INTERNATIONAL RESEARCH JOURNAL OF PHARMACY

www.irjponline.com ISSN 2230 – 8407

Research Article

ISOLATION IPG3-1 AND IPG3-3, ENDOPHYTIC FUNGI FROM DELIMA (*PUNICA GRANATUM* LINN.) TWIGS AND IN VITRO ASSESSMENT OF THEIR ANTI MICROBIAL ACTIVITY

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Article Received on: 13/03/13 Revised on: 01/04/13 Approved for publication: 01/05/13

DOI: 10.7897/2230-8407.04611

IRJP is an official publication of Moksha Publishing House. Website: www.mokshaph.com

ABSTRACT

IPG3-1,2,3,4,5 were endophytic fungi isolated from Delima twigs via direct seed inoculation on Potato Dextrose Agar (PDA). Macroscopic identification of the isolates showed clearly that IPG3-3 and 4 have radial wrinkles in the centre with black spots while IPG3-5 has flower shape in the middle. The microscopic observation on the other hand, showed that IPG3-1 and 3 has branched septate while IPG3-4 has unbranched septate. Furthermore, IPG3-2 and 5 were branched but septateless. Overall, these endophytes showed no spore formation. Secondary metabolites of IPG3-1 and IPG3-3 endophytic fungi isolates demonstrated stronger inhibition zone percentage against *Candida albicans* and than towards *Staphylococcus aureus* and *Escherichia coli* when campare to their respectively positive control amphotericin B and Chloramphenicol.

Keyword: endophytic fungi, Delima (*Punica granatum* Linn.), inhibition zone, antimicrobe activity.

INTRODUCTION

Developing remedies for infectious diseases remains a real constraint, especially in developing countries. The search for new drugs is a time-consuming process and slow in comparison to the emergence of bacterial resistance, therefore, there is an urgency to look for alternatives substances that could be developed into medicines.

Endophytic fungi are the microorganisms that are present in living tissues of various plants, establishing a mutual relationship without causing any symptom of diseases. Endophytes are rich sources of bioactive metabolites, which have medicinal benefits in medical, agricultural and industrial field. The production of secondary metabolites by endophytes is associated with environmental factors [1]. Recent findings revealed the existence of endophytic microbes that produce secondary metabolites. Numerous studies were then performed utilizing endophytic microbes as potential producers of secondary metabolites. Previous studies demonstrated that secondary metabolites are made up of alkaloids. The fact that these microbes produced secondary metabolites that matches its host plant is an interesting breakthrough, because this allowed shortcut for isolation of secondary metabolites, which is directly from host plants. Once metabolites were isolated, bio – screening can be done to reveal bioactive compounds with same medicinal effect as those demonstrated in secondary metabolites produced by endophytes. Then, there was no need to cut down the actual plant to be used as simplicia, as it will take decades for such plant to grow and ready for next harvest time[2].

One of the plants that are used widely as traditional medicine for many generations to treat infectious diseases was called Delima (*Punica granatum* Linn.). *Punica granatum* have many medicinal effect including bactericidal, anti-viral, anti – spasmodic, anti-helmintic and also an immune modulatory effect [3,4]. Every single parts of this plant can be used as an alternative form of medicine - from its flowers, fruit, root bark, bark and fruit peel. Case in point, its pounded peeled roots was used as de-worming medication, with selective effect against tapeworms. The flowers, after boiling in water, may be chewed for treatment of sore gums. Furthermore, the fruit, is a remedy for treatment of dysentery, that is to stop the release of slimy excrement discharge. Last but not least, its peeled ripe fruit is used as astringent and is a potent herbal medicine for treatment of dysentery, diarrhoea and vaginal discharge[5].

The aim of the study was to isolate endophytic microbes from twigs of delima tree and ultimately isolate bioactive compound produced by the secondary metabolite of the isolated microbes that have anti-microbial activity.

MATERIAL AND METHODS

Material

The twigs with diameter range from 0.3 – 0.6 cm , ethanol 75 %, NaOCl 5,3 %, paper disc (5 mm diameter) (OXOID), amphotericine B disc, Potato Dextrose Agar (PDA) (Pronadisa), Potato Dextrose Yeast (PDY) Broth (Pronadisa), Nutrient Agar (NA) (Oxoid), Nutrient Broth (Oxoid), Potato Dextrose Broth (Pronadisa), *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Candida albicans* ATCC 1023, *n*-hexane, ethyl acetate (EtoAC), *n* butanol (n-ButOH).

Laminar air flow, (Gelman Sciences PTY, LTD), autoclave (Hirayama), Oven (Memmert),Spectrometer UV-Vis (Shimadzu UV-1601), Microscope (Olympus) Orbital shaker Centrifuge (Kokusan H-103n).

