ISOLATION OF ENDOPHYTIC FUNGI FROM *OROXYLUM INDICUM* (L.) BENTH (KYAUNG-SHA) FRUITS AND THEIR BIOLOGICAL ACTIVITIES

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Abstract

This research focuses on the chemical and biological aspect of crude extracts of endophytic fungi isolated from *Oroxylum indicum* (L.) Benth (Kyaung sha) fruits. The endophytic fungi have been isolated from *O. indicum* (Kyaung sha) fruit by the direct method, followed by cultured in potato agar medium (PGA). Four endophytic fungi (EFK1, EFK2, EFK3, and EFK4) have been isolated from *O. indicum* (Kyaung sha) fruit. Preliminary phytochemicals, total phenolic contents (TPC) and total flavonoid content (TFC) have been carried out as chemical investigations. Showing the presence of alkaloids, α–amino acids, carbohydrates, flavonoids, glycosides, organic acids, phenolic compounds, reducing sugars, saponins, steroids, tannins, and terpenoids, and the absence of starch in all tested samples. The total phenol contents of isolated fungi were determined by the Folin-Ciocalteu reagent (FCR) method. Gallic acid (3,4,5-trihydroxy benzoic acid), was used to construct a standard calibration curve for total phenol. TPC were expressed as a microgram of gallic acid equivalent per milligram of crude extract (μg GAE/mg). TPC (μg GAE/mg) were found to be highest in EFK2 (216.86 ± 0.66). The total flavonoids content (TFC) of isolated fungi were determined by using spectrophotometric method with aluminum chloride. The content of flavonoids was expressed in term of quercetin equivalent, mg of QE/g of extract. TFC (mg QE/g) was found to be highest in EFK3 (67.47 ± 1.40). The four isolated fungi were observed to possess antioxidant capacity by the DPPH assay method. Among them, EFK 2 has more potent antioxidant activity (IC_{50} = 12.15 μg/mL) than other tested samples. Moreover, anti-diabetic activity (expressed in terms of α-amylase inhibitory) indicated that EFK 3 and EFK 4 possessed higher anti-diabetic activity (IC_{50} = 4.94 μg/mL) than other tested samples. The four endophytic fungi were found to possess high activity against all tested microorganisms with the inhibition zone diameters ranging between 20 mm ~ 30 mm. All endophytic fungi have more portent antioxidant, antidiabetic and antimicrobial effects.

Keywords: endophytic fungi, *Oroxylum indicum* (L.) Bent, phytochemical constituents, biological activities

Introduction

Natural products and their derivatives have been recognized for many years as a valuable source of therapeutic agents and structural diversity. Recently, there has been renewed interest in the bioprospecting for natural products with potential therapeutic applications due to the failure of alternative drug discovery methods to deliver lead compounds in therapeutic areas such as immunosuppression, anti-infectives and metabolic diseases (Deka *et al*., 2013). Endophytes are microorganisms that live within plants for at least a part of their life cycle without causing any visible manifestation of disease. Bioprospection of endophytes is considered a new frontier for the discovery of useful natural products as endophytes have been shown to produce a broad variety of bioactive secondary metabolites with potential agricultural, pharmaceutical and industrial applications (Newman and Cragg, 2020). The number of secondary metabolites produced by fungal endophytes is larger than that of any other group of endophytic

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microorganisms and to date the vast majority of microbes isolated as endophytes have been fungi. Fungal metabolites have served as lead compounds for the development of anticancer, antifungal and antibacterial agents (Calixto, 2019).

Family	*Bignoniaceae*
Botanical name	*Oroxylum indicum* (L.) Benth
English name	midnight horror, Indian trumpet flower
Myanmar name	Kyaung sha
Part used	Fruits

Figure 1. Photographs of *Oroxylum indicum* (L.) Benth (Kyaung-sha) Fruits

The main aim of this research was to investigate some phytochemical constituents from crude extracts of endophytic fungi isolated from the fruits of *Oroxylum indicum* (L.) Benth (Kyaung-sha) and to evaluate some biological activities such as antimicrobial activity, antioxidant activity and antidiabetic activity.

