior. Capsule linear-oblong. Seeds orbicular, glabrolus.

Surface Sterilization Method (NITE 2004)

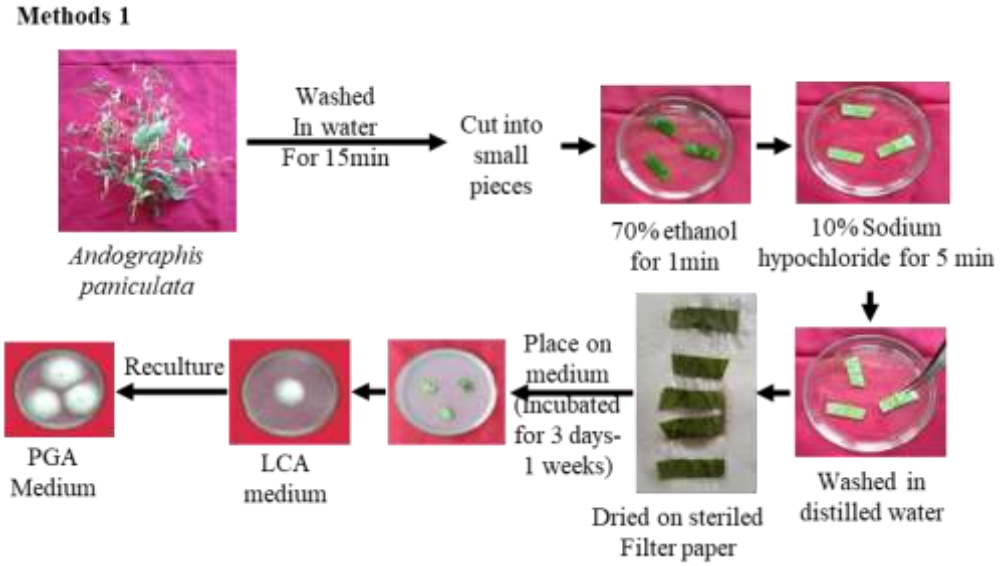


Figure 2 Procedure of Isolation Endophytic Fungi on Leaf, Stem and Root of *Andrographis paniculata*

Baiting Method (NITE-2004)

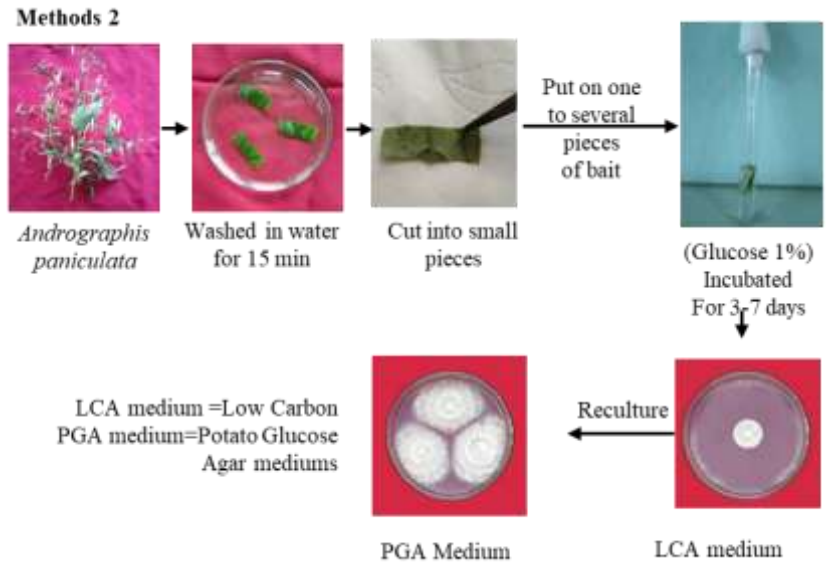


Figure 3 Procedure of Isolation Endophytic Fungi on Leaf, Stem and Root of *Andrographis paniculata*

Medium Used for the Isolation of Fungi

LCA medium
(Low Carbon Agar medium, Ando, 2004)