Methods

Twigs of Delima (*Punica granatum* Linn) plant (diameter of 0.3 – 0.6 cm) were selected in this study. Leaves were removed, cut into approximately of 2 – 3 cm long and washed under running tap water for about 10 minutes. The sections of the twigs were sterilized in the laminar air flow by first soaking in 75% EtOH for 1 minute, then in 5.3% solution of NaOCl for 5 minutes and finally soaked in 75% ethanol for another minute. After that, samples were air-dried on sterile tissue paper for 1 minute and were placed on a sterile glass slide and cut longitudinally, with a scalpel, into two equal parts. Each part was placed on an PDA (Potato Dextrose Agar) that had been treated with Chloramphenicol (0,005%) as an antibiotic. Each sample was positioned longitudinally in which the surface of the cut was placed in

contact with the medium. They were put into the incubator for 5 to 7 days with a temperature between 27° and 29°C[6].

Observation of fungal colony

Taxonomic identification of the fungi was based on their morphological characters and spore production mechanism. Determination of fungal colony were carried out through macroscopic observation of the shape of the colony and colour contrast[7]. Samples that matched with the observation criteria were named as same isolate on the other hand, for those that did not match with the criteria were called different isolate. Moreover, individual colony with different morphology was separated and classified as an individual isolate. Observation of the morphology was conducted after 5 to 7 days of incubation, each of the pure isolate was transferred into two different types of culture media (stock and working culture). Stock culture was kept in slant agar added with paraffin liquidum, while working culture was kept in slant agar

Fermentation of endophytic fungi

A section of the fungal isolate aged 7 to 10 days was isolated by using corbork, diameter 1 cm. In total there 5 section of isolate collected and added into 50 ml liquid fermentation medium of PDY (Potato Dextrose Yeast Extract Broth) in a 250 ml Erlenmeyer flask. Next, the fermentation process was conducted by using an orbital shaker for 5 days with a rotating speed of 170 rpm. Fermentation process was continued using the 200 ml in 1 L Erlenmeyer for another 7 days. The product was centrifuged at a speed of 3000 rpm for 20 minutes. Supernatant was then, extracted with organic solvent (n-hexane, EtOH and n-BuOH)[8].

Extraction of secondary metabolite

The fermentation product was extracted in n-hexane. Supernatant was collected and dried using the rotary vacuum evaporated to yield n-hexane concentrate. Residues generated were collected, and extracted further with EtOAc. Once again, supernatant collected were dried as mentioned previously to obtain EtOAc concentrate. Residues generated was extracted further in n butanol, obtain n-butanol concentrate.

Test for antimicrobial activity

The in vitro anti-microbial screening assay was performed on paper disc. The Gram (+) bacterial test microbes were *Staphylococcus aureus* and the Gram (-) bacterial test microbes were *Escherichia coli*. The yeast test microbe was *Candida albicans*. Nutrient Agar was used as medium for *Staphylococcus aureus* and *Escherichia coli* test Microbe. PDA was used as the medium for *Candida albicans* yeast test microbe. Disc was dipped into the suspension until saturation was reached, then placed on agar medium that had been inoculated with test microbes. Then, the next step was the incubation. Bacterial test samples were incubated at a temperature of 35-37° C, while the yeast test samples were incubated at a temperature 20-25 °C for 18-24 hours respectively. Macroscopic observations were carried out to see a clear zone around the disc[9].

RESULTS

From the twigs of fresh Delima (*Punica granatum* Linn.) plant, five isolates of endophytic fungi were successfully isolated; they were IPG3-1, IPG3-2, IPG3-3, IPG3-4 and IPG3-5.

Figure 1 showed the morphology observation of the isolate obtained from the tree branches

Macroscopic observation showed clearly the morphology, which includes colours, shapes, edges and surfaces of the colonies, of all isolated endophytic fungi. Of the five isolates, IPG3-1 isolate was the only isolate with different shape and color. The other four fungi has white cotton like colonies, with formation of concentric circles, flat edges and reverse golden yellow colour. Our most interesting isolation results were fungi IPG3-3, IPG3-4 and IPG3-5. Both IPG3-3 and IPG3-4 have unique characteristics, such radial wrinkles in the middle and black spots while IPG3-5 has flower like shape in the center of the colony. Apart from those mentioned above differentiation in colour morphology of the IPG3-3 and IPG3-4 was also illustrated in Figure 1. IPG3-4 colony's reverse opposite colour was more darkish orange than IPG3-3, it has more black spots in the center of its colony but no reverse opposite colour. Additionally, it is also interesting to be able, based on microscopic observation, to distinctly differentiate the morphology and the growth rate of these two fungi isolates. IPG3-3 was found to have higher growth rate than IPG3-4, Moreover, IPG3-3's morphology has a "branched " septate while IPG3-4 has un – branched septate. The morphology of IPG3-1,2 and 5 were branched however. Overall, all endophytic fungi have no spore.

