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Alkaloid (Meleagrine and Chrysogine) from Endophytic Fungi (*Penicillium* sp.) of *Annona squamosa* L.

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Abstract: Several endophytic fungal strains from Srikaya plants (*Annona squamosa* L.) have been isolated and one of them was identified as *Penicillium* sp. *Penicillium* has been proven as an established source for a wide array of unique bioactive secondary metabolites that exhibit a variety of biological activities. The aim of this study is isolation of secondary metabolite from *Penicillium*, an endophytic of *A. squamosa* L. *Penicillium* sp. from endophytic of *A. squamosa* L. was fermented in Wicherham media. The whole extract from both liquid media and mycelium was partitioned by ethyl acetate and evaporated to obtain crude ethyl acetate extract. The ethyl acetate extract was then brokedown using column chromatography with silica as stationary phase and mixture of ethyl acetate/methanol (98%:2%) as mobile phase and then was separated by sephadex column. Structure elucidation of isolated compounds were mainly done by analysis of one and two dimensional NMR (Nuclear Magnetic Resonance) data and supported by HPLC (High performance Liquid Chromatography) and MS-TOF (Mass Spectrometer-Time of Flight). Isolated secondary metabolites were tested using *in vitro* assays for anticancer and antimicrobial activity. For anticancer activity, the metabolites were tested against breast cancer cells (MCF-7) using MTT assay, while for antimicrobial activity was performed using disk diffusion assays. From these physical, chemical and spectral evidences that the secondary metabolites were confirmed as Chrysogine and Meleagrine. Chrysogine and Meleagrine have no activity as anticancer and antimicrobial.

Key words: Endophytic fungi, meleagrine, chrysogine, srikaya/sugar apple (*Annona squamosa* L.), *Penicillium* sp.

INTRODUCTION

Endophytic microbes are bacteria or fungi that live in plant tissue for certain time period, forming colonies without harming its host (Tan and Zou, 2001). Bioactive natural compounds produced by endophytes are promising with potential application in wide area such as medicine, agriculture and industry (Joseph and Priya, 2011).

It has been previously reported that extract of Srikaya (*Annona squamosa* L.) seed, also called sugar apple or sweetsop or soursop, was found to be more active as antiinsecticide than extract of *Annona muricata* (Sirsak) seed (Leatemia and Isman, 2004; Prijono *et al*., 1997). In addition, annonaceae acetogenin isolated from the seed of Srikaya was also reported as natural substance with potential activity as antitumor, pesticides and other bioactive as a barrier to electron transport in mitochondria (McLaughlin and David, 2001; Liaw *et al*., 2008). Endophytes from Annonaceae is predicted to contain similar metabolites as those extracted from the host, so it is expected that the extraction from the endophytes is more economical than extraction from the seeds of the host fruit.

In previous study, several endophytic fungal strains from Srikaya plants (*Annona squamosa* L.) have been isolated and one of them was identified as *penicillium* sp. (Yunianto *et al*., 2012). The *Penicillium* species are widely distributed in nature and with more than 200 species (Kim *et al*., 2012). As a living organisms, the *Penicillium*

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can be found on foods (Vinokurova *et al*., 2003; Samson, 2010), in indoor environments (Nielsen, 2003), in marine that associated with mangrove (Shabab *et al*., 2011) and marine organism (Bhatnagar and Kim, 2010; Meng *et al*., 2011) and associated in plants as endophytic fungus (Oliveira *et al*., 2011). The *Penicillium* has been proven as an established source for a wide array of unique bioactive secondary metabolites that exhibit a variety of biological activities (Keller *et al*., 2005). Frisvad *et al*. (2004) have studied 58 species of *Penicilium* that produce secondary metabolites. Several secondary metabolites are unique for one species, but most of the metabolite are produced by more than one species. Among those reported secondary metabolites. Roquefortine C, cyclopenins, patulin, penilic acid, terrestric acid and 2-methyl isoborneol have been reported as the most widespread metabolites were produced by *Penicillium* sp. With the reason isolation of secondary metabolite from *penicillium* sp., an endophytic of *Annona squamosa* L. is still promising to be done.