Materials and Methods

Sample Collection

The fruits sample of *O. indicum* (L.) Benth (Kyaung-sha) was collected from Pathein Township, Ayeyarwady Region in October, 2020. After being collected, the scientific name of the sample was identified by authorized botanists at Botany Department, Pathein University.

Tissue preparation

Endophytic fungi were isolated from the different tissues collected from *O. indicum* (L.) Benth (Kyaung-sha) fruits using a modified surface sterilization procedure (Ando *et al*., 2004) Briefly, the plant tissue samples were removed from storage and thawed by briefly putting them under running tap water and then washed using 0.1% (v/v) for 15 minutes followed by another wash for 1 30 min using running water. The cleaned plant tissues were then transferred to a laminar airflow cabinet. The plant tissue samples were dipped in 70% ethanol; 30 sec for leaf samples and 2 min for stem samples. The ethanol was drained out after the required amount of time and the plant tissue was then washed using a 2% sodium hypochlorite solution for 15 min which was then followed by four washes using double distilled water after which the samples were dried using sterile paper towels. The effectiveness of the sterilization procedure was tested by plating 0.1 mL of the final sterile water rinse onto Petri dishes containing potato dextrose agar (PDA) and by rolling the sterilized sample onto Petri dishes containing PDA.

Isolation of endophytic fungi

The surface sterilized tissues were cut into smaller segments (1-2 cm) using sterile razor blades and placed onto Petri dishes containing PDA supplemented and incubated at 28±2 °C until fungal growth was initiated. The growing hyphal tips which grew out of the samples were isolated and sub cultured onto Petri dishes containing PDA. The cycle of sub culturing was repeated until pure cultures of the isolates were obtained (Chandra, 2012).

Preparation of crude extracts by direct extraction methods

The culture was centrifuged (20 minutes, 4 ºC, 3500 rpm). The cell-free supernatant was extracted with ethyl acetate for 10 times to get the ethyl acetate soluble compounds by liquid-liquid partition between ethyl acetate and the culture solution 1:1 v/v. This process was done according to the ultrasound-assisted extraction to give ethyl acetate extract which was applied to investigate the chemical constituents and some biological activities. (Ando and Inaba, 2004).

Preliminary Phytochemical Test

A few gram of crude extract of selected sample was subjected to the tests of alkaloids, α-amino acids, carbohydrates, flavonoids, glycosides, organic acids, phenolic compounds, reducing sugars, saponins, starch, tannins, steroids, terpenoids according to the standard procedure. (Harborne, 1984)

Chemical Constituents of the Ethyl Acetate Extract of Endophytic Fungi Isolated from *Oroxylum indicum* (L.) Benth (Kyaung-sha) Fruits

(a) Determination of total phenol content assay

The total phenolic content (TPC) of ethyl acetate extract of endophytic fungi isolated from *O. indicum* (L.) Benth (Kyaung-sha) fruits was estimated by Folin-Ciocalteu (FC) method according to the procedure described by (Song *et al*., 2010). The extract solution (1000 µg/mL) was mixed with 5 mL of F-C reagent (1:10) in a test tube and incubated for about 5 min. To each test tube, 4 mL of 1 M sodium carbonate was added and the test tubes were kept at room temperature for 15 min and UV absorbance of reaction mixture was read at λ_{max} 765 nm. The blank solution was prepared as the above procedure by using distilled water instead of sample solution. Total phenol content was estimated as milligram gallic acid equivalent per gram (mg GAE/g) of extract.

(b) Determination of total flavonoid content assay

The total flavonoid content (TFC) of ethyl acetate extract of endophytic fungi isolated from *O. indicum* (L.) Benth (Kyaung-sha) fruits was estimated by Aluminium Chloride method according to the procedure described by (Song *et al*. 2010). The extract solution (1000 µg/mL) was mixed with 1.5 mL of methanol, 0.2 mL of 1 % $AlCl_3$ solution and 2.8 mL of distilled water. The absorbance of reaction mixture was at λ_{max} 415 nm. The blank solution was prepared as the above procedure by using distilled water instead of sample solution. Total flavonoid content was estimated as milligram quercetin equivalent per gram (mg QE/g) of extract.