Glucose	0.5 g
Yeast	0.1 g
K_2HPO_4	0.001 g
Agar	1.8 g
DW	100 ml

(after autoclaving chloramphenicol was added to the medium)

PGA medium
(Potato Glucose Agar Medium)

Glucose	0.5 g
Yeast	0.1 g
K_2HPO_4	0.001 g
Agar	1.8 g
Polypeptone	0.1 g
Potato+DW	20+80 =100 ml

(after autoclaving chloramphenicol was added to the medium)

Figure 4 Procedure for the Antimicrobial Activity Test

Seed Medium Used for the Endophytic Fungi

Glucose	-	2.0 g
Glycerol	-	1.0 g
Yeast extract	-	1.04 g
$MgSO_4$	-	0.003 g
K_2HPO_4	-	0.002 g
DW	-	100 ml
pH	-	6.5

Fermentation Medium Used For the Endophytic Fungi

Glucose	-	2.5 g
Glycerol	-	1.5 g
Yeast extract	-	1.2 g
Polypeptone	-	0.8 g
K_2HPO_4	-	0.002 g
$MgSO_4$	-	0.003 g
$FeSO_4$	-	0.001 g
DW	-	100 ml
pH	-	6.5

Assay Medium

Glucose	-	1 g
Polypeptone	-	0.1 g
Agar	-	1.8 g
D.W	-	100 ml

Study on the Effects of Age and Size of Inoculum for the Fermentation (Omura, 1985, Crueger and Crueger, 1989)

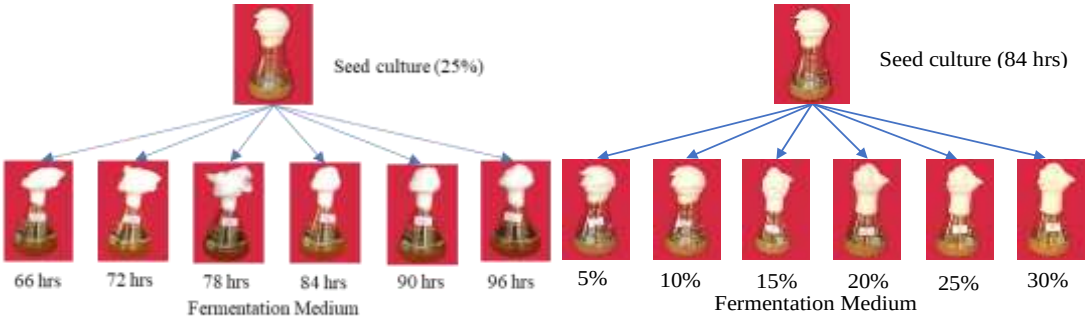


Figure 5 Procedure for the Study on the Effects of Ages and Sizes of Inoculums

Medium Optimization for Fermentation and Production of Antimicrobial Metabolites

Fermentation medium is also important for the production of metabolite (Crueger and Crueger, 1989). Therefore, fermentation was undertaken with suitable conditions of 25% sizes and 84 hrs age of inoculum with five different media.

Fermentation Medium Used for the Endophytic Fungi

FM-1			**FM-2**			**FM-3**		
Glucose	-	2.5 g	Sucrose	-	1.8g	Glucose	-	1.8 g
Glycerol	-	1.5 g	Glycerol	-	1.5g	Tapioca	-	1.5 g
Yeast extract	-	1.2 g	Yeast extract	-	1.6g	Meat extract	-	1.2 g
Polypeptone	-	0.8 g	Polypeptone	-	0.5g	Yeast extract	-	1.2 g
K_2HPO_4	-	0.002 g	K_2HPO_4	-	0.003g	Polypeptone	-	0.8 g
$MgSO_4$	-	0.003 g	$MgSO_4$	-	0.001g	K_2HPO_4	-	0.002 g
$FeSO_4$	-	0.001 g	$FeSO_4$	-	0.001g	$MgSO_4$	-	0.003 g
DW	-	100 ml	DW	-	100 ml	$FeSO_4$	-	0.001 g
						DW	-	100 ml

