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ORIGINAL ARTICLE

Evaluation of Antibacterial Properties from Endophytic Fungi of *Chrysanthemum indicum* (L.) Flowers against *Escherichia coli* and *Staphylococcus aureus*

Titah Amelia Ratte[1], Fatimawali[1,2,*], Trina Ekawati Tallei[3], Elly Juliana Suoth[1], Irma Antasionasti[1], and Paulina V.Y. Yamlean[1]

[1]Pharmacy Study Program, Faculty of Mathematics and Natural Sciences, Sam Ratulangi University, Manado, Indonesia; [2]Department of Chemistry, Faculty of Medicine, Sam Ratulangi University, Manado, Indonesia; [3]Department of Biology, Faculty of Mathematics and Natural Sciences, Sam Ratulangi University, Manado, Indonesia

* Correspondence: fatimawali@unsrat.ac.id

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Abstract

Uncovering the therapeutic potential of secondary metabolites produced by plants, animals, and microbes constitutes the foundation for the development of novel medications. The objective of this investigation is to discern the classes of secondary metabolites and assess the antibacterial properties of endophytic fungal extracts obtained from *Chrysanthemum indicum* L. flowers. Through the isolation process, five isolates designated as JEC1, JEC2, JEC3, JEC4, and JEC5 were identified. The cultivation of endophytic fungal isolates spanned a three-week period before undergoing extraction with ethyl acetate. The phytochemical tests revealed the presence of alkaloids, flavonoids, steroids, terpenoids, saponins, and tannins in the ethyl acetate extract. Antibacterial activity was determined using the agar well diffusion method, with ciprofloxacin serving as a positive control. Notably, all ethyl acetate extracts from endophytic fungi exhibited antibacterial activity. The most substantial inhibitory diameter against *Staphylococcus aureus* was recorded as 19.1±0.8 mm for the JEC3 endophytic fungi, while *Escherichia coli* exhibited an inhibitory diameter of 16±1.1 mm for the JEC2 endophytic fungi.

Introduction

Chrysanthemum indicum L., commonly referred to as Chrysanthemum, is a flowering plant highly sought after for ornamental purposes due to its diverse array of rich colours, sizes, shapes, and notable durability [1,2]. Beyond its ornamental value, Chrysanthemum is integral to traditional medicinal practices within communities, being employed for the treatment of various ailments such as hypertension, infections, and immune-related diseases [3]. This multifaceted usage is attributed to the presence of numerous chemical compounds in Chrysanthemum plants, including tannins, saponins, alkaloids, flavonoids, and terpenoids [4]. The chemical composition of Chrysanthemum underscores its medicinal potential, serving as a reservoir of bioactive compounds with antibacterial, anti-inflammatory, and antioxidant properties [5,6].

Endophytic fungi are microorganisms that live in plant tissues such as seeds, leaves, flowers, twigs, stems and roots, characterized by their ability to inhabit plant tissues without causing harm to the host [7,8], the presence of endophytic fungi is very important for the host plant balance because it can protect the host from pathogens, predators, and increase plant resistance to drought [9]. Endophytic fungi play a pivotal role in bolstering host resistance against pathogenic microbial assaults by producing bioactive compounds [7]. The host specificity of endophytic fungi is a crucial determinant in their capacity to generate bioactive compounds, a phenomenon often attributed to coevolutionary genetic transfer [9].

The bioactive compounds produced by endophytic fungi, considered secondary metabolites, encompass enzymes and various bioactive compounds [10,11]. These compounds, with potential applications in medicine and pharmacy, notably serve as antimicrobial agents, some examples include pyrrocidine A and B [12], perionicin A and B [13], phomol [14], and berefeldin [15]. The production of bioactive compounds by endophytes, especially those exclusive to their host plants, is not only important from an ecological perspective but also from a biochemical and molecular standpoint [16].

In light of the aforementioned background, the primary objective of this study was to discern the composition of secondary metabolites within endophytic fungal isolates from Chrysanthemum flowers and to assess their antibacterial efficacy.

Materials and Methods

Sample Preparation

The Chrysanthemum plant specimens used in this study were sourced from the flowers cultivated in the Green House Isye 9R7F+J9F, located in Kinilow, North Tomohon sub-district, Tomohon City, North Sulawesi, Indonesia. The floral samples were carefully collected and placed inside a double-lock plastic zipper bag for subsequent transport to the Pharmacy Laboratory at Sam Ratulangi University.

Isolation of Endophytic Fungi

Chrysanthemum flower specimens underwent surface sterilization through sequential immersion in 70% ethanol for 30 seconds, followed by exposure to 5.25% sodium hypochlorite for 1 minute, and subsequent rinsing with sterile distilled water for an additional 1 minute. Following sterilization, the samples were dried, cut into small fragments measuring 1 cm × 1 cm, and subsequently placed onto Potato Dextrose Agar (PDA) media. Triplicate inoculations were performed, with each petri dish accommodating three distinct sample pieces. The entire sterilization and inoculation procedures were conducted within a Laminar Airflow (LAF) environment. The prepared samples were then incubated in a controlled environment at 30°C for a duration ranging from 3 to 31 days [17].