Biological Activities of Ethyl Acetate Extracts of Endophytic Fungi

(a) Investigation of antioxidant activity assay

In this experiment, DPPH (2 mg) was thoroughly dissolved in EtOH (100 mL). This solution was freshly prepared in the brown coloured reagent bottle and stored in the fridge for no longer than 24 h. The crude extracts of *O. indicum* (2 mg) and 10 mL of EtOH were thoroughly mixed by shaker. The mixture solution was filtered and the stock solution was obtained. By adding with EtOH, the sample solutions in different concentrations of 200, 100, 50, 25, 12.5, 6.25 and 3.125 μg/mL were prepared from the stock solution. The effect on DPPH radical was determined by using the method of Marinova and Batchvarov (2011). The control solution was prepared by mixing 1.5 mL of 50 μM DPPH solution and 1.5 mL of EtOH using shaker. The test sample solution was also prepared by mixing thoroughly 1.5 mL of 50 μM DPPH solutions and 1.5 mL of each sample solution. The mixture solutions were allowed to stand at room temperature for 30 min. Then, the absorbance of each solution was measured at 517 nm by using UV-1650 spectrophotometer. Absorbance measurements were done in triplicate for each concentration and then mean values so obtained were used to calculate percent inhibition of oxidation (Basma *et al*., 2011). The capability to scavenge the DPPH radical was calculated by using the following equation:

$$\% \text{ RSA} = \frac{A_{control} - (A_{sample} - A_{blank})}{A_{control} \times 100}$$

Where, %RSA = Radical Scavenging Activity

$A_{control}$ = absorbance of the control (DPPH only) solution

A_{blank} = absorbance of the blank (EtOH + Test sample solution) solution

A_{sample} = absorbance of the test sample solution

(b) Determination of antidiabetic activity by α-amylase inhibition assay

In alpha amylase assay, the starch-iodine was used. First 2 mL of (0.5%) substrate starch solution and 1 mL of tested solution (Acarbose standard drug and crude) of seven different concentration such as 200, 100, 50, 25, 12.5, 6.25 and 3.125 μg/mL were added in a bottle and this mixture was incubated for 3 min at room temperature. To start the reaction, 1 mL of α-amylase was added in above solution followed by incubated for 15 min at room temperature. To stop the reaction, 4 mL of 0.1M HCl was added in this mixture and to detect the reaction, 1 mL of Iodine-iodide indicator (1 mM) was added in the mixture. Absorbance was read at 650 nm by UV spectrophotometer in the visible region. The control solution was prepared as above procedure by using phosphate buffer (0.02M, pH 6.5) instead of drug solution.

All the experiments were done in triplicate. Percent inhibition of each sample solution was calculated by using the following formula. Standard deviation (SD) and 50% inhibition concentration (IC_{50}) value in μg/mL were calculated by computer excel program.

$$\% \text{ Inhibition} = \frac{A_{Sample} - A_{Control}}{A_{Sample}} \text{ x } 100$$

Where,

$A_{control}$ = the absorbance of the control solution

A_{sample} = the absorbance of sample solution

(c) Determination of antimicrobial activity of ethyl acetate extracts of endophytic fungi