FM-4			**FM-5**		
Glucose	-	2.0 g	Glycerol	-	2.0 g
Potato powder	-	1.5 g	Tapioca	-	0.8 g
Meat extract	-	0.8 g	Malt extract	-	0.8 g
Yeast extract	-	1.6 g	Yeast extract	-	1.2 g
Polypeptone	-	0.5 g	Polypeptone	-	0.8 g
K_2HPO_4	-	0.003 g	K_2HPO_4	-	0.002 g
$MgSO_4$	-	0.001 g	$MgSO_4$	-	0.003 g
$FeSO_4$	-	0.001 g	$FeSO_4$	-	0.001 g
DW	-	100 ml	DW	-	100 ml

Preliminary Study for the Extraction of Bioactive Compound

Preliminary study for the extraction of bioactive compound was undertaken by the method of Thomashow *et al.*, 2008 and Jain and Pundir (2011).

Purpose: The purpose of their preliminary study to know how to extract the active metabolite from the fermented broth by which solvent system. The solvents used are

1. Chloroform $CHCL_3$
2. Dichloromethane CH_2CL_2
3. Toluene
4. Hexane

The fermented broth (5ml) was added with each solvent (5ml). After shaking till separation two layers, each layer was examined the activity against *Escherichia coli.*

The Macroscopial and Microscopical Character of Endophytic Fungus TF-02

The macroscopial and microscopical were observed by the methods of Domasch, 1993, Taxonomy and significance of black Aspergilla. The morphological and microscopical characters were observed by the methods of Ando and Inaba, 2004. Microscopical characters were studied by microscope. Comparison of these characters with reference keys was undertaken to identify.

Results

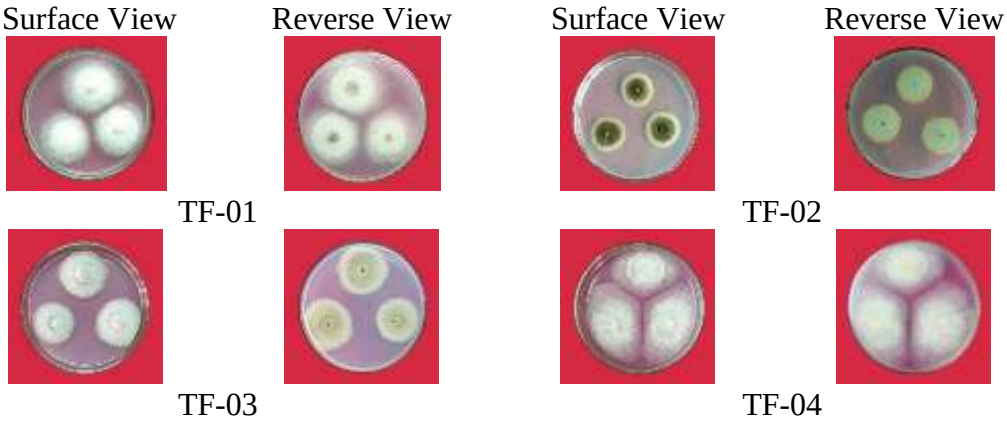
Isolation of Endophytic fungi from *Andrographis Paniculata* Parts Sample

In the isolation of Endophytic fungi, 14 fungi were isolated from *Andrographis paniculata* parts sample collected from Magway University Campus. Fungus TF-01, TF-02, TF-03, TF-04 isolated from leave of *Andrographis paniculata*. Fungus TF-07, TF-08, TF-09, TF-10 from stem of *Andrographis paniculata.* Fungus TF-11, TF-12, TF-13, TF-14 from root of *Andrographis paniculata* respectively. In this study Nine fungi were collected by surface sterilization method (NITE, 2004). Five fungi were collected by Baiting method (NITE, 2004).