In this study, isolation and elucidation structure of secondary metabolites from endophytic fungi of sugar apple plants are described. Isolated secondary metabolites was tested using *in vitro* assays for activity as anticancer and antimicrobial. For activity as anticancer, the metabolite was tested against breast cancer cells (MCF-7) using MTT cytotoxicity method, while for antimicrobial assay using the activity of metabolite was performed using disk diffusion methods.

MATERIALS AND METHODS

Isolation of endophytic fungi: Twigs of Sugar apple plants used in this study were collected from Tangerang, Banten-Indonesia. The twigs were washed with distilled water, cut into small pieces (1-2 cm). The surface of twigs was sterilized by three times immersion. The first was immersed in ethanol 70% v/v for 1 min, the second was in 5.25% NaOCl solution for 5 min and the last, was immersed in 70% ethanol solution v/v for 0.5 min. Moreover the outer skin of twigs was discarded using a sterile knife and then placed on an agar medium in a sterilized petri dish. The agar medium was prepared by adopting the procedure in literature (Rusman, 2006). The media composition was agar (15 g), dry powder of the host plant leaves (15 g), chloramphenicol (0.2 g) and up to 1 L distilled water. The media was incubated at room temperature for 20 days. After that the fungi colony formed was then moved to new media with the composition as follow : agar (15 g), Malt extract (15 g) and distilled water up to 1 L. The pH of media was adjusted so pH 7.4 to 7.8 was obtained.

Liquid fermentation of isolated fungal: Single isolate of endophytic fungus (the macroscopic and microscopic) was used as mother culture for cultivation. This isolate was firstly incubated for 3 to 7 days before being used as starter inoculums for the cultivation/subsequent fermentation.

Liquid fermentation was carried on 8 L of Wickerham medium, where for 1 L of this medium was composed of distilled water (1 L), yeast extract (3 g), malt extract (3 g), peptone (5 g), glucose monohydrate (20 g) and pH adjusted to 7.2 to 7.4. Furthermore the prepared media was divided into 10 Erlenmeyer flasks 2 L and each flask contains 800 mL. The starter (5 % v/v) was added to each flask containing media and then incubated at room temperature for 20 days (Ebel *et al*., 2006).

Ethyl acetate extracts preparation: A 500 mL ethyl acetate was added into each fungal culture in erlenmeyer flask and the mixture was kept overnight to ensure that the fungal cell died. The mixture was then Sonicated three times for 15 min (for cell destruction) and followed by filtration using Büchner vacuum.

The ethyl acetate phase was then separated from water phase (medium) using separation funnel. The ethyl acetate phase was then washed with water two times with destillated water to remove the remaining salts and other polar constituents. Just after evaporation, the ethyl acetate extracts was diluted in 90% methanol and extracted with n-hexane to remove fatty acids and other non-polar constituents. After that the methanol phase was evaporated as ethyl acetate extract. At the same time, the water phase (medium) was extracted with water-saturated n-Butanol to collect the polar constituents.

Isolation of secondary metabolite: The extract of ethyl acetate phase was isolated by passing through on a silica column (60 - 120 mesh) with mobile phase mixture of ethyl acetate:methanol (98:2% v/v). Two fractions obtained (denoted F7 and F9) were applied on TLC plate using ethyl accetate:methanol (98:2 % v/v) as mobile phase. These two fractions were then evaporated followed by purifying using sephadex column with methanol as mobile phase. Purified F7 and F9 fraction were single spotted on TLC plate.