The microscopically of the endophytic fungi was shown in Figure 2.

Observation results:

Figure 1

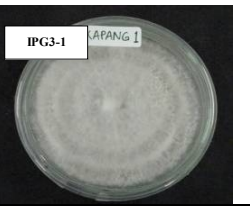
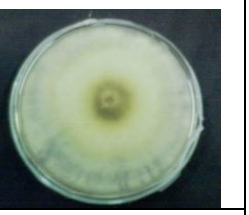
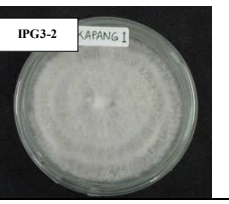
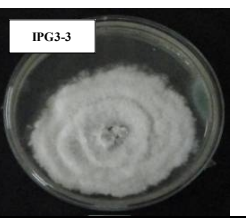
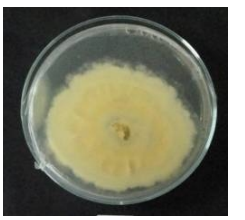
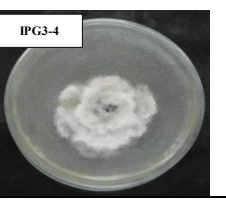
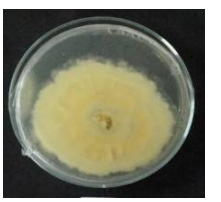
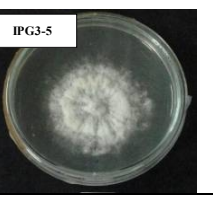
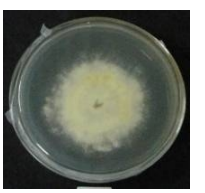
No.	Isolate Code/ Type of Isolates	Source of Isolates	Diameter of Fungi (cm) on seventh day	Morphological Characteristics of Macroscopic Fungi	
				Front appearance	Reverse color
1.	IPG3-1 / Fungi 1	Third branch	8,8	The shape of the colony is round, its color is white, the edge of the colony is flat, and the colony surface is cotton like, forming a concentric circle, with rapid colony growth (2 days). 	Gold en yellow 
2.	IPG3-2 / Fungi 2	Third branch	8,8	The shape of the colony is round, the color is white, the edge of the colony is flat, surface of the colony is cotton like, forming concentric circle, with rapid growth (3 days). 	Yellowish white 
3.	IPG3-3 / Fungi 3	Third branch	7,9	The shape of the colony is round, the color is white, the edge of the colony is wavy, the surface of colony is cotton like, and there are black dots in the center of the colony, with rapid growth (2 days). 	Yellow-light orange 
4.	IPG3-4 / Fungi 4	Third branch	4,5	The shape of the colony is round, the color is white, the edge of colony is wavy, the surface of the colony is cotton like, and there are black dots in the center of the colony, with slow colony growth (6 days). 	Darkish-orange 
5.	IPG3-5 / Fungi	Third branch	5,5	The shape of the colony is round, the color is white, the edges of the colony are wavy, and the colony surface is cotton like, with a slow colony growth (5 days) 	Light yellow 

Figure 2

No.	Isolate Code/ Type of Isolates	Source of Isolates	Microscopic Morphological Characteristics
1	IPG3-1 / Fungi 1	Third branch	With septate and branched 
2	IPG3-2 / Fungi 2	Third branch	Branched, without septate 
3	IPG3-3 / Fungi 3	Third branch	With septate and branched 
4	IPG3-4 / Fungi 4	Third branch	With septate, without branch 
5	IPG3-5 / Fungi	Third branch	Branched, without septate 

Anti microbial activity

Table 1: The antimicrobial test of secondary metabolite of endophytic microbe from Delima Tree branches. (*Punica granatum* Linn.) plant