Sampling Sites and Plant Parts Collection

Table 2 Isolated Endohytic Fungi from Plant Parts Samples

No.	Plant parts sample	Isolation Method	Number of endophytic fungi	Isolated endophtic
1.	Leaf	Surface sterilization method	TF-01, TF-02, TF-03, TF-04	4
		Baiting Method	TF-05, TF-06	2
2.	Stem	Surface sterilization method	TF-07, TF-08, TF-09	3
		Baiting Method	TF-10	1
3.	Root	Surface sterilization method	TF-11, TF-12	2
		Baiting Method	TF-13, TF-14	2
		Total Isolated endohpytic fungi		14



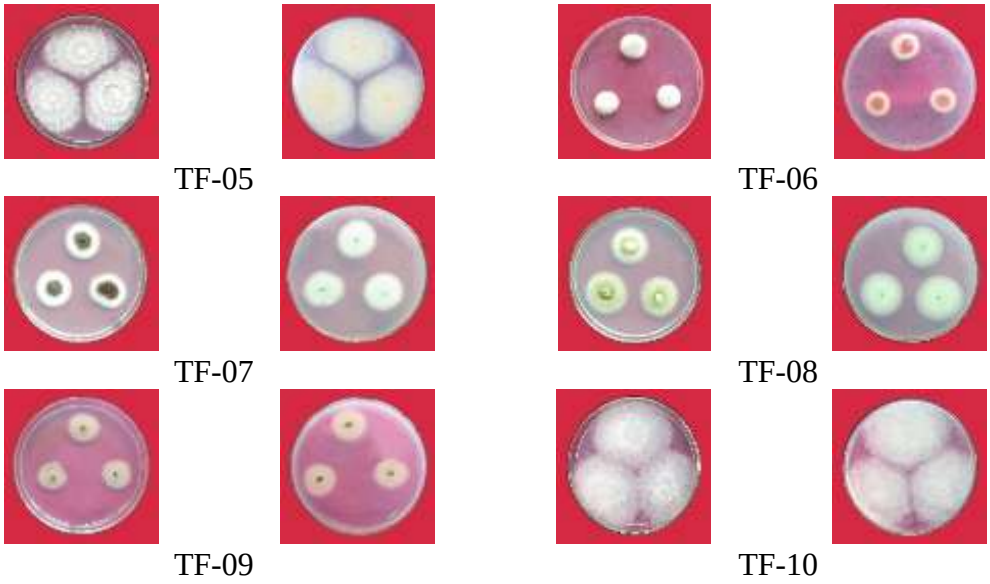


Figure 6 Morphology of Fungus TF-01 and TF-10

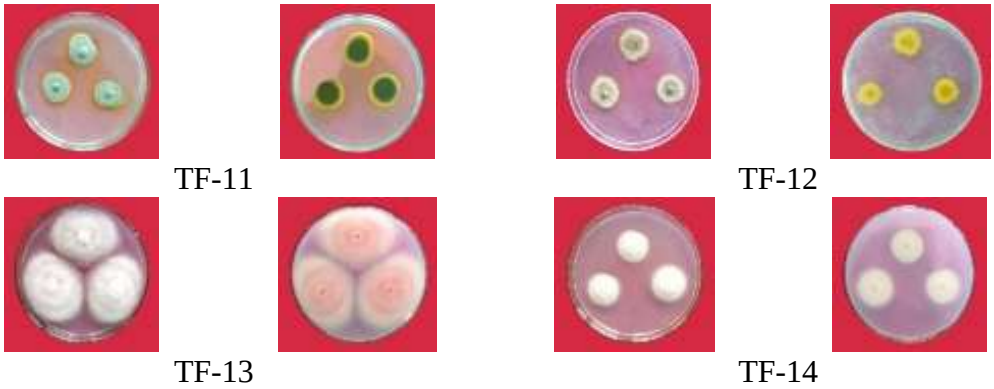


Figure 7 Morphology of Fungus TF-11 and TF-14

Preliminary Study for Antimicrobial Activities by Paper Disc Diffusion Assay NITE, 2004