Structure elucidation of secondary metabolite: Profile analysis and the UV spectrum of secondary metabolite were recorded using a Knauer HPLC-DA (High performance Liquid Chromatography- Diode Array) Diode Array Detector K-2800 and Sunfire column C-18, 150×4.6 mm, 5 μm using mixture of methanol and water as mobile phase. The gradient elution was set gradually by increasing the methanol composition from 0% at 0 min

until 100% at 30 min. The molecular weight of secondary metabolites were identified using Waters high resolution Mass Spectrometer-Time of Flight (MS-TOF). Spectra of NMR (^{1}H-NMR,^{13}C-NMR,COSY, HMQC, HMBC) were recorded on Jeol-500 MHZ.

Cytotoxicity test using *in vitro* with MTT (Methyl Thyazole Tetrazolium) assay: The secondary metabolite were tested using *in vitro* MTT assay against cancer cells,MCF-7 (breast cancer cell line) by adapting the method in literature (CCRC, 2008).

MCF-7 cells were cultured in Medium containing Fetal Bovine Serum (FBS) 10% (v/v). Cells (5×103 cells/well) were transferred to 96-well plate and incubated for 24 h. Cells were treated by test compound of secondary metabolite (100 μg mL^{-1}). Both treated and untreated on MCF-7 cell line were incubated for 24 h. At the end of the incubation, 5 mg mL^{-1} solution of MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] were added to each wells and the cells were incubated for 4 h in 37°C. Viable cells reacts with MTT to form purple formazan crystal. After 4 h, stopper sodium dodesil sulphate 10% in 0,1 N sulphuric acid solution were added to dissolve formazan crystal. Cells were incubated over night and protected from light. Cells were shaken for 10 min before read by ELISA reader at λ 570 nm. Cell viability in treated cells was expressed as the amount of dye reduction relative to that of untreated control cells. The wells which contained only medium and MTT were used as blanks.

Antimicrobial assay: Disk diffusion (Zone inhibition) methods was used to investigate the antimicrobial activity of sample. The samples were seeded into respective medium by spread plate method 10 μL (10^{6} cells mL^{-1}) with the 24 h cultures. The procedure is as follow:samples were dissolved in methanol with a concentration 100 μg mL^{-1} and sterilized with UV irradiation for 30 min. After solidification the filter paper discs (5 mm in diameter) impregnated with the samples were placed into 5 petri dish containing a microbial culture *Salmonella tyhpi*, *Escherichia coli*, *Bacillus subtilis*, *Candida albicans* and *Staphylococcus aureus* and incubated at 37°C for 1×24 h. Tetracycline (10 μg mL^{-1}) used as positive control and methanol solvent (100 μg mL^{-1}) used as negative control. The diameters of the inhibition zones were measured in mm.

RESULTS

Isolation of endophytic fungi: Several endophytic fungal strains were isolated from Srikaya plants (*Annona squamosa*.L) (Yunianto *et al*., 2012) and one of them was identified as *Penicillium* sp. by observing on macrocospic and microscopic as shown in Fig. 1.

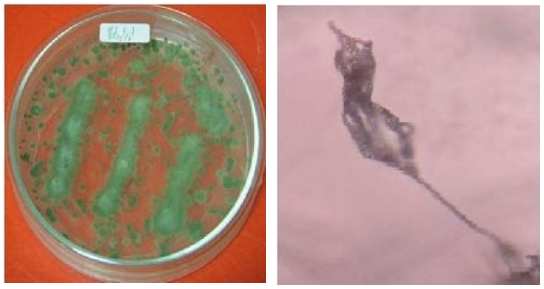

Fig. 1: Morphology of *Penicillium* sp. of endophytic fungi isolated from twig sugar apple (*Annona squamosa*), 5 days old colonies at PDA media

Structure elucidation of isolate F9, The compound was isolated as a light yellow powder generating an orange TLC spot under 366 nm. It has maximum UV absorption at: 229, 269, 346 nm as recorded on HPLC-DA (Fig. 2).