Purification of Endophytic Fungi

Endophytic fungi cultivated individually on PDA media were extracted using a sterile osse needle and subsequently transferred to fresh PDA media. The examination of purified isolates was conducted after an incubation period of 5-7 days. Throughout this period, if macroscopically distinct colony growth persisted, a separation procedure was implemented until pure isolates were achieved.

Cultivation of Endophytic Fungi

The purified isolates were cut into pieces measuring 1 cm x 1 cm and subsequently introduced into rice-based media. Subsequent to this, an incubation period spanning 2-3 weeks ensued, with careful observation of the growth dynamics conducted at room temperature within the range of 20-25°C [18].

Extraction of Secondary Metabolites from Endophytic Fungi

The cultivated fungi were extracted utilizing ethyl acetate solvent through a triple extraction process. One hundred milliliters of ethyl acetate solvent were applied to saturate the fermentation medium, followed by filtration. The resulting filtrate was subsequently subjected to drying in a water bath set at 70°C for a duration of 3 days. The resultant extract was stored at 4°C (refrigerator) for subsequent utilization in testing.

Identification of Secondary Metabolite Groups

Various analysis was conducted to analyze the presence of secondary metabolite groups in the samples. For the alkaloid test, 2.5 mL of 2% HCl was added to several samples, and Dragendroff reagent and Mayer reagent were used for testing. Alkaloid compounds were identified by the formation of an orange precipitate with Dragendroff reagent or a yellowish-white precipitate with Mayer reagent. In the flavonoid test, samples were subjected to the addition of Mg powder and 5 drops of concentrated HCl, followed by heating. The presence of flavonoids was indicated by a red or orange coloration in the solution. For the saponin test, a specific amount of the sample was added to distilled water, shaken, and allowed to stand. The addition of 10% HCl and shaking again confirmed the presence of saponin group compounds if the foam remained stable. In the tannin test, several samples were treated with 5 drops of 1% $FeCl_3$, and a blue or black coloration in the solution signified the presence of tannin compounds. The steroid test involved treating samples with chloroform, concentrated acetic acid, and dilute sulfuric acid, with a blue or green coloration indicating the presence of steroid compounds. In the terpenoid test, samples treated with chloroform, concentrated acetic acid, and dilute sulfuric acid showed the absence of terpenoid compounds if the solution turned blue or green.

Antibacterial Activity Test

The method used in the test is the agar well diffusion method. The experimental technique employed in this study is the hole/spout method. Staphylococcus aureus and Escherichia coli bacteria were the test organisms utilized, sourced from the stock culture collection of the Pharmaceutical Microbiology Laboratory at Sam Ratulangi University. The testing procedure involved adding 20µl of the test bacteria to 30mL of Nutrient Agar (NA), homogenizing the mixture, and pouring it into a Petri dish. Subsequently, the solidified media was perforated using a punching tool. Ten microliters of each extract from the five samples were applied to the perforations at equidistant intervals. Additionally, two perforations served as positive (ciprofloxacin) and negative (left blank) controls. The incubation period lasted 24 hours at 37°C. Post-incubation, the formed inhibition zones were observed, and their diameters were measured using a vernier caliper. Endophytic fungal isolates demonstrating a positive inhibition zone are considered potential candidates for the production of antibacterial compounds [19].

Results and Discussion

Characterization of Isolated Fungal Isolates

The isolation of endophytic fungi from Chrysanthemum plant flowers on PDA medium yields fungi that exhibit distinct macroscopic characteristics, with variations observed in terms of color, including white, orange, and grayish hues, as well as diverse morphologies. The individual growth of each fungus, particularly its occurrence on the periphery of flower samples cultivated on PDA media, suggests its endophytic nature. A total of five fungal isolates were successfully isolated and purified from the flower parts of Chrysanthemum (*Chrysanthemum indicum* L.), as indicated in Table 1. Following the purification process, all isolates exhibited uniform white coloration with different colony shapes, as illustrated in Table 1.

Secondary Metabolite Production by Endophytic Fungi

The manifestation of color on the surface of the rice media serves as an indicator that endophytic fungi are producing secondary metabolites. This occurrence is attributed to the secretion of secondary metabolites by fungi either intracellularly or into the surrounding media environment [20].

The selection of a solvent for the extraction process significantly influences the effectiveness of extraction, contingent upon the solubility of natural material compounds in the chosen solvent [21]. Ethyl acetate emerges as a suitable solvent for extraction due to its ease of evaporation, non-hygroscopic nature, low toxicity, and semi-polar characteristics. Its semi-polarity enable the

attraction of both polar and nonpolar compounds, contributing to its versatility in extracting compounds with varying polarities [22]. The results of the extraction process using ethyl acetate solvent yield an extract containing compounds spanning a spectrum of polarity, ranging from semi-polar to non-polar.

Table 1. Characteristics of Chrysanthemum flower endophytic fungi.