Table 3 Antimicrobial Activities of Isolated Fungi on Test Organisms

Isolated fungi	Fermation day and inhibitory zone (mm)				
	Pseudomonas fluorescence	***Bacillus subtilis***	***Escherichia coli***	***Saccharomyces cerevisiae***	***Candida albicans***
TF-01	-	19.58	-	-	-
TF-02	-	-	35.32	-	-
TF-03	-	-	-	23.89	-
TF-04	-	-	-	-	28.60
TF-05	16.42	-	19.78	-	-
TF-06	-	23.29	-	-	-
TF-07	-	-	21.57	18.44	-
TF-08	-	-	23.89	-	-
TF-09	24.12	-	16.49	-	-
TF-10	-	-	-	-	21.67
TF-11	-	-	13.85	-	-
TF-12	-	19.21	-	-	-
TF-13	-	-	-	14.54	-
TF-14	-	-	-	-	16.40

Figure 8 Antimicrobial Activities of Isolated Fungi TF-01 to TF-14 on *Escherichia coli*

Figure 9 Antimicrobial Activities of Isolated Fungi TF-01 to TF-14 on *Bacillus subtilis*

Figure 10 Antimicrobial Activities of Isolated Fungi TF-01 to TF-14 on *Pseudomonas fluorescence*

Figure 11 Antimicrobial Activities of Isolated Fungi TF-01 to TF-14 on *Saccharomyces cerevisiae*

Figure 12 Antimicrobial Activities of Isolated Fungi TF-01 to TF-14 on *Candida albicans*

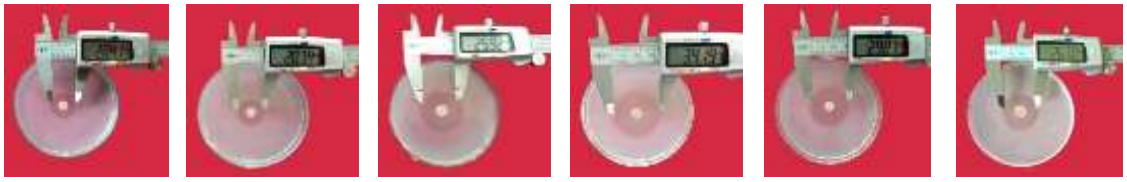
The Effects of Ages and Sizes of Inoculums for the Fermentation

Table 4 The Effects of Ages of Cultures on Fermentation

Growth Hour (hrs)	**Inhibitory Zone (mm) on *Escherichia coli***
66 hrs	20.41
72 hrs	20.74
78 hrs	25.92
84 hrs	34.19
90 hrs	29.87
96 hrs	26.70

Table 5 The Effects of Sizes of Cultures on Fermentation

Culture Times (Sizes of Culture, %)	Antibacterial activity on *Escherichia coli* (Clear zone, mm)
5%	23.83
10%	25.79
15%	27.38
20%	29.49
25%	33.55
30%	23.95



66hrs 72hrs 78hrs 84hrs 90hrs 96hrs

Figure 13 The Effects of Ages of Cultures on Fermentation (4 days)

5% 10% 15% 20% 25% 30%

Figure 14 The Effects of Size of Cultures on Fermentation (4 days)

Medium Optimization for Fermentation and Production of Antibacterial Metabolite

Table 6 Selection of Medium Based on the Results of Antimicrobial Activity

Sizes of Culture (%)	Antimicrobial Activity (Clear Zone, mm)
FM-1	23.07mm
FM-2	26.26mm
FM-3	23.60mm
FM-4	37.69mm
FM-5	36.13mm

FM-1 FM-2 FM-3 FM-4 FM-5

Figure 15 Antimicrobial Activity of Fungus *Aspergillus versicolor* (at Fermentation 5 Days)

Preliminary Study for the Extraction of Bioactive Compound (Jain and Pundi 2011)

Table 7 Activity of Pre-extraction with 4 Solvents System

No.	Solvent	Upper Layer	Lower Layer
1.	Chloromethane $CHCl_3$	21.25mm	16.17mm
2.	Dichloromethane CH_2Cl_2	24.29mm	17.46mm
3.	Toluene	19.31mm	14.26mm
4.	Hexane	21.98mm	10.70mm

In this study, it was found that the highest activity was shown in the solvent Dichloromethane CH_2CL_2.