No.	Isolate	Fraction	Diameter of inhibition zone (mm)		
			Staphylococcus aureus	*Escherichia coli*	*Candida albicans*
1.	IPG3-1	*n*-hexane ehyl acetate *n*-butanol	0.0 15.67 0.0	0.0 18.67 0.0	0.0 15.33 0.0
2.	IPG3-2	*n*-hexane ehyl acetate *n*-butanol	0.0 10.00 0.0	0.0 10.00 0.0	0.0 0.0 0.0
3.	IPG3-3	*n*-hexane ehyl acetate *n*-butanol	0.0 15.67 0.0	0.0 17.00 0.0	0.0 19.33 00
4.	IPG3-4	*n*-hexane ehyl acetate *n*-butanol	0.0 0.0 0.0	0.0 8.33 0.0	0.0 0.0 0.0
5.	IPG3-5	*n*-hexane ehyl acetate *n*-butanol	0.0 10.67 0.0	0.0 9.33 0.0	0.0 0.0 0.0
Positive control Chloramphenicol			23.44	27.22	-
Positive control Amphotericyn B Diameter of the paper disc			-	-	13.78 6.00 mm

Based on the in vitro anti microbial test, n hexane and n ButOH extract of IPG3-1, IPG3-2, IPG3-3 IPG3-4 and IPG3-5 showed no activity toward to *Staphylococus aureus, Escherichia coli* and *candida albicans*. The EtOAc extract of all isolates except for IPG3-4 showed strong activity against both *Staphylococcus aureus* and *Escherichia coli*, IPG3-4 selectively, inhibit *Escherichia coli* with percentage activity of 30.60 %. IPG3-1 and 3 demonstrated strong activity to *Candida albicans,*

DISCUSSION

In the current study, identification of endophytic fungi isolated was performed using the well known conventional method (macro and microscopic observation). These allowed for identification of morphology of the isolated fungi and ability to see that there were not any spore productions. Study however was continued with an extra 4 weeks endophyte isolate incubation; yet, spora formation was not observed but only mycells were formed, hence, accurate genotype identification was successful but not the phenotype classification of individual endophytic fungi[7]. A more advanced technique using r DNA sequencing method, as endophytic fungi underwent fast phenotype mutation[10] .however, has been considered for future analysis of the endophytes.

Surface sterilization method employed within the study was also different from those described in Jaggaonwala *et al* [11]. in the step where sample was not rinsed with sterile water. after soaking in sterilization solution (EtoH and Na HClO3). Additional washing with sterile water is not necessary[12].

The next part of work, isolation of secondary metabolites. Isolation of metabolites of interest was carried out by way of fermentation in media containing rich source of carbon and nitrogen compound as well as essential organic salt $CaCO_3$, followed by extraction in organic phase (n-hexane, EtoAc and n-BuOH). PDY media was used in this experiment because the potato and dextrose contain high percentage of carbon while, the yeast extract contained within PDY media has high percentage of nitrogen related compound.

The result was, secondary metabolite from isolate IPG3-1 and IPG3-3 were very active aginst *candida albicans* with percentage activity of 111.25 and 140.28 % resepectively, when compared to amphotericine B treated control. In addition, IPG3-1has an antimicrobial property against *Staphylococcus aureus* with a percentage of 66.85% and against *Escherichia coli* with a percentage of 68.59%, meanwhile a comparison between IPG3-3 to chloramphenicol reveals an antimicrobial activity of 66.85 % and 62.45% against *Staphylococcus aureus* and *Escherichia coli* respectively. These results are obtained by comparing to the diameter of the inhibition zone of the extracts with the diameter of the inhibition zone of comparator antibiotics.

These above results are in line with previous finding by Purwantini [13].; whom stated that extract of delima fruit have potent anti fungal activity towards *Candida albicans*. Furthermore, the fact that endophytes live within its host plant and these microbes produced secondary metabolites that matches its host plant (as mentioned in introduction, Strobel[14]. It became a clear and interesting scientific evidence that secondary metabolites isolated endophytes living in delima tree has potent anti microbial activity.

Overall, Endophytic fungi from Delima (*Punica granatum* Linn.) tree branches have potent antimicrobial components. This is a very exciting preliminary study. It require advance findings in terms of bio screening of endophytes against wide range of bacterial test organism, isolation and anti-mirobial assessment (IC_{50}) of individual secondary metabolites obtained.

CONCLUSION

The IPG3-1 and IPG3-3 isolate both have the anti microbial potency towards *Staphylococcus aureus* (66.85%). On the other hand, towards *Escherichia coli*, IPG3-1 and 3 have potent inhibitory activity of 68.59% and 62.45% respectively when compare to Chloramphenicol. Additionally, they also have potent inhibitory activity towards fungi (*Candida albicans*) 111.25% and 140% when compare to Amphotericin B control.

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Cite this article as:
Shirly Kumala and Hayatul Izzati. Isolation IPG3-1 and IPG3-3 , endophytic fungi from Delima (Punica granatum Linn.) twigs and in vitro assessment of their anti microbial activity. Int. Res. J. Pharm. 2013; 4(6):49-53