The antimicrobial activity of ethyl acetate extracts of endophytic fungi isolated from *O. indicum* (L.) Benth (Kyaung-sha) fruits was determined against eight strains of microorganisms such as *Agro tumefaciens* (NITE 09678), *Bacillus pumilus* (IFO 12092), *Bacillus subtilis* (IFO 90517), *Candida albicans* (NITE 09542), Escherichia coli (AHU 5436), *Micro luteus* (NITE 83297), *Pseudomonas fluoresens* (IFO 94307) and *Staphylococus aureus* (AHU 8465) by employing agar well diffusion method. To prepare the agar plate, firstly, peptone (0.5 g) and sodium chloride (0.25 g) were mixed in distilled water and made up to 100 mL with distilled water. The pH of this solution was adjusted at 7.2 with 0.1 M sodium hydroxide solution and 1.5 g of agar was added. Nutrient agar was prepared according to method described by (Ando and Inaba, 2004). Briefly nutrient agar was boiled and 20-25 mL of the medium was poured into a test tube and plugged with cotton wool and autoclaved at 121 ºC for 15 minutes. Then the tubes were cooled down to 60 ºC and poured into sterilized petri-dish and 0.1 mL of spore suspension was also added into the dishes. The agar was allowed to set for 30 minutes after which 10 mm plate agar well was made with the help of sterilized cork border. After that, about 0.1 mL each of the prepared extract solution was introduced into the agar well and incubated at 37 ºC for 24 h. The inhibition zone (clear zone) appeared around the agar well indicating the presence of antimicrobial activity. The extent of antimicrobial activity was measured from the zone of inhibition diameter.

Results and Discussion

The four endophytic fungi (EFK 1, EFK 2, EFK 3 and EFK 4) have been isolated from *O. indicum* (L.) Benth (Kyaung-sha) fruits by the direct method, followed by cultured in potato agar medium (PGA). The phytochemical tests revealed that alkaloids, α-amino acids, carbohydrates, flavonoids, glycosides, organic acid, phenolic compounds, reducing sugars, saponins, steroid, tannins and terpenoids were found to be present but and starch was absent in selected sample. The total phenol content of the ethyl acetate extract of four endophytic fungi isolated from *O. indicum* (L.) Benth (Kyaung-sha) fruits was determined with spectrophotometric method by using Folin-Ciocalteu reagent. TPC (μg GAE/mg) were found to be highest in EFK-2 (216.86 ± 0.66). The total flavonoid content of sample was determined with spectrophotometric method by aluminum chloride reagent and was found to be highest in EFK-3 (67.47 ± 1.4 mg QE/g). The results are shown in Table1.

Table 1. Total Phenol and Total Flavonoid Contents of Four Endophytic Fungi

Fungi	Total Phenol Content (mg GAE ± SD)/g of extract	Total Flavonoid Content (mg QE ± SD)/g of extract
EFK-1	16.95 ± 0.28	14.68 ± 0.22
EFK-2	126.86 ± 0.14	59.77 ± 0.11
EFK-3	44.79 ± 0.21	67.47 ± 1.40
EFK-4	52.90 ± 0.14	34.61 ± 0.22

According to the experimental results, phenol and flavonoid compounds were detected in all endophytic fungi. Besides their established antioxidant activity, many phenolic compounds may exhibit significant antimicrobial activity. Since many plant extracts are rich in phenolic compounds, this is of particular interest for the development of natural alternatives to synthetic preservatives in food and cosmetic applications. Flavonoids are also present as a potent water-soluble antioxidant and free radical scavengers, which prevent from the oxidative cell damage and also have strong anticancer activity. It also helps in managing diabetes induced oxidative stress.

The antioxidant activity was measured in terms of hydrogen donating or radical scavenging ability of the four endophytic fungi by using the stable radical DPPH. The results are shown in Figure 2 and Table 2. The EFK-2 was found to be the low (IC_{50}) value 12.15 μg/mL than the other endophytic fungi, low IC_{50} value indicate the more potent antioxidant activity. However, the selected sample was weaker activity than the standard ascorbic acid (IC_{50} = 4.22 μg/mL).