Molecular weight of 433,1552 g moL^{-1} was deduced from ES (-) and ES (+) MS-TOF spectra showing a molecular ion peak at m/z 432.1659 $[M-H]^-$ and 434.1445 $[M+H]^+$. Further analysis of NMR data suggested that the compound F-9 was meleagrine (Fig. 3) which has been previously reported by Kawai *et al*. (1984). Table 1 shows NMR data of the isolated meleagrine and its comparison to the reported from Kawai *et al*. (1984) and Effendi (2004).

Structure elucidation of isolate F7, Analysis of HPLC-DA showed that the compound F7 has UV maximum at: 211, 249 and 294 nm (Fig. 4).

The molecular weight of 190,0955 g moL^{-1} was established from ES (-) and ES (+) MS-TOF experiments generating molecular ion peak at m/z 207.0963 $[M+H_2O]^-$ and m/z 191.0947 $[M+H]^+$. Compound F7 was identified as 2-(1-Hydroxyethyl)-4(3H)-quinazolinone (Chrysogine) (Fig. 5.) based analysis of one and two dimensional NMR data and its comparison to the previously reported data (Kettering *et al*., 2004) are listed in Table 2.

Cytotoxicity assay *in vitro* with MTT method: Cytotoxicity assay of Meleagrine and Chrysogine at level 100 μg mL^{-1} was tested against MCF-7 (breast cancer cell) using MTT viability test and the result is shown in Fig. 6. As can be seen from Fig. 6 that Meleagrine and Chrysogine at concentration extract of 100 μg mL^{-1} could not inhibit the grow of MCF-7 cell more than 50% compared to that of untreated control cells (negative control).

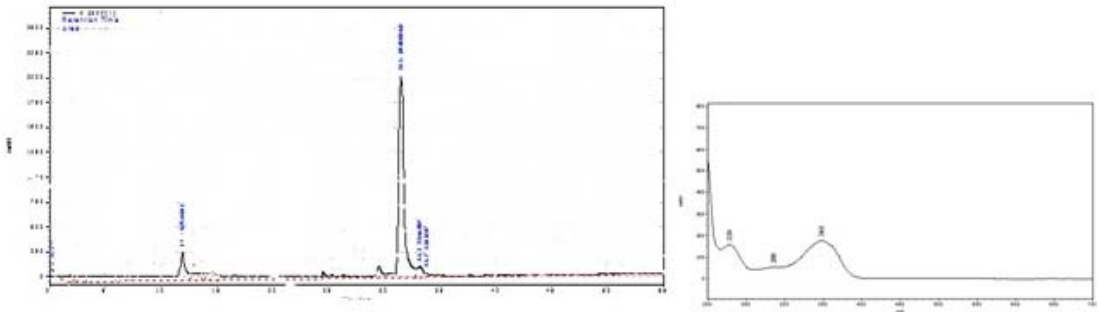

Fig. 2: Profile of chromatogram and UV spectrum of isolate F-9

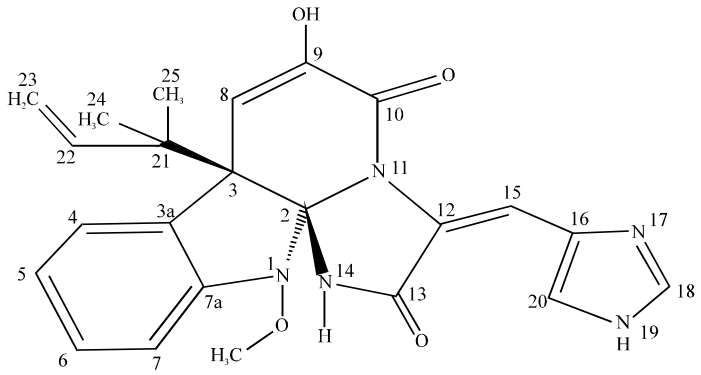

Fig. 3: Structure of Meleagrine

Table 1: Comparison of H-NMR compound of F-9 with H-NMR from Kawai *et al.* (1984) and Effendi (2004)