In this study, it was observed that found that Dichloromethane CH_2CL_2 extract exhibited the highest activity although all extracts showed the activity. Therefore, if was assumed that the bioactive compound can be extracted twice with CH_2Cl_2 from the fermented broth.

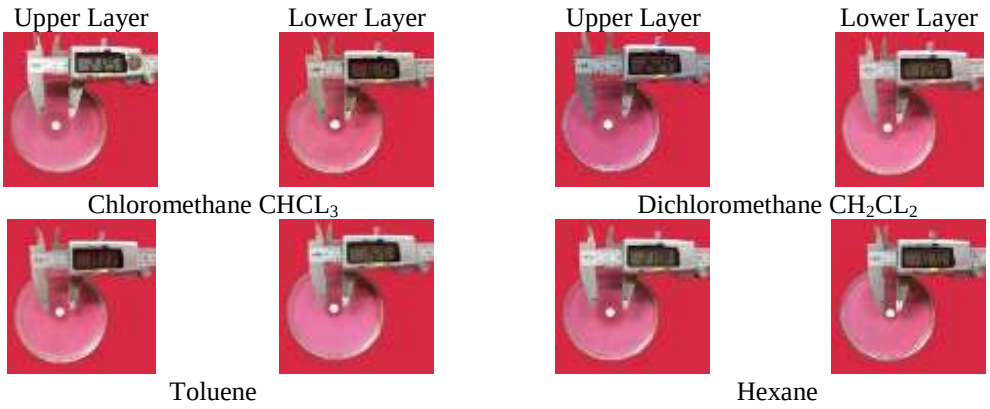


Figure 16 Solvent Based on Antimicrobial Activity

Morphological Characters of Endophytic Fungi TF-02

Colonies are usually fast growing, white yellow and then Shade of Yellow brown, brown to black, mostly consisting of a dense flat or erect conidiophores. Texture is velvety or cottony. Reverse is white, goldish or brown.

(X200) (7 days old culture) **(X200) (7 days old culture)**

Figure 17 Morphology and Photomicrograph of Fungus TF-02

Microscopic Characters of Endophytic Fungi TF-02

Hyphae are long, septate hyphae than appear glassy and transparent conidio -phores, which are specialized hyhphal stakes for asexual reproduction, typically measure 120-700 μm in length. Conidiophores terminate is small vesicles (10-15 μm in diameter), that are biseriate (i.e) with two successive layers of cells interposing the vesicle and conidia. The first layers of cells are called metulae upon which phialides are born. Hulle cells present and globose. The vesicles are variable in shape but often described as spoon shaped. Conidia are spherical approximately 2.5-3.5 μm in diameter and may have smooth or slightly roughened surfaces.

KEY TO GENUS

(Domsch *et al*, 1993; Klich, 2002; Mc Clenny, 2005; Nyongesa *et al*, 2015)

KEY TO GROUP

1. Conidium lacking septum…. ……..Ameroconidium
2. Conidium with 1 septum ….. ……..Didymoconidium
3. Conidium with more than 1 septum and only transverse septa ……... .…….. ……...Phragmoconidium
4. Conidium body subdivided by intersecting septa in more than one plane …….. …….....Dictyoconidium

Ameroconidium

A. Conidiophore not produced
B. Conidiophore not produced or not clear
C. Conidiophores with or without septa develop single, not branched
   1. Conidia holoblastic
   2. Conidia enteroblastic
      i. Phialo-conidium
      ii. Multi-phialides with parallel arrangement
         - *Penicillim*
         - *Aspergillus*
         - *Purpureocillium*
         - *Paecilomyces*

According to references key, TF-02 fungus may be the genus of *Penicillium, Aspergillus, Purpureocillium* and *Paecilomyces*.