Isolates code	Endophytic Fungi	Descriptions
JEC 1		White fungal colony
JEC 2		White fungal colony
JEC 3		White fungal colony
JEC 4		White fungal colony
JEC 5		White fungal colony

Secondary Metabolite Groups

The outcomes delineated in Table 2 elucidate the characterization of secondary metabolite groups within ethyl acetate extracts derived from distinct endophytic fungal isolates (JEC1, JEC2, JEC3, JEC4, and JEC5). The analysis focused on various phytochemical components present in the extracts. Tannins were identified in extracts JEC1 and JEC3, while they were absent in extracts JEC2, JEC4, and JEC5. Steroids were absent in JEC1, JEC2, and JEC5 but present in JEC3 and JEC4. Terpenoids were found in JEC1 and JEC2 but were absent in JEC3, JEC4, and JEC5. Alkaloids and flavonoids were consistently present in all extracts. Saponins were present in JEC2, JEC3, JEC4, and JEC5 but absent in JEC1. The notation of presence (+) or absence (-) of secondary metabolites is specified in the respective columns. The overall analysis reveals a varied composition of secondary metabolites across the different extracts, emphasizing the diversity of phytochemical constituents in the ethyl acetate extracts from various endophytic fungal isolates.

Table 2. Secondary metabolite group of ethyl acetate extract of endophytic fungi.

Secondary Metabolites	Extract JEC1	Extract JEC2	Extract JEC3	Extract JEC4	Extract JEC5
Tannins	+	-	+	-	+
Steroids	-	-	+	+	-
Terpenoids	+	+	-	-	+
Alkaloids	+	+	+	+	+
Flavonoids	+	+	+	+	+
Saponin	-	+	+	+	+

Description :
(+) indicates the presence of secondary metabolites in the extract tested,
(-) indicates the absence of secondary metabolites in the extract tested.

Antibacterial Activity

Table 3 presents the findings from the measurement of the inhibition zone diameter concerning *S. aureus* and *E. coli* bacteria. The antibacterial activity assessment employs the diffusion method, where the clear zone formed around the well serves as an indicator of the extracts' and antibiotics' inhibitory effects on bacterial growth. Result interpretation involves measuring the inhibition zones around the wells using a caliper [23].

Table 3. Antibacterial activity of ethyl acetate extract of endophytic fungi against *Staphylococcus aureus* and *Escherichia coli* bacteria.

Bacteria		Inhibition Zone Measurement Results (mm)						
		JEC1	JEC2	JEC3	JEC4	JEC5	(+)	(-)
S. aureus	I	13.8	15	20	14.3	11.5	24.8	-
	II	12.8	12.5	18.8	12	12	24.5	-
	III	12.5	13	18.5	12	12	24	-
	SD	**13±0.7**	**13.5±1.3**	**19.1±0.8**	**12.8±1.3**	**11.83±0.3**	**24.4±0.4**	**-**
E. coli	I	14.8	15.8	12.5	12.8	12.3	27	-
	II	14	15	17.5	13.8	14	28.3	-
	III	14	17.3	12.8	12.8	13.5	25	-
	SD	**14.3±0.4**	**16±1.1**	**14.3±2.8**	**13.1±0.6**	**13.3±0.9**	**26.8±1.6**	-

Description:
(+): Ciprofloxacin Positive Control
(-): Negative Control -
SD: Mean diameter of inhibition zone ± Standard Deviation

The ethyl acetate extract concentration utilized from the endophytic fungal isolates was 50 mg/mL, employing DMSO as the solvent. For comparative analysis, the positive control employed was the antibiotic ciprofloxacin, with a concentration of 11.8 mg/mL dissolved in distilled water.

Based on the acquired data, it is evident that metabolite compounds derived from fungal isolates JEC1, JEC2, JEC3, JEC4, and JEC5 exhibit antibacterial activity, as evidenced by the formation of inhibition zones against both Gram-Positive bacteria, specifically *S. aureus*, and Gram-Negative bacteria, particularly *E. coli*.

The observed disparity in inhibition zone values can be attributed to variations in the fungal species, each producing distinct secondary metabolites. Additionally, the quantity of produced secondary metabolites is influenced by nutrient absorption during fermentation by endophytic fungi. Notably, the inhibitory efficacy of the isolates is more pronounced against the growth of *E. coli* bacteria in comparison to *S. aureus* bacteria. The secondary metabolites, specifically flavonoids, alkaloids, and tannins, play a significant role in inhibiting bacterial activity [24].

Conclusions

This investigation focused on the isolation, cultivation, extraction, identification of secondary metabolites, and antibacterial activity testing of endophytic fungi sourced from Chrysanthemum flowers (*C. indicum* L.) located in Kinilow, North Tomohon sub-district, Tomohon City, North Sulawesi, Indonesia. The outcomes of phytochemical assessments indicate the presence of alkaloids, flavonoids, steroids, terpenoids, saponins, and tannins in the ethyl acetate extract obtained from the endophytic fungus *C. indicum* L. Moreover, all ethyl acetate extracts from the endophytic fungi manifest an inhibition zone against both *S. aureus* and *E. coli* bacteria. Nonetheless, further investigation is warranted to explore the potential of JEC3 endophytic fungal isolates as antibacterial agents against other microbial strains.

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