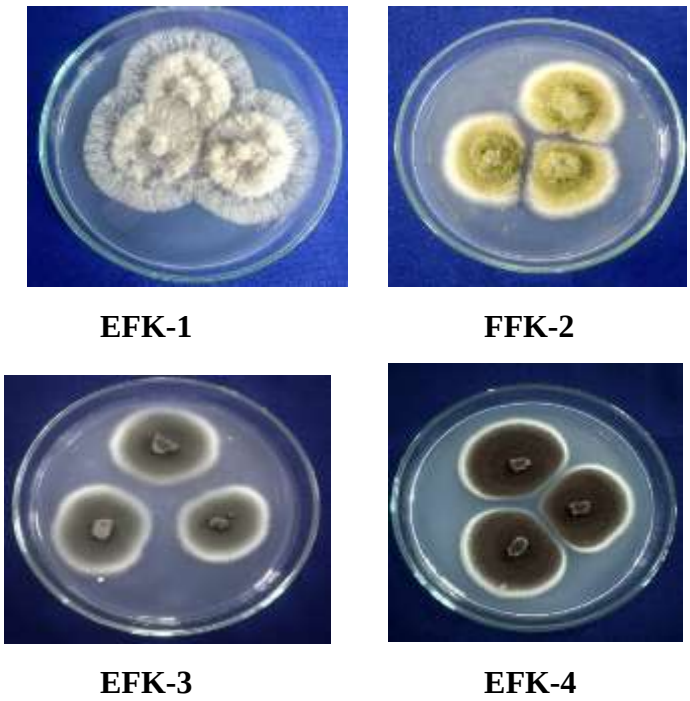


Figure 2. Colony characteristic of endophytic fungi from (Kyaung sha) fruit

Hyperglycemia has been a classical risk in the development of diabetes and the complications associated with diabetes. Therefore, control of blood glucose levels is critical in the early treatment of diabetes mellitus and reduction of macro and microvascular complications. One therapeutic approach is the prevention of carbohydrate absorption after food intake, which is facilitated by inhibition of enteric enzymes including *α*-glucosidase and *α*-amylase present in the brush borders of intestine. In this study, the *α*-amylase inhibitory activity of *O. indicum* (L.) Benth (Kyaung-sha) fruit was investigated. The inhibitory effect of crude extracts of four isolated fungi were analyzed. The percentage inhibition of *α*-amylase by crude extract were studied in a concentration range of 3.125-200 μg/mL. The percentage inhibition of the samples on *α*-amylase enzyme activity increased with increasing the concentrations. From the percentage inhibition, the respective IC_{50} values for the crude extracts were calculated and the results are respectively

tabulated in Table (3). The four endophytic fungi isolated from *O. indicum* (L.) Benth (Kyaung-sha) fruit also explored for the in *vitro* *α*-amylase inhibition and their activity was compared with standard anti-diabetic drug, acarbose. The 50% α–amylase inhibition potency (IC_{50}) of crude extracts of the four endophytic fungi was ranging between 4.97-12.36 μg/mL, indicating that crude extracts possessed potent α–amylase inhibition activity. However, the selected sample was weaker activity than the standard acarbose (IC_{50} = 3.47 μg/mL).

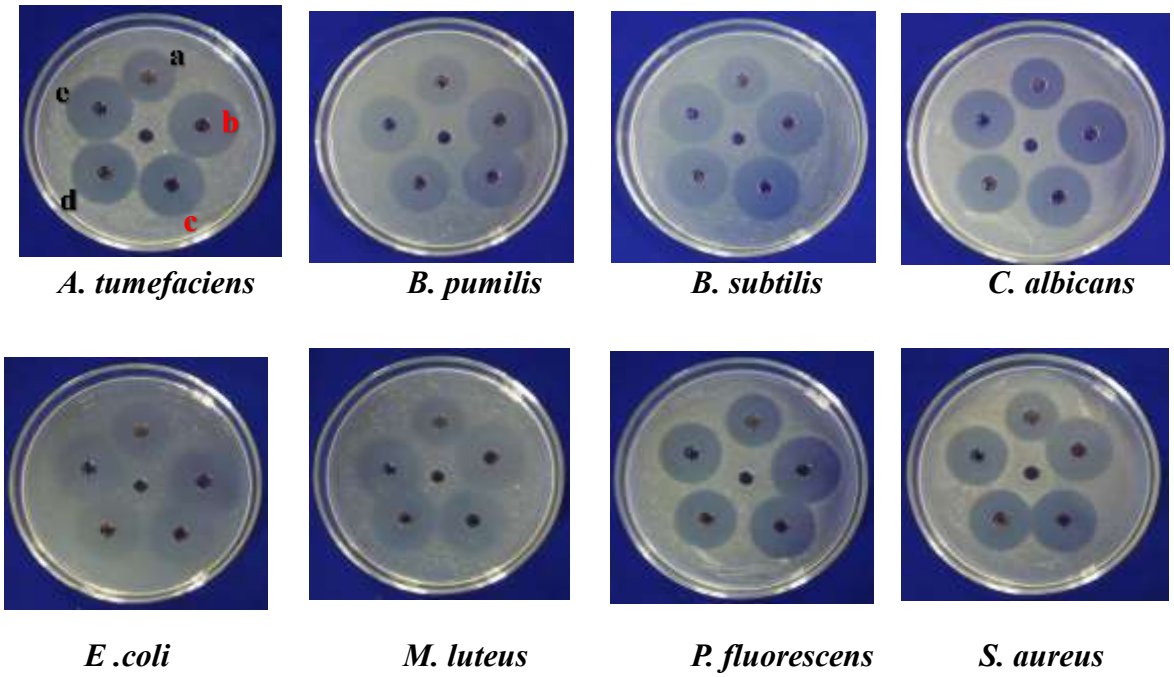
Table 2. Antioxidant Activity of Four Endophytic Fungi by DPPH Assay