Table 8 Comparison of Microscopical characters of *Penicillium, Aspergillus, Paecilomyces, Purpureocillium* and fungus TF-02

Genus of Fungi	Distinct Characters
Penicillium *	Phialides have thicker apices
Aspergillus *	Vesicle present
Paecilomyces *	Phialides are basically swollen, taper towards their apices are slightly apart from each other
Purpureocillium *	Phialides are basically swollen, taper towards their apices are slightly apart from each other
TF-02	Vesicle present

* (Domsch *et al*, 1993; Klich, 2002; McClenny, 2005; Nyongesa *et al*., 2015)

In the study on the comparison of microscopical characters, fungus TF-02 was designated to the genus *Aspergillus* due to the presence of vesicle.

Discussion and Conclusion

In the present study, *Andrographis paniculata* (Say-Kha-Gyi) collected from Magway University campus from isolation of endophytic fungi. Endophytic fungi were isolated by the method of Surface sterilization method and Baiting method. In this study, Nine fungi were isolated from surface sterilization method and five fungi were isolated from Baiting method. Fungi TF-01, TF-02, TF-03, TF-04, TF-05 and TF-06 were isolated from Say-Kha-Gyi leaves. Fungi TF-07, TF-08, TF-09 and TF-10 were isolated from Say-Kha-Gyi stem. Fungi TF-11, TF-12, TF-13 and TF-14 were isolated from Say-Kha-Gyi root. According to results, more fungi were isolated by surface sterilization method than Baiting method.

For the investigation of antimicrobial activities of isolated endophytic fungi, five kinds of test organisms were used by paper disc diffusion assay method. Fungus TF-02, TF-08 and TF-11 showed that antimicrobial activities against *Escherichia coli.* TF-01, TF-06 and TF-12 showed

that antimicrobial activities against *Bacillus subtilis*. TF-05 and TF-09, showed that antimicrobial activities against *Pseudomonas fluorescence*. TF-03, TF-07 and TF-13 showed that antimicrobial activities against *Saccharomyces cerevisiae*. TF-04, TF-10 and TF-14 showed that antimicrobial activities against *Canidida albicans*. According to the results, Fungus TF-02 was showed the highest antibacterial activity against *Escherichia coli*.

For the optimal age of inoculum 66hrs, 72hrs, 78hrs, 84hrs, 90hrs and 96hrs and for the size of inoculum 5%, 10%, 15%, 20%, 25% and 30% were used. By the results, the age of inoculum 84hrs (34.19mm) and the size of inoculum 25% (33.55mm) at 9 day cultured was best for TF-02 of fermentation. For the selection of fermentation, five kinds of fermentation media FM-1, FM-2, FM-3, FM-4, FM-5 were used for antibacterial activity. Among them, FM-4 showed the best antibacterial activity (37.69mm) at 7 days fermentation. The fermentation study is to know optimum size and age of inoculum and suitable fermentation medium for the production of antibacterial metabolize from fungus TF-02.

The purpose that preliminary study for the extraction of bioactive compound was to know how to extract the bioactive compound from fermented broth. The study for the extraction of bioactive compound from the fermented broth by using four solvents, Chloroform, Dichloromethane, Toluene and Hexane. In this study it was found that the highest activity was shown in the solvent Dicholoromethane. The study of morphological fungus TF-02 on MEA medium at 7 days' culture was excellent growth. In the investigation of identification, fungus TF-02 was identified as *Aspergillus versicolor* (vuill) Tirabosahi 1926 base on its morphological characters, microscopical characters and reference keys.

In conclusion, Endophtic fungus TF-02 were isolated from Say-Kha-Gyi and its was identified as *Aspergillus versicolor*. Endophytic fungus TF-02 was antibacterial activity against *Escherichia coli*. By the literature references, *Escherichia coli* can cause diarrhea was known. Therefore, this isolated endophytic fungus TF-02 will more or less help on against pathogenic microbes *Escherichia coli*.

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