Position	δ ^{1}H (ppm) J_{H-H} (in Hz) (Kawai *et al.*, 1984) in $CDCl_3$	δ ^{1}H (ppm) J_{H-H} (in Hz) (Effendi, 2004) in DMSO	δ ^{1}H (ppm) J_{H-H} (in Hz) (This study, in CD_3OH)
1			
2			
3			
3a			
4	7,58 (br d,7.5)	7,52 (d, 7,0)	7,59 (d, 7.9)
5	7.07 (br t, 7,5)	7,02 (br t, 7,6)	7,05 (t, 7.9)
6	7,30 (br t, 7,5)	7,25 (br t, 7,6)	7,25 (t. 7,9)
7	6,95 (br d, 7,5)	6,96 (d,7,0)	6,99 (d, 7,9)
8	5,50 (s)	5,24 (s)	5,38 (s)
9 (OH)		10,0 (br s)	
10			
11			
12			
13			
14 (NH)		9,20 (s)	
15	8,27 (s)	8,17 (s)	8,28 (s)
16			
17			
18	7,61 (s)	7,75 (s)	7,81 (s)
19 (NH)	12,72 (br s)	12,78 (br s)	
20	7,25 (s)	7,52 (s)	7,36 (s)
21			
22	6,12 (dd, 17,2; 10,6)	6,00 (br s)	6,00 (br.s)
23	5,10 (d, 17,2) H23A; 5,05 (d, 10,6) H23B	5,00 (br d, 18,3) H23A; 4,98 (br d, 18,3) H23B	5.00 (t, 17,2)
24	1,35 (s)	1,18 (s)	1,31 (s)
25	1.34 (s)	1,18 (s)	1.28 (s)
1'-OCH_3	3,73 (s)	3,65 (s)	3.09 (s)