Samples	% RSA (mean ±SD) in different concentrations (μg/mL)							IC_{50} (μg/mL)
	3.125	6.25	12.5	25	50	100	200	
EFK-1	34.63 ± 0.54	42.83 ± 0.91	46.72 ± 0.79	57.94 ± 1.82	79.36 ± 1.56	91.19 ± 0.26	99.83 ± 0.75	16.15
EFK-2	32.82 ± 0.40	45.60 ± 0.26	50.26 ± 0.26	51.73 ± 1.82	70.55 ± 0.75	77.29 ± 0.15	80.83 ± 0.69	12.15
EFK-3	44.30 ± 0.26	46.98 ± 0.30	49.48 ± 0.26	55.96 ± 0.52	57.69 ± 0.40	67.01 ± 0.40	80.14 ± 0.15	13.5
EFK-4	34.72 ± 0.45	37.31 ± 0.52	45.85 ± 0.26	47.93 ± 0.26	57.08 ± 0.54	66.49 ± 0.15	72.11 ± 0.54	30.66
Ascorbic acid	45.05 ± 0.82	56.35 ± 0.35	68.42 ± 0.45	73.13 ± 0.33	81.95 ± 0.12	88.18 ± 0.23	97.40 ± 0.65	4.22

Table 3. Antidiabetic Activity of Four Endophytic Fungi by α-Amylase Inhibition Assay

Samples	% Inhibition in different concentrations (μg/mL)							IC_{50} (μg/mL)
	3.125	6.25	12.5	25	50	100	200	
EFK-1	27.64 ± 0.59	35.66 ± 0.54	65.23 ± 0.60	75.82 ± 0.07	80.85 ± 0.02	83.79 ± 0.03	85.48 ± 0.02	9.28
EFK-3	33.86 ± 0.25	41.45 ± 0.13	50.19 ± 0.09	52.58 ± 0.08	54.95 ± 0.12	60.60 ± 0.09	64.71 ± 0.07	12.36
EFK-3	37.47 ± 0.15	59.05 ± 0.06	60.96 ± 0.06	70.06 ± 0.05	75.88 ± 0.02	77.89 ± 0.03	81.40 ± 0.05	4.97
EFK-4	20.61 ± 0.49	52.12 ± 0.10	58.73 ± 0.06	60.39 ± 0.06	65.99 ± 0.04	72.34 ± 0.04	77.33 ± 0.03	4.97
Acarbose	48.03 ± 0.12	58.11 ± 0.30	72.49 ± 0.39	81.22 ± 0.32	89.11 ± 0.26	91.30 ± 0.41	98.19 ± 0.59	3.74