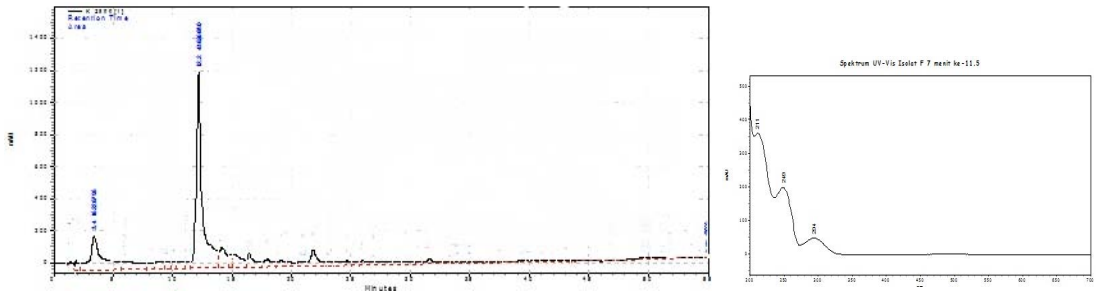

Fig. 4: Profile of chromatogram and UV spectrum of isolate F-7

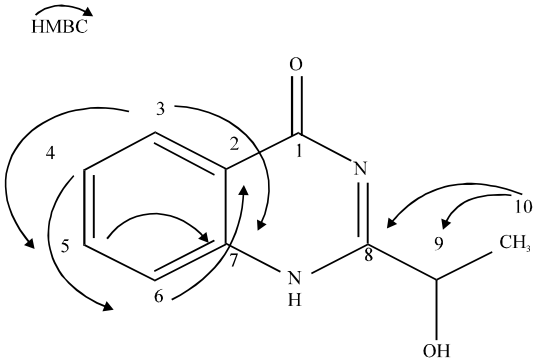

Fig. 5: Structure of Chrysogine

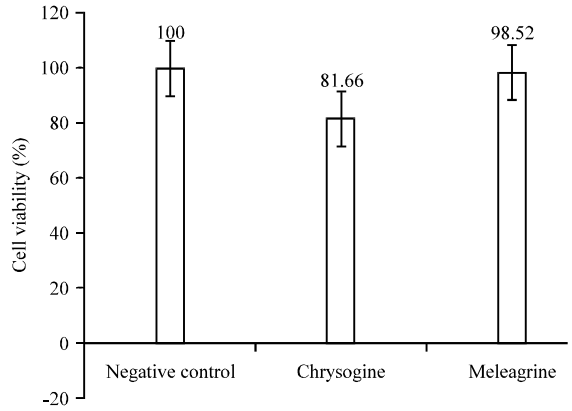

Fig. 6: Effect of Meleagrine and Chrysogine at 100 μg mL^{-1} on % viability of breast cancer cell MCF-7 as determined by the MTT cell viability assay

Antimicrobial assay: Antimicrobial assay of Meleagrine and Chrysogine are shown in Table 3.

DISCUSSION

In this study, the isolated filamentous fungi from twigs of Srikaya plants (*Annona squamosa.* L) was identified as genus *Penicillium.* Traditionally, taxonomic classification of *Penicillium* is difficult, since the traditional classification just relies on macroscopic and morphology, which are not clearly defined for *Penicillium.* The species of *Penicillium chrysogenum*, known as penicillin producer, was firstly classified by Fleeming. Later, this *Penicillium chrysogenum* was re-identified as *Penicillium rubens* by Houbraken *et al.* (2011) based on the differences in their secondary metabolite, although both species have similarity in phenotype and morphology.

The ethyl acetate fraction extracts, instead of the butanol extracts as previously conducted by Kumala *et al.* (2006), were screened for their cytotoxicity. The use of ethyl acetate extracts was intended to facilitate further isolation of single compound compared to that of butanol extract, which is more polar solvent.

Based on the comparison of H-NMR data of compound in F9 with H-NMR data of the literature from Kawai *et al.* (1984) and Effendi (2004), it can be observed a similarity on chemical shift, as shown in Table 1. Although H-NMR data on some chemical shifts that do not show up, such as the position of H in 9 (OH), 14 (NH) and 19 (NH), therefore, an H-NMR analysis using solvent CD3OD was conducted. According to Sharif *et al.* (2007), exchangeable protons (NH or OH) of compounds will be replaced by 2H of deuteration solvent (D2O or CD3OD) when such compound are measured in protic solvents such as water or methanol. There are 3 un-identified chemical shifts of H position, however, UV spectra and physical properties of compound obtained under this study show a similar to the literature Effendi (2004). For chemical shifts in position 9 (OH) is very difficult to prove because of the solvent effect during the H-NMR analysis. On the other hand, the existence of NH at position 14 (NH) and 19 (NH) can be proved by verifying them using reagents Dagendroff where brown spots were observed which indicate the existence f such nitrogen (N) as NH in the compound. By comparing the physical properties, UV-max, molecular weight, spectra data H-NMR of compound in F9 with

Table 2: The HMBC data and comparison of H-NMR and C-NMR of compound F-7 with H-NMR and C-NMR from Kettering *et al.* (2004)

	Kettering *et al.* (2004)		This study		
Position	δ ^{1}H (ppm), J_{H-H} (Hz) in $CDCl_3$ (500 MHZ)	δ ^{13}C (ppm), in $CDCl_3$	δ ^{1}H (ppm), J_{H-H} (Hz) in CD_3OD (500 MHZ)	δ ^{13}C (ppm), in CD_3OD (500 MHZ)	HMBC (H to C)
1	-	170.2	-	172.1	
2	-	120.9	-	124.6	
3	7.74 (dd; 1.4 , 7.8)	128.4	7.74 (dd; 7.95, 7.95)	132.5	C7, C5
4	7.11 (dt, 1.1,7.8)	122.3	7.16 (dt; 7.35, 7.95)	129.6	C6
5	7.45 (ddd, 1.4, 7.8, 8.4)	131.8	7.51 (ddd; 1.2, 7.35, 8.55)	133.4	C7, C4
6	8.57 (dd, 1.1, 8.4)	119.8	8.54 (dd; 7.35, 8.55)	122.3	C2
7	-	138.7	-	138.1	
8	-	174.1	-	176.2	
9	4.10 (dq, 3.8, 6.8)	67.9	4.21 (dq; 6.7, 7.35)	69.9	
10	1.30 (d, 6.8)	20.8	1.42 (d; 6.75)	21.3	C9, C8
7-NH	11.98 (s)		-		
9-OH	5.95 (d, 3.8)		-		