Screening of antimicrobial activity of four endophytic fungi isolated from *O. indicum* (L.) Benth (Kyaung-sha) fruits was done by agar well diffusion method (Table 4 and Figure 3). In this study, the samples were tested on eight pathogenic microorganisms such as *Agro tumefaciens, Bacillus pumilus, Bacillus subtilis, Candida albicans, Escherichia coli, Micro luteus, Pseudomonas fluoresens* and Staphylococus aureus. From these results, it was found that selected sample exhibit antimicrobial activity against all tested microorganisms. The four endophytic fungi exhibited inhibition zone diameters ranged between in 23 mm ~ 30 mm respectively against all tested microorganisms.



a= EFK1, b= EFK2, c= EFK3, d= EFK4 & e= Standard

Figure 3. Screening of antimicrobial activity of the crude extracts by agar well diffusion method

Table 4. Inhibition Zone Diameters of Crude Extracts Against Eight Microorganisms by Agar Well Diffusion Method

Microorganism	Inhibition zone diameters (mm)				
	EFK-1	**EFK-2**	**EFK-3**	**EFK-4**	**STD**
A. tumefaciens	23.29 ± 0.12	29.20 ± 0.08	26.47 ± 0.11	27.41 ± 0.12	29.29 ± 0.03
B. pumiliu	23.77 ± 0.17	30.21 ± 0.31	29.39 ± 0.37	29.07 ± 0.08	29.48 ± 0.01
B. subtilis	24.13 ± 0.08	32.79 ± 0.02	31.26 ± 0.25	31.16 ± 0.03	28.89 ± 0.05
C. albicans	24.51 ± 0.16	30.17 ± 0.25	29.53 ± 1.19	28.51 ± 0.43	30.34 ± 0.09

Microorganism	Inhibition zone diameters (mm)				
	EFK-1	**EFK-2**	**EFK-3**	**EFK-4**	**STD**
E .coli	24.55 ± 0.10	30.81 ± 0.26	29.42 ± 0.07	29.23 ± 0.06	29.29 ± 0.03
M. luteus	24.50 ± 0.04	29.85 ± 0.12	29.25 ± 0.06	29.36 ± 0.04	29.48 ± 0.01
P. fluorescens	23.73 ± 0.01	29.43 ± 0.36	28.90 ± 0.12	29.37 ± 0.51	28.89 ± 0.05
S. aureus	25.09 ± 0.05	30.00 ± 0.03	29.66 ± 0.03	29.42 ± 0.04	30.34 ± 0.09

Agar well diameter (8 mm)
10 mm – 14 mm = weak activity (+)
15 mm – 19 mm = modreate activity (++)
20 mm and above = potent activity (+++)
STD = chloramphenicol

Conclusion

The four endophytic fungi isolated from *O. indicum* (L.) Benth (Kyaung-sha) showed. Phytochemical results, such as alkaloids, α-amino acids, carbohydrates, flavonoids, glycosides, organic acid, phenolic compounds, reducing sugars, saponins, steroid, tannins and terpenoids were found to be present and starch was absent. The ethyl acetate extract of EFK-2 contains significant high TPC (126.86 ± 0.14 mg GAE/g) and EFK-3 contains significant high TPC (67.47 ± 1.40 mg QE/g). It showed high antimicrobial activity (23 mm ~ 30mm) against *Agro tumefaciens, Bacillus pumilus, Bacillus subtilis, Candida albicans, Escherichia coli, Micro luteus, Pseudomonas fluoresens* and *Staphylococus aureus* due to presence the flavonoids and phenols. The crude extracts of four isolated fungi also showed DPPH free radical scavenging activity assay (IC_{50} = 16.15 μg/mL, 12.15 μg/mL, 13.50 μg/mL and 30.66 μg/mL respectively) has antioxidant activity. The antidiabetic activity due to its α-amylase inhibitory effect (IC_{50}= 9.28 μg/mL, 12.36 μg/mL and 4.97 μg/mL respectively). The results from this research strongly indicated that tested crude extracts of four endophytic fungi isolated from *O. indicum* (L.) Benth (Kyaung-sha) fruit may play a role as medicinal constituents.

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