Table 3: Activity on antimicrobial assay of meleagrine and chrysogine

Test of antimicrobial and antifungal	Meleagrine	Chrysogine	Positive control (tetracycline)
Escherichia coli	-	-	+
Salmonella tyhpi	-	-	+
Bacillus subtilis	-	-	+
Staphylococcus aureus	-	-	+
Candida albicans	-	-	-

(-) Not activity, (+) Active

the data from literature, it can be sufficient to prove that the compound is Meleagrine.

In vitro cytotoxicity assay of Meleagrine at concentration level of 100 μg mL^{-1} indicated that this compound was inactive for inhibiting the MCF-7 cancer cells. Swanson and Pezzuto (1990) claimed that a compound with LC_{50} less than 20 μg mL^{-1} is considered as an active compound. In this study, the MTT test was carried out at concentrations 100 μg mL^{-1}. Based on the data in Fig. 6, the Meleagirne has no activity as anticancer, because the percentage viability of MCF-7 cancer cells was more than 50%. In addition, Meleagrine has also no activity against microbial and fungus. The similar result has been observed by Rusman (2006) when the meleagrine was tested against L 5178Y cell line as well as for its antimicrobial and antifungal activity testing towards *B. subtilis* and *C. herbarum*. However, Meleagrine has been reported as an active substance as an anti tumor (Bhatnagar and Kim, 2010). This Meleagrine has worldwide marketed as antitumor and antibiotic agent by Bioaustralis Fine Chemical with CAS no 71751-77-4. Kawai *et al.* (1984) suggested that owing to its un-stable structure can be the reason for the different in its activity.

From Table 2 shows that the data H-NMR and C-NMR of compounds F7 is similar to literature data NMR data from Kettering *et al.* (2004), namely 2-(1-Hydroxyethyl)-4 (3H)-quinazolinone (Chrysogine). Although in the chemical shift H-NMR does not appear in H at position 7-NH and 9-OH. This can be due to the solvent (-CD_3 OD) suppresion effect during the H-NMR analysis lead to lost of NH and OH signal. Data from two-dimensional NMR (HMBC) supported the structure, as shown in Fig. 5. According to literature from Kettering *et al.* (2004) from fermented *Penicillium* strains, the compounds Chrysogine have [α] D +183 - +186° (in ethyl acetate), UV λmax = 224, 263 (in methanol) and 302 nm (log å 4.05, 3.56, 3.28) with weight of 190 g moL^{-1}. While compounds of F7 has UV spectrum (in methanol) at λmax = 211, 249 and 294 nm, with molecular weight as 190.0955 g moL^{-1}.

By comparing the data from NMR, UV spectrum and its molecular weight, it can concluded that the compound in F7 is Chrysogine. According to Fig. 6 and Table 3, it can be observed that the Chrysogine has no activity as anticancer and antimicrobial.

CONCLUSION

From these physical, chemical and spectral evidences that the secondary metabolites (F7 and F9) were confirmed consecutive as Chrysogine (Fig. 4) and Meleagrine (Fig. 3), The secondary metabolites were isolated from *Penicillium* sp., an endophytic of *Annona squamosa* L. Chrysogine and Meleagrine has no activity as anticancer and antimicrobial.

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